MICROPROPAGATION OF *TULBAGHIA* SPECIES

VIWE NOMZAMO PRECIOUS NGUNGE

Submitted in fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Research Centre for Plant Growth and Development

School of Biological and Conservation Sciences

University of KwaZulu-Natal, Pietermaritzburg

March 2011

This work is dedicated to my brother Esethu Ngunge

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STUDENT DECLARATION

Micropropagation of *Tulbaghia* species

I, Viwe Nomzamo Precious Ngunge*,* 205504156 (Student Number) declare that :

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

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

Student's Full Name: Viwe Nomzamo Precious Ngunge
Student Number: 205504156
Thesis Title: Micropropagation of *Tulbaghia* species

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

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PUBLICATIONS FROM THIS THESIS

NCUBE, B., NGUNGE, V.N.P., FINNIE, J.F. & VAN STADEN, J. 2011. A comparative study of the antimicrobial and phytochemical properties between outdoor-grown and micropropagated *Tulbaghia violacea* Harv. plants. *Journal of Ethnopharmacology*. 2011 doi: 10.1016/j.jep.2011.01.039.

Contributions: The micropropagation of plants was done by the second author, the experiments on the antimicrobial and phytochemical properties were done by the first two authors, the second author did part of the writing. This work was undertaken under the supervision of Dr. J.F. Finnie and Prof J. van Staden.

CONFERENCE CONTRIBUTIONS FROM THIS THESIS

NGUNGE, V.N.P., FINNIE, J.F. & VAN STADEN, J. 2010. *In vitro* seed germination of *Tulbaghia* species. Twelfth Annual Meeting of the Research Centre for Plant Growth and Development (RCPGD). University of KwaZulu-Natal, Pietermaritzburg.

NGUNGE, V.N.P., FINNIE, J.F. & VAN STADEN, J. 2010. Micropropagation of *Tulbaghia* species. Thirty-sixth Annual Conference of the South African Association of Botanists (SAAB). University of North-West, Portchefstroom.

NGUNGE, V.N.P., FINNIE, J.F. & VAN STADEN, J. 2009. Micropropagation of *Tulbaghia* species. Eleventh Annual Meeting of the Research Centre for Plant Growth and Development (RCPGD). University of KwaZulu-Natal, Pietermaritzburg.

Signed: _____________________

ACKNOWLEDGEMENTS

I am grateful to My Father, Jehovah for being with me through this journey.

My research would not have been possible if it were not for my supervisor Professor J. van Staden who not only afforded me the opportunity to conduct this research, but guided me through it. I sincerely thank my co-supervisor Dr. J.F. Finnie for all he has done to make my research a success.

I would like to extend a word of gratitude to Professor M. Strnad (Laboratory of Plant Growth Regulators, Palacky University and Institute of Experimental Botany Olomouc, Czech Republic) for the cytokinin analysis. The advice and encouragement received from my Research Committee members (Dr. M. Light, Dr. M. Bairu and Dr. S. Amoo) is really appreciated. Dr. M. Moyo and Dr. M. Kulkarni, I thank you deeply for your help. Jane Koetle, thank you for letting me follow you around the laboratories. My sincere thanks goes to all RCPGD members for their support and encouragement. Rofhiwa, Malefsane, Halima, Zamantshali, Ashwell, Bhekumthetho and Balungile, I will forever be grateful to you for your support. My sincere gratitude goes to the administrative staff (Mrs. J. Magnussen and Mrs. L. Warren) for ensuring that everything ran smoothly.

It gives me great delight to express my gratitude to the ladies in the Centre for Electron Microscopy (CEM) – Mrs. S. Mackellar, Ms. T. Xuma, and Mrs. P. Donnely for their assistance with the microscopy work.

A special thanks goes to my family and friends. My mother Lindiwe “Lindi” Ngunge, you have always been the pillar of my strength. Thank you for not giving up on me.

I will forever be indebted to the National Research Foundation (NRF) for funding this research.

ABSTRACT

Tulbaghia (Alliaceae) is a genus of plants with medicinal, ornamental and nutritive value. Different plant parts such as roots, bulbs, leaves and flowers are used in the treatment of a variety of conditions. The bulbs of *Tulbaghia violacea* are used as a remedy for pulmonary tuberculosis as well as an anthelmintic. Due to the extensive harvesting of plants in this genus, the genus is susceptible to overexploitation and may eventually become threatened with extinction. It was therefore the aim of this study to systematically examine the micropropagation of *Tulbaghia ludwigiana* and *Tulbaghia violacea*, as well as to evaluate the antimicrobial and phytochemical properties of micropropagated plants.

Seeds of *T. ludwigiana* and *T. violacea* were successfully decontaminated using 70% ethanol, 1% Benlate and 3.5% NaOCl. Temperature played a significant role in the germination of both species while light did not play a significant role in this process. Light did not play a significant role in the stomatal density of *T. violacea* seedlings.

Hypocotyls were the regenerative part of the seedlings in both species. A low number of shoots was yielded by the combination of various concentrations of NAA and *m*TR in the growth medium in both species. There were more isoprenoid cytokinins than there were aromatic cytokinins in each of the seedling sections of *T. violacea* that were analysed, with *t*Z being the predominant isoprenoid cytokinin, while BA was the predominant aromatic cytokinin. Shoots of both species were successfully rooted in a medium with IBA, while some shoots had simultaneously rooted during shoot multiplication. Potting soil and vermiculite were used in the acclimatization of both species, where *T. violacea* plantlets acclimatized successfully. This was not the case for *T. ludwigiana*. Micropropagated *T. violacea* plantlets contained higher concentrations of phytochemical compounds and displayed better antibacterial activity than outdoor-grown plants.

LIST OF ABBREVIATIONS

2.4-D	2.4-dichlorophenoxyacetic acid
ANOVA	analysis of variance
B5	Gamborg B-5
BA	benzyladenine
BAP	benzylaminopurine
BAP9G	benzylaminopurine-9-glucoside
BAPR	benzylaminopurine riboside
BAPR5′MP	benzylaminopurine riboside-5′-monophosphate
CTE	catechin equivalents
*c*Z	*cis*-zeatin
*c*Z9G	*cis*-zeatin-9-glucoside
*c*ZOG	*cis*-zeatin-O-glucoside
*c*ZR	*cis*-zeatin riboside
*c*ZR5′MP	*cis*-zeatin riboside-5′-monophosphate
*c*ZROG	*cis*-zeatin riboside-O-glucoside
DCM	dichloromethane
DE	diosgenin equivalent
DHZ	dihydrozeatin
DHZ9G	dihydrozeatin-9-glucoside
DHZOG	dihydrozeatin-O-glucoside

DHZR	dihydrozeatin riboside
DHZR5′MP	dihydrozeatin riboside-5′-monophosphate
DHZROG	dihydrozeatin riboside-O-glucoside
DMRT	Duncan’s multiple range test
EtOH	ethanol
GAE	gallic acid equivalents
IAA	indoleacetic acid
IBA	indolebutyric acid
iP	isopentenyladenine
iP9G	isopentenyladenine-9-glucoside
iPR	isopentenyladenosine
iPR5′MP	isopentenyladenosine -5′-monophosphate
LCE	leucocyanidin equivalents
MGT	mean germination time
MIC	minimum inhibitory concentration
MFC	minimum fungicidal concentration
MS	Murashige & Skoog
*m*T	*meta*-topolin
*m*T9G	*meta*-topolin-9-glucoside
*m*TOG	*meta*-topolin-O-glucoside
*m*TR	*meta*-topolin riboside

*m*TR5′MP	*meta*-topolin riboside-5′-monophosphate
*m*TROG	*meta*-topolin riboside-O-glucoside
NAA	napthaleneacetic acid
NN	Nitsch & Nitsch
*o*T	*ortho*-topolin
*o*tOG	*ortho*-topolin-O-glucoside
*o*TR	*ortho*-topolin riboside
*o*TROG	*ortho*-topolin riboside-O-glucoside
*o*T9G	*ortho*-topolin-9-glucoside
*o*TR5′MP	*ortho*-topolin riboside-5′-monophosphate
PE	petroleum ether
Picloram	3,5,6-trichloro-2-pyridinecarboxylic acid
*p*T	*para*-topolin
*p*TOG	*para*-topolin-O-glucoside
*p*TR	*para*-topolin riboside
*p*TR5′MP	*para*-topolin riboside-5′-monophosphate
*p*TROG	*para*-topolin riboside-O-glucoside
SEM	scanning electron microscopy
SH	Schenk & Hilderbrandt
TDZ	thidiazuron
TTC	2,3,5-triphenyl tetrazolium chloride

*t*Z	*trans*-zeatin
*t*Z9G	*trans*-zeatin-9-glucoside
*t*ZOG	*trans*-zeatin-O-glucoside
*t*ZR	*trans*-zeatin riboside
*t*ZR5′MP	*trans*-zeatin riboside-5′-monophosphate
*t*ZROG	*trans*-zeatin riboside-O-glucoside
WPM	woody plant medium

CHAPTER 1
Introduction

Traditional medicine is highly valued by people in South Africa. According to **FENNELL (2002)**, approximately two thirds of the South African population use traditional medicine. **DOLD & COCKS (2002)** noted that many people, especially in the rural areas rely heavily on traditional medicine, either because primary healthcare is hard to obtain, or modern healthcare services are out of their reach because of the high cost of western pharmaceuticals. **MANDER (1998)** noted that for some, traditional medicine forms part of their cultural belief. The author goes on to mention that as the human population increases, this reliance on traditional medicine also increases, which leads to extensive harvesting of medicinal plants from the wild. Most often, bulbs and roots are used for traditional medicine **(DOLD & COCKS, 2002)** and this puts pressure on medicinal bulbous plants. The use of bulbs for traditional medicine results in destructive harvesting because the whole plant has to be uprooted. It is this destructive harvesting that poses a threat to biodiversity as it leads to the extinction of plant species.

The genus *Tulbaghia* is a group of plants that is of nutritive, ornamental and medicinal value to people in South Africa. Due to the diverse uses of plants in this genus, the genus is susceptible to overexploitation and will become threatened with extinction **(SCOTT-SHAW, 1999)**. Thus, the development of a means to ensure that the availability of plants meets the demands is crucial. This is not only important for human use, but for the conservation of the species. Conventional methods can be used to propagate *Tulbaghia*, but there are more advantages in using micropropagation, since these are plants which multiply slowly **(PHELAN et al., 2007)**. Micropropagation ensures mass production of high quality, virus-free plants in less time than conventional propagation **(BRYAN, 2002)**. This is so because environmental conditions of plant growth can be manipulated **(TETYANA, 2000)**. It was therefore the purpose of this study to develop a micropropagation protocol for *Tulbaghia.*

1.1 DISTRIBUTION AND MORPHOLOGY

The Alliaceae is predominantly a South American family, comprising about 13 genera and approximately 600 species **(HUTCHINGS et al., 1996; KUBITZKI, 1998; BRYAN, 2002)**. Genera of this family were once included in the Amaryllidaceae and Liliaceae, which is why the Alliaceae is said to be an intermediate between the Amaryllidaceae and the Lilliaceae **(MAOELA, 2005)**. *Tulbaghia,* whose common names are "wild garlic", "sweet garlic" and "pink agapanthus" (because of its close relation to agapanthus), is a small genus that belongs to the family Alliaceae. It is the second largest genus in the family, the largest being *Allium*. It was named after Ryk Tulbagh, the Dutch Governor of the Cape of Good Hope, who sent specimens of this genus to Linnaeus **(POOLEY, 1998; BRYAN, 2002)**. *Tulbaghia* occurs in tropical Africa including South Africa **(HUTCHINGS et al., 1996; KUBITZKI, 1998; BRYAN, 2002)**. In South Africa, it is indigenous to the Eastern Cape, southern KwaZulu-Natal and the Transvaal. *Tulbaghia* consists of 26 species **(USHER, 1974; KUBEC et al., 2002)**, the majority of which (22 species) are found in South Africa **(KUBITZKI, 1998)**. Some of these species are found in dry or damp rocky grasslands, while some are found in marshy streambanks **(POOLEY, 1998)**.

A prominent characteristic of this genus is the alliaceous odour, which comes from sulphur compounds released from wounded or decaying tissue. Plants in this genus have a height that ranges from 150-600 mm. They are herbaceous, acaulescent, perennial geophytes with compact tuberous rhizomes, rarely corms or bulbs with thick roots **(HUTCHINGS et al., 1996; KUBITZKI, 1998; POOLEY, 1998)**. The rhizome is swollen with an irregular shape and is covered by dry, fibrous leaves. It has a short, closed sheath that is formed at the base. The leaves are basal and strap-shaped. The number of flowers found in plants of this genus ranges from 3 to 40 **(KUBITZKI, 1998)**. The colour differs with every species. The flowers of some species like *T. acutiloba* and *T. ludwigiana* have a sweet scent **(POOLEY, 1998; BRYAN, 2002)**. The flowers are generally small and are borne on an umbel of 6 to 12. They have a corona or a fleshy ring at the mouth of the

tube. In most species, the short branches in the inflorescence or the individual pedicels are occasionally subtended by smaller membranous bracts. The pollen is sulcate and reticulate, with most species pollinated by insects **(KUBITZKI, 1998)**. Seeds are flat and elongate, with each mature seed having an embryo that is almost straight and an endoderm that is helobial. The anther tapetum is secretory with 2-4 nuclear tapetum cells **(KUBITZKI, 1998)**.

1.2 MEDICINAL AND OTHER USES

1.2.1 Medicinal uses

Different plant parts, namely: roots, bulbs, leaves and flowers are used in the treatment of a variety of conditions **(WATT & BREYER-BRANDWIJK, 1962)**. Flower essences produced from *Tulbaghia* are used for healing throughout South Africa, in Canada, Japan and the United States of America **(SPIES, 2004)**.

Tulbaghia acutiloba is cooked by the Sotho of South Africa to make a lotion used to wash incisions **(WATT & BREYER-BRANDWIJK, 1962)**. Bruised roots of *T. alliacea* are used in the Transkei region to prepare a medicated bath to treat rheumatism, fits, and paralysis as well as to reduce body temperature in cases of fever. The root is also taken in small doses as a purgative **(WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS et al., 1996; BRYAN, 2002)**. Infusions of *T. alliacea, T. ludwigiana* and *T. violacea* are used as a love charm medicine **(HUTCHINGS et al., 1996)**. The bulbs of *T. alliacea*, *T. cepacea* and *T. violacea* are used as a remedy for pulmonary tuberculosis as well as an anthelmintic **(WATT & BREYER-BRANDWIJK, 1962; VAN WYK et al., 1997; KUBEC et al., 2002; FENNELL et al., 2004)**.

Roots, bulbs and leaves of *T. violacea* are used in South Africa to make tea **(BATTEN & BOKELMANN, 1966; ROBERTS, 1990)**. The tubers of *T. violacea* are used in the Transkei region by diviners to rub on the body as a protective charm against evil spirits. Plant infusions made from *T. violacea* are used for the treatment of colic and restlessness in young children, while the leaves are rubbed

on the head for the relief of sinus headaches **(HUTCHINGS et al., 1996; BRYAN, 2002).** The bulb of *T. violacea* is used for the relief of pain and fever as well as to treat gastrointestinal ailments, constipation, oesophageal cancer, hypertension and asthma **(WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS et al., 1996; KUBEC et al., 2002)**.

Tulbaghia alliacea, T. acutiloba, T. natalensis, T. leucantha and T. violacea are grown by the Zulu and Xhosa around their homes as a protective charm to repel snakes **(WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS et al., 1996; POOLEY, 1998; KUBEC et al., 2002)**. The Sotho use *T. dieterlenii* in a decoction they drink to rid the body of a "snake" believed to have been introduced by witchcraft **(WATT & BREYER-BRANDWIJK, 1962)**.

Pharmacological screenings were conducted on *Tulbaghia* for anthelmintic, anti-inflammatory, antibacterial, antifungal, antihypertensive, and antioxidant activities **(McGAW et al., 2000; MOTSEI et al., 2003; FENNELL et al., 2004; LINDSEY & VAN STADEN, 2004; VAN DEN HEEVER et al., 2008)**. **McGAW et al. (2000)** and **FENNELL et al. (2004)** reported *T. violacea* to have anthelmintic activity against the nematode *Caenorhabditis elegans.* Anti-inflammatory activity was reported in *T. violacea* by **GAIDAMASHVILI & VAN STADEN (2006)**. *Tulbaghia violacea* was reported to have antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* as well as *Escherichia coli* **(WATT & BREYER-BRANDWIJK, 1962; GAIDAMASHVILI & VAN STADEN, 2002)**. *Tulbaghia violacea* and *T. alliacea* exhibited antifungal activities against *Candida albicans* **(MOTSEI et al., 2003; LINDSEY & VAN STADEN, 2004)**, while *T. violacea* was found to have antifungal activity against *Botrytis cinerea, Pythium ultimum* and *Rhizoctonia solani* **(LINDSEY & VAN STADEN, 2004)**. *Tulbaghia alliacea* extracts were found to be fungicidal against *Saccharomyces cerevisiae.* The antifungal as well as the antibacterial activity demonstrated by *T. alliacea* and *T. violacea* could be due to the presence of allicin, which is a compund that was isolated from *Tulbaghia* and *Allium* **(MOTSEI et al., 2003)**. *Tulbaghia violacea* was reported by **ZSCHOCKE & VAN STADEN (2000)** to contain anti-hypertensive properties, using an angiotensin converting enzyme assay. **ZHENG & WANG (2001)** reported *T.*

violacea to have antioxidant activity. This activity as well as the anti-inflammatory activity could be due to the presence of the flavonoids quercetin and kaempferol **(HUTCHINGS et al., 1996)**.

1.2.2 Horticultural and nutritive uses

WATT & BREYER-BRANDWIJK (1962) reported on the horticultural potential of *T. violacea*. This species is used as a bedding plant in South African gardens, street landscapes and parks due to its attractive foliage as well as its pretty flowers **(KUBITZKI, 1998; BRYAN, 2002)**.

Tulbaghia dieterlenii and *T. leucantha* are mixed together with tobacco in order to "strengthen" the tobacco **(WATT & BREYER-BRANDWIJK, 1962; POOLEY, 1998)**. These authors have also reported *T. leucantha, T. natalensis, T. acutiloba* and *T. violacea* to be used as culinary herbs. *T. ludwigiana* is used as fodder for livestock **(DLAMINI, 2002)** and its leaves and rhizomes are edible **(LONG, 2005)**. In some cultures, the leaves of *T. violacea* are used as a substitute for chives and garlic **(KUBEC, 2002)**, while young plants can be eaten as vegetables **(HUTCHINGS et al., 1996)**.

1.2.3 Bioactive compounds

Unlike the family Amaryllidaceae, Alliaceae is characterized by an absence of alkaloids **(WATT & BREYER-BRANDWIJK, 1962; KUBUTZKI, 1998)**. Steroidal saponins were found in Alliaceae and these were isolated from *T. violacea* **(HUTCHINGS et al., 1996; KUBITZKI, 1998)**, but it is not known whether they are responsible for the plant's biological activity **(McGAW et al., 2000)**.

1.3 CONSERVATION STATUS

Flowering bulbous plants always have been and will continue to be important to humans. This is not only due to their beauty, but they are associated with medicine and are a source of food to many. Due to these reasons, their conservation may

be compromised. Three species of *Tulbaghia* are found on the list of threatened or rare species. *Tulbaghia tenuior* is found in Namibia but there is not enough data to make an informed decision about its status **(GOLDING, 2002)**. *Tulbaghia ludwigiana* is listed under local scale extinctions **(SCOTT-SHAW, 1999)**. According to **SPARG et al. (2005)**, many plant species of medicinal value are severely threatened in the wild and are often difficult to find outside protected areas. This is so for *T. montana*, which is listed under threatened plants and is therefore only found in protected areas. It is considered rare with a narrow distribution and low abundance. As for its urgency for conservation, it is of medium priority **(SCOTT-SHAW, 1999)**.

1.4 PROPAGATION

1.4.1 Conventional propagation

Propagation is a technique used to multiply plants by both sexual and asexual means. The sexual means is propagation by seeds. The asexual (vegetative) means involves a number of methods including cuttings, layering, budding, grafting and micropropagation **(BRYANT, 2003)**.

According to **BRYAN (2002),** *Tulbaghia* can be propagated from seed or vegetatively, by dividing larger clumps. The seeds need to be sowed in containers in a cold frame in a sandy soil mix that is barely covered. This should be done as soon as the seeds ripen. Seedlings can be transplanted when they reach an adequate size and can be planted out in the second year in summer. The advantage of this method is that it is easy for seeds to germinate and the seedlings reach flowering size quickly. In propagation by dividing larger clumps, the ideal season for dividing most species of *Tulbaghia* is spring, while for *T. alliacea* it is late summer. When the clumps have been planted, it is best to leave them undisturbed for as long as possible and first flowering can generally be expected in the second or third year. Moisture is required for the development of the foliage, but at the appearance of flower spikes, the amount of water given to the plant can be reduced. *Tulbaghia* is not prone to pests and diseases, but the

foliage can be damaged by slugs and snails **(JOFFE, 1993; BRICKELL, 1996; BRYAN, 2002)**.

1.4.2 Micropropagation

Micropropagation, also known as *in vitro* propagation, is a technique used to rapidly multiply plants in an artificial medium under sterile conditions, from very small plant parts such as stems, seeds, embryos, shoot tips, pollen grains, root tips as well as single cells and callus. In 1902, Haberlandt advocated the use of micropropagation in research laboratories throughout the world, when he first attempted to isolate and grow plant tissue in aseptic culture **(HARTMANN & KESTER, 1975)**. There are advantages and disadvantages to using micropropagation **(GEORGE & DEBERGH, 2008)**.

Advantages

- Very small pieces of plants are required to initiate cultures, from which small shoots or embryos are propagated. These plants may be maintained or multiplied in a small amount of space.
- There is reduced or no loss of plants through diseases as these plants are grown under sterile conditions.
- Environmental factors such as light and temperature as well as other factors like nutrient and plant growth regulator levels can be manipulated to suit plant growth. The manipulation of such factors ensures independence of plants from seasonal changes, which means that plant production can continue all year round.
- Clones of plants that are difficult or slow to propagate vegetatively are produced.
- New cultivars with more desirable characteristics are produced.
- Plant material produced vegetatively can in most instances be stored over long periods.

- Less labour is required for this technique, in that, between subcultures plant material requires little attention. Labour and materials required in conventional propagation for watering, weeding or spraying are not required in micropropagation **(GEORGE & DEBERGH, 2008)**.

Disadvantages

- Micropropagation may be expensive as it requires advanced skills in a specialized production facility.
- The plantlets produced may initially be small and in some instances may have undesirable characteristics.
- Plants produced *in vitro* are not autotrophic, because they are supplemented with a source of carbon, mostly sucrose. They may take a while before they are capable of independent growth.
- Upon being transferred to the external environment, the plantlets may be susceptible to water loss. They may therefore need to be subjected to conditions where there is a slow decrease in humidity and an increase in light. This may increase the chances of a production of genetically abnormal plants **(GEORGE & DEBERGH, 2008)**.

There are more benefits in using micropropagation than there are in using conventional propagation.

1.4.2.1 Stages of micropropagation

Three stages of micropropagation were suggested by **MURASHIGE (1974),** which are stages I-III. A fourth stage was later proposed by **DEBERGH & MAENE (1981),** which is now stage 0. Another stage was added so that currently, five stages for successful micropropagation are recognized **(KANE et al., 2008)**.

Stage 0: Selection and preparation of the mother plant

According to **GEORGE & DEBERGH, (2008)** and **KANE et al. (2008)** it is of the utmost importance that before micropropagation begins, careful attention is given to the selection as well as the maintenance of stock plants to be used as a source of explants. **HARTMANN et al. (1997)** mention that the type of explant, where and how it is collected varies with the purpose of the culture, the species and sometimes the cultivar. The selected stock plants should be kept under optimal cultivation conditions in order to reduce the risk of diseases, but allowing active growth **(GEORGE & DEBERGH, 2008; KANE et al., 2008)**.

Stage I: Initiation of aseptic cultures

Pathogens can sometimes inhibit growth and rooting **(HARTMANN et al., 1997)**. This is why the goal of this stage is to establish and maintain plant cultures that are free of contamination. This is done by surface sterilization of the plants using chemicals that are toxic to microorganisms and non-toxic to plant material. Such disinfectants include alcohol (methanol, ethanol or isopropanol) and bleach (calcium hypochlorite and sodium hypochlorite). A few drops of a surfactant or detergent (Tween 20) are usually added to the bleach to improve surface coverage.

Stage II: Multiplication of propagules

This stage is concerned with the production of new plant outgrowths which will eventually give rise to complete plants. Cultures from this stage are usually subdivided and subcultured for further multiplication. Multiplication is carried out until the desired quantity of plant material is achieved, which will be used in subsequent stages **(HARTMANN et al., 1997)**.

Stage III: Rooting

The rooting of shoots is an important stage as this prepares the shoots for successful transfer to the soil. This is done by transferring the shoots to rooting media, which in most cases has reduced or no cytokinin with or without auxin.

Rooting may be done *in vitro* or *ex vitro* **(HARTMANN et al., 1997)**. Some shoots, however, do not require a rooting stage as they develop roots spontaneously in stage II. In some instances, elongation of shoots may be required prior to rooting **(GEORGE & DEBERGH, 2008; KANE et al., 2008)**. Rooting is not the only way shoots are prepared for successful transfer to the soil. They can be conditioned in order to increase the rate of success during transfer, by for example, increasing the light intensity that they are subjected to **(HARTMANN et al., 1997)**.

Stage IV: Transfer to the natural environment

The final stage of micropropagation involves the transfer of plants from an *in vitro to* an *ex vitro* environment. This stage can be carried out once the plantlets are well rooted. Upon removal of plantlets from culture vessels, the agar needs to be completely washed from the plants to remove potential sources of new infection **(HARTMANN et al., 1997)**. The way in which the transfer is carried out determines the success or failure of this stage. Plants need to be acclimatized to conditions of slowly decreasing humidity to prevent dehydration **(GEORGE & DEBERGH, 2008; KANE et al., 2008)**, which is why the mist house is a good option. Even though these acclimatization procedures are followed carefully, poor survival rates are often encountered. This is so because the transition of *in vitro* grown plants from a heterotrophic to a photoautotrophic state is not immediate **(KANE et al., 2008)**. *In vitro* grown plants exhibit a variety of anatomical as well as physiological features which contribute to their limited ability to regulate water loss upon transfer to the natural environment. Such features include abnormal stomata functioning, reduction in leaf epicuticular wax, poorly differentiated mesophyll as well as poor vascular connections between shoot and roots. Pesticide application may be required in this stage **(KANE et al., 2008)**.

1.4.2.2 Media constituents

In vitro grown plants obtain nutrients for growth from prepared artificial media containing nutrients. The nature of the culture medium used to grow plants *in vitro* greatly influences the success of plant tissue culture **(GEORGE & DE KLERK, 2008)**. It is mentioned by **HARTMANN & KESTER (1975)**, that the kind of plant

and the purpose for producing the culture determines the nutrients required in the culture medium. Some of the commonly used tissue culture media include the B5 medium **(GAMBORG et al., 1968)**, NN medium **(NITSCH & NITSCH, 1969)**, SH medium **(SCHENK & HILDERBRANDT, 1972)**, White medium **(WHITE, 1963)**, WPM **(LLOYD & MCCOWN, 1980)** as well as the MS medium **(MURASHIGE & SKOOG, 1962) (Table 1.1)**. The latter, according to **BEYL (2008)**, is the most suitable and most commonly used for the regeneration of plants from tissues and callus.

Table 1.1: The constituents of a modified **MURASHIGE & SKOOG (1962)** basal medium.

Components	**Salts**	**Stock solution mass (g/litre)**	**Volume stock (ml/L) final medium**
Macronutrients	NH_4NO_3	165	10
	KNO_3	95	20
	$CaCl_2.2H_2O$	44	10
	$MgSO_4.7H_2O$	37	10
	NaFe EDTA	4	10
	KH_2PO_4	17	10
Micronutrients	H_3BO_3	0.62	
	$MnSO_4.4H_2O$	2.23	
	$ZnSO_4.7H_2O$	0.86	10
	KI	0.083	
	$Na_2MoO_4.2H_2O$	0.025	
	$CuSO_4.5H_2O$	0.0025	10
	$CoCl_2.6H_2O$	0.0025	

Vitamins	Thiamin HCl	0.01	10
	Nicotinic acid	0.05	
	Pyridoxin HCl	0.05	
	Glycine	0.2	

Optional: Sucrose 30 g/L; Agar 8 g/L; Myo-inositol 0.1 g/L

Macronutrients are inorganic medium constituents required in large quantities by plants, while micronutrients are inorganic medium constituents required in small quantities by plants.

Vitamins are organic constituents that form part of enzymes or cofactors essential for metabolic functions. Myo-inositol, once characterized as one of the B complex vitamin group, is a sugar alcohol. It was discovered to be essential in the micropropagation of all monocotyledons **(KAUL & SABHARWAL, 1975)**, some dicotyledons and gymnosperms **(BEYL, 2008)**.

Sugar is a very important organic constituent for *in vitro* growth and development of a culture. It is not only a frequently used carbon or energy source, but an osmotic agent as well. Sucrose has constantly been found to be the best carbohydrate. This is supported by **GAUTHERET (1945)** as cited by **THORPE et al. (2008)**, who found sucrose to be the best source of carbon followed by glucose. Glucose, being more expensive than sucrose is preferred in cases where it supports growth better than sucrose.

Plant growth regulators (PGRs) are chemicals, which may naturally occur in a plant (endogenous) or may be applied (exogenous), that have a regulatory role in the growth as well as the development of a plant. Plant growth regulators are used in very low concentrations in the media, but exert dramatic effects, depending on the concentration used, their activity and the target tissue. Apart from stimulating cell division and expansion, they are responsible for the regulation of the initiation as well as the growth and development of shoots and roots on explants and embryos whether on semi-solid or in liquid media. Until recently, only five groups of plant growth regulators were recognized and they are auxins, cytokinins, gibberellins, ethylene and abscisic acid. In micropropagation, auxins and

cytokinins are by far the most important for regulating growth and morphogenesis **(BEYL, 2008)**, while gibberellins have been used in some instances to promote shoot elongation **(HARTMANN et al., 1997)**.

According to **BAJGUZ & PIOTROWSKA (2009)**, auxins were the first plant growth regulators discovered. They are very widely used in plant tissue culture and usually form an inherent part of the nutrient media **(MACHAKOVA et al., 2008)**. Auxins are associated with many developmental processes which include cell elongation, swelling of tissue, apical dominance, somatic embryogenesis and adventitious root formation. In general, root initiation is favoured when there is a low auxin concentration and callus is formed when there is a high auxin concentration **(Fig. 1.1)**. Auxins are generally stable in culture with the exception of IAA, which accounts for its low effectiveness compared to synthetic auxins such as 2.4-D or NAA. **BEYL (2008)** reported IAA to be the weakest auxin. Indoleacetic acid (IAA) and indolebutyric acid (IBA) are the naturally occurring auxins used, while 1-naphthaleneacetic acid (NAA), 2.4-dichlorophenoxyacetic acid (2.4-D) and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) are synthetic auxins.

Cytokinins promote cell division as well as stimulate the initiation and growth of shoots *in vitro*. In higher concentrations, cytokinins promote axillary shoot formation by opposing apical dominance regulated by auxin. They inhibit root formation and induce adventitious shoot formation. Benzyladenine (BA), zeatin, kinetin, thidiazuron (TDZ) and isopentenyladenine (iP) are the most commonly used cytokinins. Zeatin and iP are the naturally-occuring cytokinins **(BEYL, 2008; VAN STADEN et al., 2008)**. Despite BA being recently identified as a naturally-occuring cytokinin, it is still viewed as a synthetic cytokinin **(VAN STADEN & CROUCH, 1996)**, while kinetin and TDZ are synthetic. Benzyladenine is a stronger cytokinin compared to zeatin, while TDZ is stronger than BA **(BEYL, 2008; VAN STADEN et al., 2008)**. Benzyladenine has been extensively used in experiments and **STRNAD (1997)** speculates that this could be because it is the most active and cheapest cytokinin. It has, however, been reported to cause hyperhydricity **(LESHEM et al., 1988)** in many species. The topolins, as discovered by **STRNAD et al. (1997)**, are derivatives of BA and are aromatic and naturally occurring

cytokinins. These plant growth regulators are expensive, hence their limited use **(VAN STADEN et al., 2008)**. Topolins are being explored as an alternative to BA in shoot multiplication for bulbous species. This is so because they have been reported to result in less hyperhydricity than BA. In a study conducted by **BAIRU et al, (2007)** to assess the effect of topolins on hypehydricity, it was observed that hyperhydricity was most severe with BA treatments than with topolin treatments.

According to **BEYL (2008)**, the effects of auxin and cytokinin ratio on the morphogenesis of cultured tissues were demonstrated by **SKOOG & MILLER (1957)**, and that has served as the basis for the manipulation of plant tissue culture until today. The auxin-cytokinin interaction can best be explained by the following diagram:

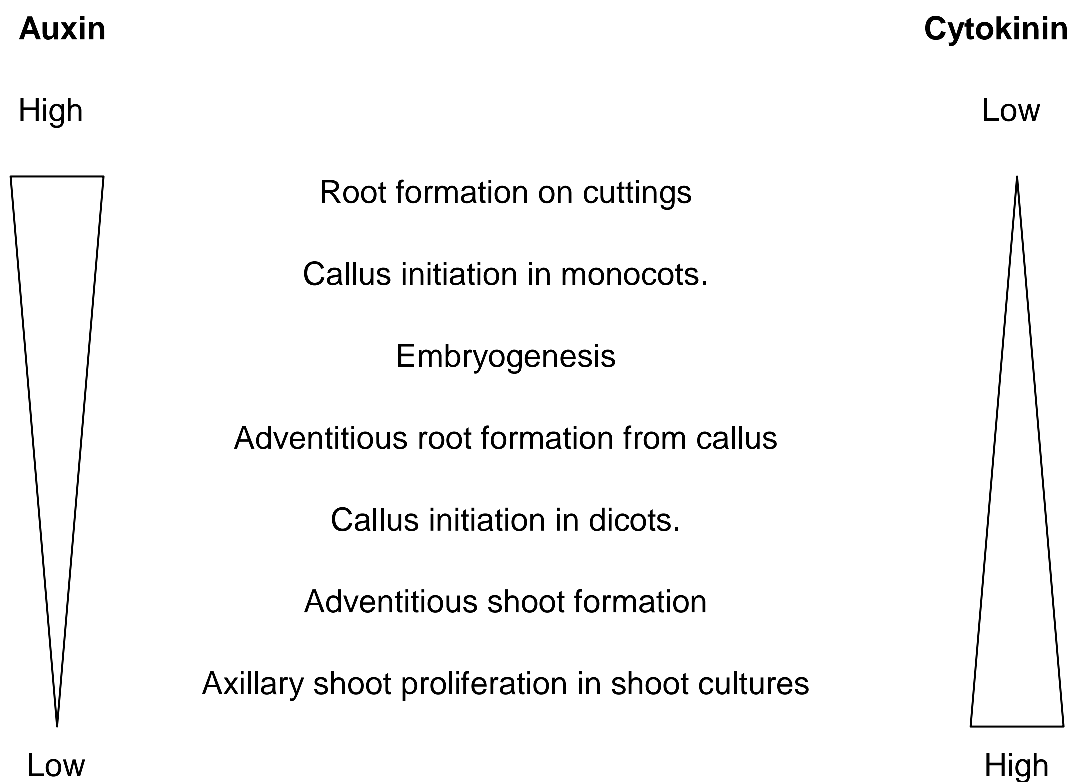

Figure 1.1: The effects of auxin and cytokinin ratio on the morphogenesis of *in vitro* cultures **(VAN STADEN et al., 2008)**.

The auxin-cytokinin interaction as shown in **Figure 1.1** does not always hold true, especially for monocotyledons. For instance, auxin alone at high levels may be required by monocotyledons to induce callus, without the presence of a cytokinin. The formation of adventitious shoots as well as root meristems depends largely on the balance between auxin and cytokinin. The type and concentration of cytokinin required varies according to the plant being cultured **(VAN STADEN et al., 2008)**.

Solidifying agents are necessary in the case of semi-solid media, to ensure that the explants are placed in precise contact with the medium and remain aerated. The most common solidifying agents are agar and gellan gums like Gelrite and Phytagel **(BEYL, 2008; THORPE et al., 2008)**. The growth of cultures can be influenced by agar. If it is too soft, hyperhydricity may result and if it is too hard, plant growth is reduced. If it is of poor quality it interferes or inhibits the growth of cultures **(BEYL, 2008)**.

1.4.2.3 The effect of environmental factors on micropropagation

Many factors, both internal and external, play a vital role in the micropropagation of plants. Temperature, humidity and light: (light intensity, light quality and photoperiod) are important factors in the environment of plants as they have an effect on plant response **(WENT, 1953)**.

Plants growing in a natural environment usually experience temperatures which fluctuate widely especially between night and day. In order to speed growth and morphogenesis of *in vitro* grown plants, cultures are usually maintained at mean temperatures, higher than those experienced by the same plants growing in their natural environment. The optimum temperature at which cultures are grown varies with different species, but the mean growth temperature was found to be 25 °C, with tropical and subtropical species having a tendency to be cultured at temperatures slightly higher than temperate species **(GEORGE & DAVIES, 2008)**.

Humidity, higher than 95% in culture vessels **(AFREEN, 2005)**, is an important environmental factor in micropropagation as it affects water relations of cultured plantlets. High humidity in tissue culture is associated with poor development of

the morphological structure of leaves as well as high mortality of plantlets upon transfer to the *ex vitro* environment **(KOZAI et al., 1993)**.

The growth and development of plants depend on light for photosynthesis, photomorphogenesis and phototropism. Species differ in their light requirements, but generally the light intensity for micropropagation is between 40 and 80 μmol m^{-2} sec^{-1}, which is extremely low compared to 600-1 200 μmol m^{-2} sec^{-1} found outdoors **(HARTMANN et al., 1997)**. In most cases, fluorescent tubes, which are the most cost-effective among the types of light sources, are used as the light source **(KOZAI & KUBOTA, 2005)**. It was noted by **HARTMANN et al. (1997)** that the impact of photoperiod on culture development has been confined to only a few systematical studies, but generally, longer day lengths (12 to 16 hours) are most often used.

1.4.3 Micropropagation of the Alliaceae

The Alliaceae is among eight monocotyledonous families outlined by **FENNELL & VAN STADEN (2004)** in their review of southern African bulbs which have been propagated through tissue culture. **ASCOUGH et al. (2008)** have also included the Alliaceae in their summary of successful *in vitro* induction of storage organs of ornamental geophytes. Some work has been done on the micropropagation of Alliaceae species, with the genus *Allium* receiving most of the attention since it is the largest genus in the family with many species used for food, medicinal purposes as well as ornamentals **(KUBITZKI, 1998)**. A limited amount of work has been done on the genus *Tulbaghia* **(Table 1.2)**.

Table 1.2: Micropropagation of some species of the Alliaceae

Plant name	Explant	Growth response	Reference
Allium aflatunense	Inflorescence sections, basal plates	Plantlets, bulblets	**EVENOR et al. (1997)**

Plant name	Explant	Growth response	Reference
Allium ampeloprasum	Shoot-tips, young florets	Plantlets, bulblets	**EVENOR et al. (1997)**
	Shoot-tips	Plantlets	**GANTAIT et al. (2010)**
Allium cepa	Shoots, basal plates	Multiple shoots	**HUSSEY (1978)**
Allium cepa	Roots	Callus	**KRIKORIAN & KATZ (1968)**
	Roots	Callus, roots	**DAVEY et al. (1974)**
	Basal discs	Callus, roots	**FREEMAN et al. (1974)**
	Basal plates	Callus	**DUNSTAN & SHORT (1977a)**
	Basal discs, Seedling radical, flower heads	Callus, shoots, roots	**DUNSTAN & SHORT (1977b)**
	Flower heads	Multiple shoots	**DUNSTAN & SHORT (1979a)**
	Twin scales	Multiple shoots	**HUSSEY & FALAVIGNA (1980)**
	Basal plates	Multiple shoots	**KAHANE et al. (1992)**
	Shoot-tips	Multiple shoots, bulbs	**MOHAMED-YASSEEN et al. (1994)**
	Inflorescence sections	Shoots, bulbs	**MOHAMED-YASSEEN et al. (1993)**
	Bulbs	Multiple shoots	**KAMSTAITYTE & STANYS (2004)**
	Aerial bulbs	Callus, plantlets	**FRIDBORG (1971)**
	Bulb scales	Callus, roots	**NANDI et al. (1977)**

Plant name	Explant	Growth response	Reference
Allium giganteum	Young leaves	Callus, shoots	**INAGAKI et al. (1992)**
	Ovaries	Multiple shoots	**SUSEK et al. (2002)**
Allium porrum	Basal plates	Multiple shoots	**DUNSTAN & SHORT (1979b)**
	Flower heads	Shoots	**NOVAK & HAVEL (1981)**
Allium sativum	Stem tips, stem segments, bulb leaf discs	Callus, plantlets	**ABO EL–NIL (1977)**
	Shoot buds	Multiple shoots	**BHOJWANI (1980)**
	Bulblet sections	Plantlets	**MASUDA et al. (1994)**
Allium sativum	Shoot-tips	Shoots, bulbs	**MOHAMED-YASSEEN et al. (1994)**
	Root tips	Callus	**HAQUE et al. (1998)**
	Bulbils	Plantlets	**LUBRACO et al. (2000)**
	Leaf bases	Callus, plantlets	**CHOWDHURY et al. (2003)**
	Shoot-tips, basal plates	Multiple shoots,	**BEKHEET (2006)**
Allium tuberosum	Shoots	Plantlets	**PANDEY et al. (1992)**
Allium victorialis	Shoot-tips, leaf discs, bulbs	Multiple shoots, callus	**LIM et al. (1998)**

Plant name	Explant	Growth response	Reference
Allium wallichii	Shoots	Plantlets	**WAWROSCH et al. (2001)**
Ipheion uniflorum	Bulb sections	Plantlets , callus	**HUSSEY (1975)**
Leucocoryne coquimbensis	Bulbs, seeds	Bulblets	**OLATE & BRIDGEN (2005)**
Tulbaghia simmleri	Leaves, peduncles, bulb scales, twin-scales	Shoots, callus	**ZSCHOCKE & VAN STADEN (2000)**
Tulbaghia violacea	Flowers, shoots	Multiple shoots	**PHELAN et al. (2007)**

1.5 AIMS

Tulbaghia is a genus of valuable plants which multiply slowly **(PHELAN et al., 2007)**. The production of large quantities of high quality plants, in less time than is the case with conventional propagation, is required for these plants as they are of medicinal, ornamental and nutritional value. It was, therefore, the aim of this study to develop an efficient protocol for the micropropagation of *T. violacea* and *T. ludwigiana* as well as to determine the viability of micropropagated plants in the production of medicinal compounds. The specific objectives were to determine the most suitable explants and plant growth regulators for the growth and development of these plants as well as to evaluate their antimicrobial and phytochemical properties.

CHAPTER 2
In vitro seed germination and seedling growth

2.1 INTRODUCTION

The selection of an explant for culture is an important stage, as it determines the failure or success of micropropagation **(GEORGE, 2008; KANE et al., 2008)**. Seeds are by far the most convenient sources of explants, with regards to ease of decontamination, because their use ensures a decrease in the risk of contamination. This is so because seeds are more tolerant to surface sterilization protocols and thus easier to establish as sterile cultures **(KITTO, 2008)**. Additionally, once a decontamination protocol has been established, contamination of the plant material arising from the seeds will be eliminated or greatly reduced **(GEORGE & DEBERGH, 2008)**.

Seed germination, as defined by **LEVINE & MILLER (1994)**, is the reactivation in the growth of a plant embryo, resulting in a plant sprouting from the seed. This process is affected by a number of environmental factors, such as temperature, light, water and oxygen **(MAYER & POLJAKOFF-MAYBER, 1982; LEVINE & MILLER, 1994)**. These factors are interactive in controlling seed germination **(BASKIN & BASKIN, 1998)**. The extent to which they are required is species specific **(MAYER & POLJAKOFF-MAYBER, 1982)**.

Germination occurs at different temperature ranges for different seeds, with alternating temperatures usually being more favourable than constant ones. Some species germinate only at alternating temperature regimes, which is similar to the alternating temperatures in their natural habitat. Very low and very high temperatures prevent germination of seeds **(BASKIN & BASKIN, 1998)**. Seeds from temperate regions usually germinate at low temperatures such as 2 or 3 °C, while those from regions with a warmer climate require temperatures from 20 °C and above **(LEVINE & MILLER, 1994)**. The optimal temperature is found in the

range of temperatures within which a particular seed germinates. This is the temperature at which the highest germination occurs in the shortest time.

It has long been recognised that light is an important factor in seed germination. It has been observed that plants growing in the wild, demonstrate variability in their behaviour towards light, with regards to seed germination. Seeds are generally divided into those which germinate only in continuous light; only in continuous dark; after a brief exposure to illumination and those which are indifferent to the presence or the absence of light during germination **(MAYER & POLJAKOFF-MAYBER, 1982)**. It was noted by **BASKIN & BASKIN (1998)** that cool white fluorescent tubes are a better light source for germination than incandescent light bulbs. The authors further explain that this is so because incandescent light bulbs emit a large amount of far-red light and heat whereas cool white fluorescent tubes emit considerable red light and very little far-red light. The absorption of far-red light converts Pfr, the active form of phytochrome, which is responsible for the promotion of germination, to the inactive Pr, which inhibits germination. The absorption of red-light has the opposite effect.

During the initial stage of germination, which is imbibition, seeds absorb a large amount of water. This water uptake causes the seed to swell and the seed coat to burst **(LEVINE & MILLER, 1994)**. Placing seeds in conditions where the amount of water is too low or too high may inhibit germination **(BASKIN & BASKIN, 1998)**. Oxygen is the main gas that affects seed germination **(BLACK et al., 2006)**. Most seeds germinate in an atmosphere where there is 20% oxygen and 0.03% carbon dioxide **(MAYER & POLJAKOFF-MAYBER, 1982)**. The growth of seedlings depends mainly on temperature, light, water and nutrient availability **(KULKARNI et al., 2007)**. It was mentioned in the previous Chapter that one of the challenges faced by *in vitro* grown seedlings is dysfunctional stomata, that is, their inability to close **(BLANKE & BELCHER, 1989)**. This leads to excessive water loss resulting in dehydration upon transfer of plants to the external environment. **KULKARNI et al. (2005b)** studied the effect of temperature on *in vivo* seed germination and seedling growth of *T. violacea*. The aim of the current study was to investigate the

optimal temperature and light conditions for the *in vitro* seed germination and seedling growth of *T. ludwigiana* and *T. violacea*.

2.2 MATERIALS AND METHODS

2.2.1 Seed collection and viability testing

Seeds of *T. ludwigiana* and *T. violacea* **(Fig. 2.1)** were purchased from Silverhill Seeds Nursery (Cape Town, South Africa) and African Bulbs (Napier, South Africa) respectively. They were tested for viability using a 1% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) prior to being used for experiments. This was done by cutting the seeds into two, placing them in pill vials containing the TTC solution. The pill vials were covered in foil and kept at room temperature for 48 h. Seeds were considered viable upon detection of a pink colour in the embryo.

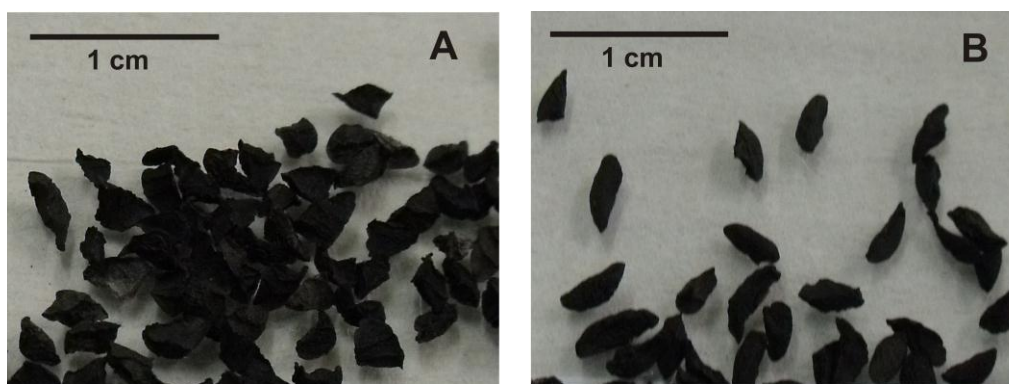

Figure 2.1: Seeds of *T. ludwigiana* **(A)** and *T. violacea* **(B)**.

2.2.2 Seed decontamination and germination

Seeds were surface decontaminated using 70% ethanol for 60 s, followed by a 1% solution of Benlate® for 10 min and 3.5% sodium hypochlorite (NaOCl) containing a few drops of Tween 20 for 15 min. The decontaminated seeds were rinsed three times with sterile distilled water to ensure that no traces of the sterilants remained on them. Seeds were germinated in culture tubes containing 10 ml of one-tenth

strength Murashige and Skoog (MS) medium **(MURASHIGE & SKOOG, 1962)**, without plant growth regulators and sucrose. The pH of the medium was adjusted to 5.8 with 1 N KOH or 1 N HCl. The medium was solidified with the addition of 8 g l^{-1} of agar (Bacteriological-Agar No.1, Oxoid Ltd., England), after which it was sterilized by autoclaving at 121 ^{o}C for 20 min. Strips of Parafilm® were used to seal the culture tubes. Germination in all experiments was considered to have occurred when the radicle protruded 2 mm. Germination counts were made daily for 30 days for all experiments. Upon termination of the germination experiments, four replicates of 10 randomly chosen seedlings were used to generate data. The leaf number, shoot length, root length and fresh weight of seedlings were recorded in order to determine the effect of temperature, photoperiod and light intensity on seedling growth.

2.2.3 Effect of temperature on germination and seedling growth

The effect of temperature on germination was determined by setting up an experiment where four replicates of 15 seeds were placed in growth rooms with constant temperatures (10, 15, 20, 25, 30, 35 ^{o}C) and an alternating temperature (30/15 ^{o}C). The photoperiod was kept constant at 16 h light and 8 h dark (16/8 h). The optimal temperature (T_o) for germination was determined for constant temperatures using the formula:

$T_o = \Sigma t_p / \Sigma p$,

where p is the percentage germination at temperature t_p **(KULKARNI et al., 2005b)**. The mean germination time (MGT) was calculated using the formula:

MGT = Σ (n x d)/N, where n is the number of seeds germinated each day, d is the number of days from the beginning of the experiment and N is the total number of seeds germinated at the termination of the experiment **(ELLIS & ROBERTS, 1981)**.

2.2.4 Effect of photoperiod on germination and seedling growth

The effect of photoperiod on germination was determined by setting up an experiment where four replicates of 15 seeds were placed in growth rooms with

different photoperiod regimes: continuous light (24 h), continuous dark and alternating light (16/8 h). The temperature was kept constant at 25 ºC.

2.2.5 Effect of light intensity on germination and seedling growth

The effect of light intensity on germination was determined by setting up an experiment where four replicates of 15 seeds were placed in growth rooms with different light intensity regimes: 40, 80 and 120 µmol m^{-2} s^{-1}. Cool white fluorescent tubes were used (Osram L 40W/205). The light intensity was measured with a quantum radiation sensor (Model Skp 215, Skye Instruments Ltd, UK). The temperature and photoperiod were kept constant at 25 ºC and 16/8 h respectively.

2.2.6 Effect of light on stomatal density of *T. violacea*

Leaves of *T. violacea* harvested from seedlings obtained from previous experiments **(2.2.4** and **2.2.5)** were used to determine the effect of photoperiod and light intensity on stomatal density. A leaf obtained from an acclimatized plant growing in a greenhouse was used as the control. One disc was cut from three different, randomly chosen leaves from each treatment. The discs were mounted on aluminium stubs with the adaxial side up and viewed under a scanning electron microscope (ZEISS EVO® MA 15 SEM, Carl Zeiss SMT, Germany), fitted with a digital imaging system driven by SmartSEM software. The microscope was set in the variable pressure mode where the chamber pressure ranged from 300-400 Torr, operating at 15 kV with a working distance (WD) of between 6.5-7.5 mm. In order to maintain the structural integrity of the cells, live material was used. Micrographs were taken at a magnification of 600x for all images and the number of mature stomata was counted. Each of these values was treated as n=1. A total of three replicates per treatment was used for statistical analysis. Leaf disc areas (454 µm x 306 µm) of 1.38924×10^5 μm^2 were used and the number of stomata was expressed per mm^2. The estimation of stomatal density was done using methods described by **MIGUENS et al. (1993)** and **SNIDER et al. (2009)** with modifications.

2.3 DATA ANALYSIS

The germination data were arcsine transformed before being subjected to one-way analysis of variance (ANOVA). Means were separated by Duncan's Multiple Range Test (DMRT) using SPSS statistical package (version 10.0) at the 5% level of significance. Graphs were drawn using SigmaPlot software (version 8.0).

2.4 RESULTS AND DISCUSSION

2.4.1 Effect of temperature on germination and seedling growth

No germination occurred at 10 °C for *T. ludwigiana* **(Fig. 2.2)**. A similar result was obtained by **KULKARNI et al. (2005a)**, where *in vivo* seed germination was inhibited for *T. alliacea* at 10 °C. This result agrees with what **BASKIN & BASKIN (1998)** indicated, that very low and very high temperatures prevent germination. The non-occurrence of germination at low temperatures, could be due to the retardation of metabolic activities which prevents germination **(DELACHIAVE & DE PINHO, 2003)**. The germination percentage was highest at 20 °C, after which it decreased with an increase in temperature until it was low (10%) at 35 °C for both species. There was a significant difference in the germination percentage at 35 °C and that of other temperatures in both species. The low germination percentage reported at high temperature could be a result of proteins being denatured and membrane permeability being altered at these temperatures **(DELACHIAVE & DE PINHO, 2003)**. There was no significant difference in the germination percentage at 15 to 30 °C in *T. ludwigiana*. In *T. violacea*, there was no significant difference in the germination percentage from temperatures 10 to 25 °C. The germination percentage for the alternating temperature (35/15 °C) in *T. ludwigiana* was lower than at temperatures 15 to 30 °C, but only significantly so when compared with that at 20 °C. In *T. violacea*, the germination percentage at alternating temperature was significantly lower than at constant temperatures 10 to 25 °C.

The shortest MGT (6 days) was recorded at 25 and 30 °C for *T. ludwigiana*, while in *T. violacea* (8 days), it was at 20, 25 and 35/15 °C. **BASKIN & BASKIN (1998)** noted that alternating temperatures are usually more favourable for seed germination than constant ones. Both *T. ludwigiana* and *T. violacea* have proven to be among the species where seed germination is favoured by constant temperatures rather than the tested alternating one. The calculated optimal temperature for the seed germination of *T. ludwigiana* and *T. violacea* was 22.8 and 19.9 °C, respectively. This result not only indicates that seeds of both plants are temperature dependent for germination, but confirms the view of **LEVINE & MILLER (1994)**, that seeds from regions with a warmer climate require higher temperatures to germinate. The slight difference in temperature requirements of these species could be attributed to their geographical distribution.

The seedling growth parameters were highest at 15, 20 and 25 °C for both species **(Table 2.1)**. The greatest seedling fresh weight (50.43 ± 2.13 mg) was obtained at 15 °C for *T. ludwigiana* and was significantly higher than other treatments except at a temperature of 25 °C. For *T. violacea*, the greatest seedling fresh weight (40.30 ± 3.10 mg) was obtained at 25 °C, which was significantly higher than other treatments except for temperatures 15 and 20 °C. A similar result was reported **(KULKARNI et al., 2005b)** for *T. violacea* where the greatest seedling fresh weight was obtained at a temperature of 25 °C for *in vivo* grown seedlings.

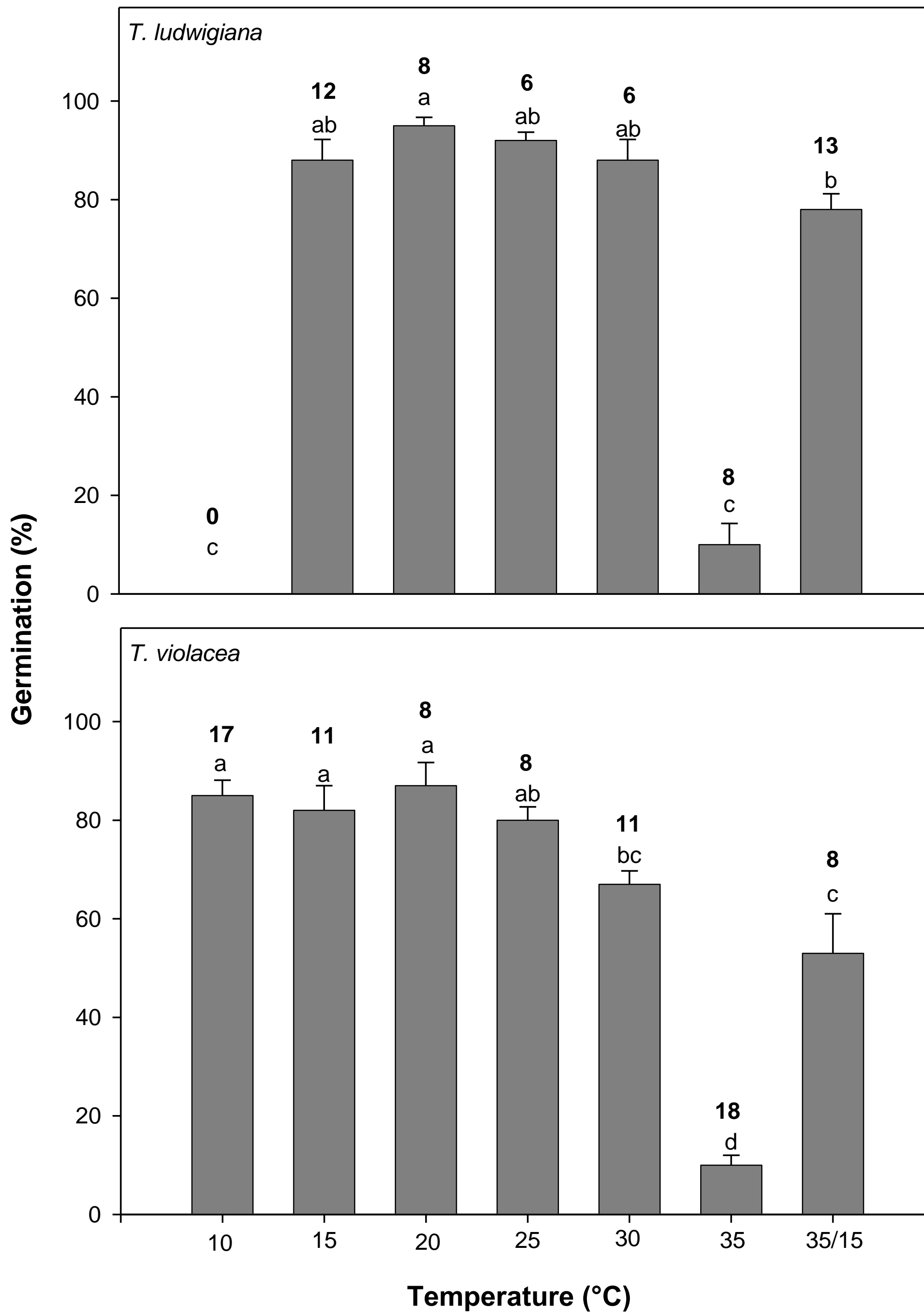

Figure 2.2: Effect of temperature on germination (%) of *T. ludwigiana* and *T. violacea*. Bars with different letters indicate significant differences between treatments (P ≤ 0.05) according to DMRT. The bold numbers above the letters represent MGT in days.

Table 2.1: Effect of temperature on the seedling growth of *T. ludwigiana* and *T. violacea*. Values with different letters in a column indicate significant differences between treatments (P ≤ 0.05) according to DMRT.

Species	Temperature	Mean number of leaves	Mean shoot length (cm)	Mean root length (cm)	Mean fresh weight (mg)
T. ludwigiana					
	10 °C	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00e
	15 °C	2.00 ± 0.00ab	8.94 ± 0.34a	3.27 ± 0.15ab	50.43 ± 2.13a
	20 °C	2.00 ± 0.00ab	8.94 ± 0.34a	3.24 ± 0.14ab	44.08 ± 2.15b
	25 °C	2.03 ± 0.03a	8.60 ± 0.51a	3.47 ± 0.34a	46.08 ± 2.53ab
	30 °C	2.00 ± 0.00ab	5.95 ± 0.29b	3.15 ± 0.14ab	33.15 ± 1.72c
	35 °C	0.00 ± 0.00c	0.00 ± 0.00c	0.02 ± 0.01c	0.10 ± 0.05d
	30/15 °C	1.98 ± 0.03b	6.34 ± 0.34b	2.72 ± 0.17b	38.45 ± 2.39c
T. violacea					
	10 °C	1.00 ± 0.00c	0.84 ± 0.04d	0.66 ± 0.05c	9.48 ± 0.59c
	15 °C	2.53 ± 0.08a	6.07 ± 0.22b	1.52 ± 0.08a	35.58 ± 1.98a
	20 °C	2.05 ± 0.03b	7.60 ± 0.28a	1.72 ± 0.07a	38.40 ± 2.18a
	25 °C	2.58 ± 0.10a	6.13 ± 0.28b	1.60 ± 0.09a	40.30 ± 3.10a
	30 °C	2.03 ± 0.06b	5.59 ± 0.38bc	1.21 ± 0.08b	26.73± 1.42b
	35 °C	0.00 ± 0.00d	0.00 ± 0.00e	0.05 ± 0.03d	0.13 ± 0.05d
	30/15 °C	1.83 ± 0.15b	4.88 ± 0.48c	1.08 ± 0.11b	21.70 ± 2.16b

2.4.2 Effect of photoperiod on germination and seedling growth

The germination percentage was highest under continuous light and lowest under alternating light for both species **(Fig. 2.3)**. In *T. ludwigiana*, there was a significant difference in the germination percentage between continuous and alternating light. The observation by **BASKIN & BASKIN (1998)**, that non-dormant seeds of numerous species germinate equally well under light and dark conditions holds true for *T. violacea* seeing that no significant difference was observed in the germination percentage for all treatments of this species. **KULKARNI et al. (2005a)** obtained a similar result for *in vivo* seed germination of *T. alliacea*. The MGT (6 days) was the same for all treatments of *T. ludwigiana*, while in *T. violacea* the shortest MGT was 6 days in continuous dark and alternating light and longer (7

days) in continuous light. According to the classification of seeds by **MAYER-POLJAKOFF-MAYBER (1982)**, seeds of both species are thus apparently indifferent to the presence or absence of light for germination.

Seedling growth parameters **(Table 2.2)** were highest in continuous light (except for the mean shoot length) in both species. However, there was no significant difference in all treatments of *T. ludwigiana* for the mean root length and the mean number of leaves. In *T. violacea*, there was no significant difference in all the treatments for the mean fresh weight. **TAIZ & ZEIGER (1991)** mentioned that even though seeds may germinate in the dark, dark-grown seedlings will differ in their morphology from light-grown seedlings. Symptoms of etiolation were observed in both species where dark-grown seedlings showed the lowest number of leaves. The highest shoot length was observed in the dark-grown seedlings of *T. ludwigiana*. Chlorosis was observed in dark-grown seedlings in both species.

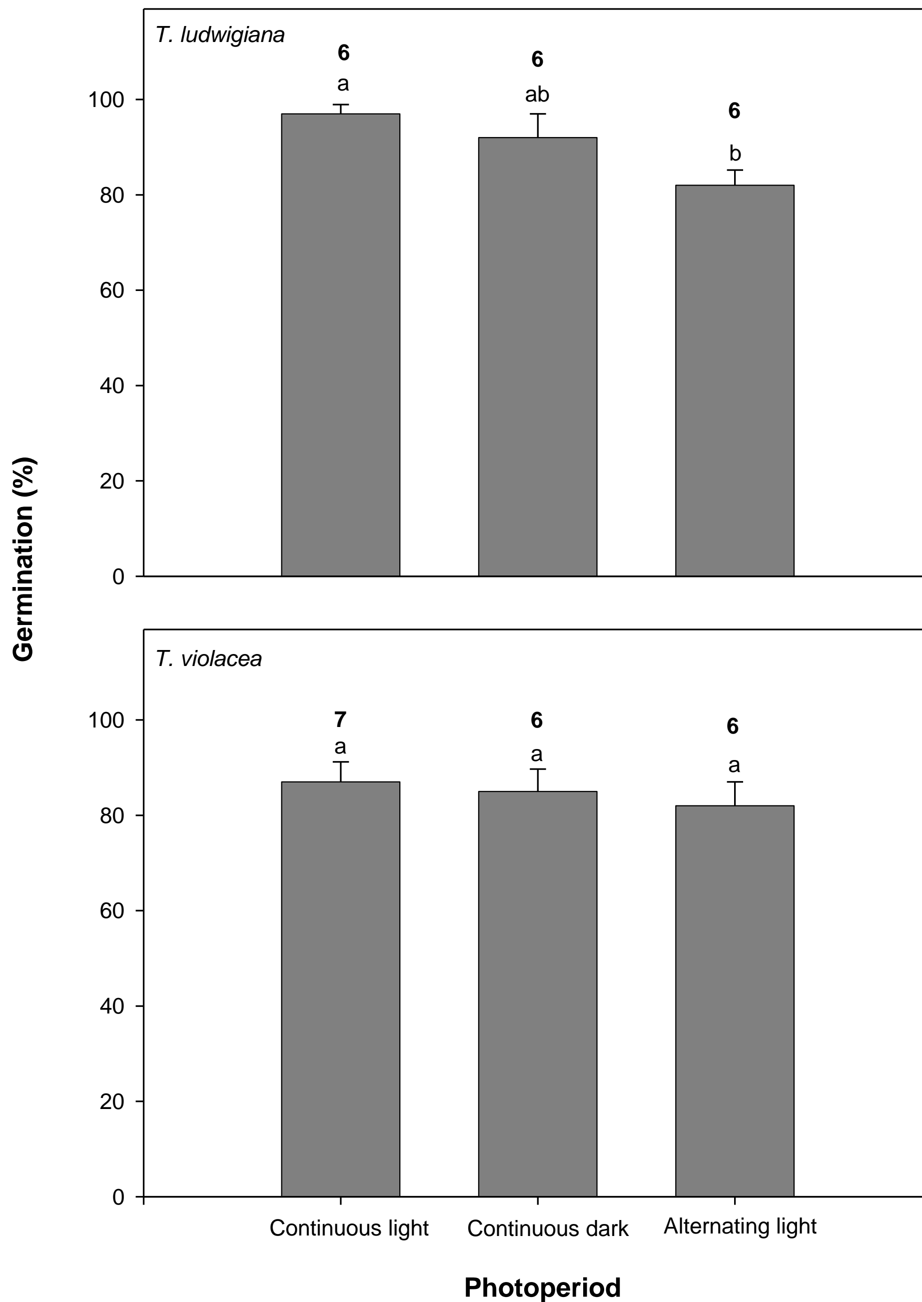

Figure 2.3: Effect of photoperiod on germination (%) of *T. ludwigiana* and *T. violacea*. Bars with different letters indicate significant differences between treatments ($P \leq 0.05$) according to DMRT. The bold numbers above the letters represent MGT in days.

Table 2.2: Effect of photoperiod on the seedling growth of *T. ludwigiana* and *T. violacea*. Values with different letters in a column indicate significant differences between treatments ($P \leq 0.05$) according to DMRT.

Species	Photoperiod	Mean number of leaves	Mean shoot length (cm)	Mean root length (cm)	Mean fresh weight (mg)
T. ludwigiana	**Continuous light**	2.08 ± 0.04a	7.23 ± 0.23b	3.97 ± 0.30a	48.70 ± 2.20a
	Continuous dark	2.00 ± 0.00a	9.06 ± 0.37a	3.63 ± 0.17a	41.98 ± 1.87b
	Alternating light	2.03 ± 0.06a	8.16 ± 0.38ab	3.69 ± 0.21a	38.28 ± 2.00b
T. violacea					
	Continuous light	3.03 ± 0.04a	5.90 ± 0.31b	2.12 ± 0.14a	43.95 ± 3.42a
	Continuous dark	2.00 ± 0.04c	7.21 ± 0.44a	1.73 ± 0.10b	38.30 ± 2.33a
	Alternating light	2.23 ± 0.07b	8.11 ± 0.35a	1.76 ± 0.08b	36.78 ± 2.43a

2.4.3 Effect of light intensity on germination and seedling growth

The germination percentage for *T. ludwigiana* was highest at 40 µmol m^{-2} s^{-1} and lowest at 120 µmol m^{-2} s^{-1} with a significant difference between these treatments **(Fig. 2.4)**. As with *T. ludwigiana*, the germination percentage of *T. violacea* was highest at 40 µmol m^{-2} s^{-1}, but lowest at 80 µmol m^{-2} s^{-1}. Light intensity, like photoperiod, does not have a significant effect on seed germination of *T. violacea* as none of the treatments were significantly different from each other. However, the MGT was shortest at 40 µmol m^{-2} s^{-1} for both species, even though it was slightly shorter for *T. ludwigiana* (5 days) than for *T. violacea* (6 days).

Plantlet growth is enhanced by high light intensity **(IBARAKI & NOZAKI, 2005)**. This is seen in the results presented in **Table 2.3,** where a trend was noticed in both species, where all growth parameters were highest at the highest light intensity (120 µmol m^{-2} s^{-1}), except for the mean shoot length of *T. violacea*, which was highest (6.09 ± 0.33 cm) at the lowest light intensity (40 µmol m^{-2} s^{-1}). In *T.*

violacea, a significant difference was observed in all treatments for the mean number of leaves and for the mean root length.

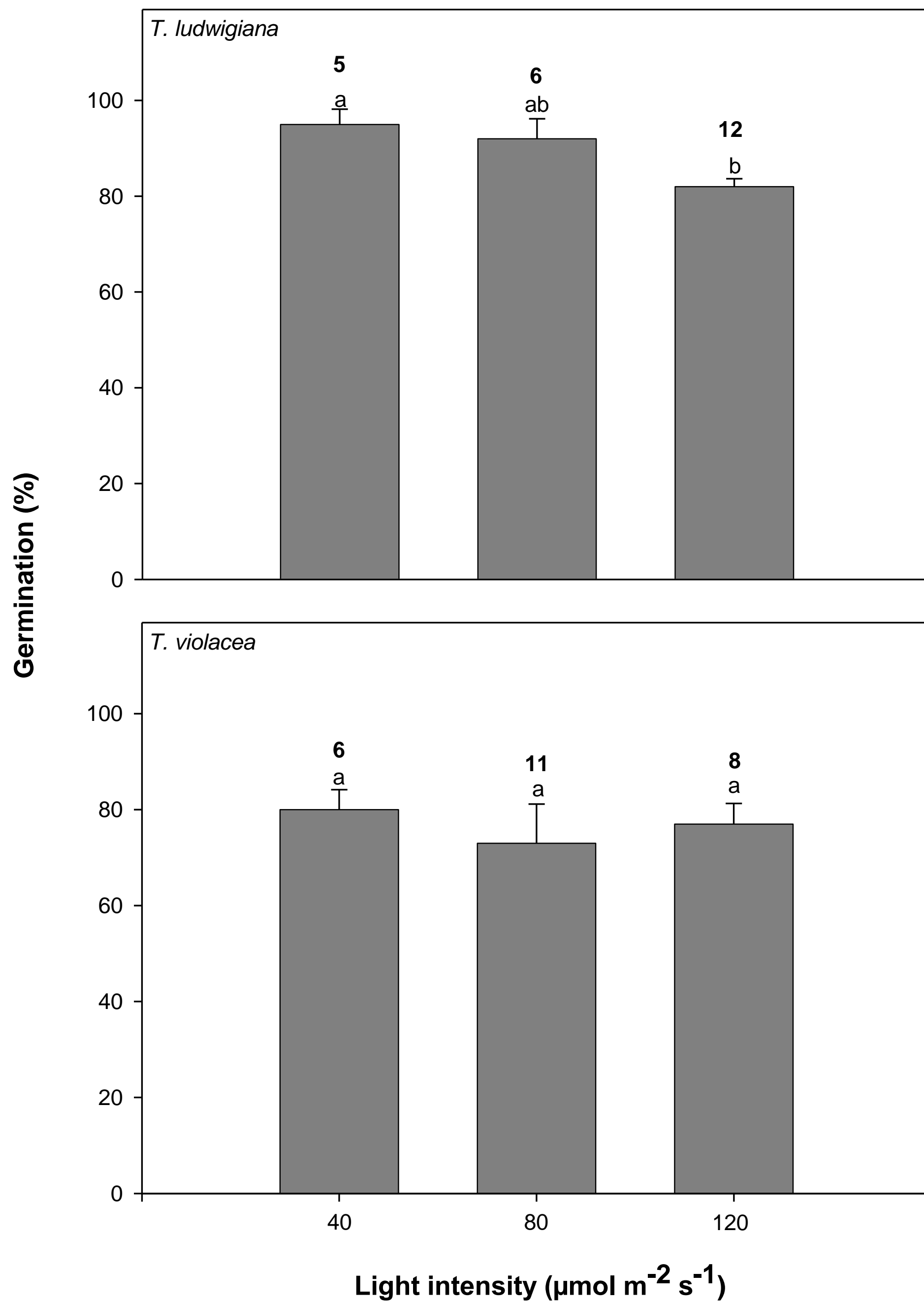

Figure 2.4: Effect of light intensity on germination (%) of *T. ludwigiana* and *T. violacea*. Bars with different letters indicate significant differences between treatments ($P \leq 0.05$) according to DMRT. The bold numbers above the letters represent MGT in days.

Table 2.3: Effect of light intensity on the seedling growth of *T. ludwigiana* and *T. violacea*. Values with different letters in a column indicate significant differences between treatments (P ≤ 0.05) according to DMRT.

Species	Light intensity (μmol m^{-2} s^{-1})	Mean number of leaves	Mean shoot length (cm)	Mean root length (cm)	Mean fresh weight (mg)
T. ludwigiana	**40**	2.03 ± 0.03b	8.00 ± 0.40a	3.72 ± 0.19ab	43.10 ± 2.18b
	80	2.08 ± 0.06b	6.60 ± 0.34b	3.25 ± 0.23b	45.95 ± 1.97b
	120	2.35 ± 0.08a	8.59 ± 0.34a	4.20 ± 0.30a	56.28 ± 2.16a
T. violacea					
	40	2.85 ± 0.09b	6.09 ± 0.33a	1.80 ± 0.08b	41.88 ± 2.46a
	80	2.20 ± 0.13c	4.00 ± 0.36b	1.14 ± 0.14c	27.43 ± 2.19b
	120	3.30 ± 0.12a	4.55 ± 0.21b	2.43 ± 0.23a	48.05 ± 4.11a

2.4.4 Effect of light on stomatal density of *T. violacea*

The highest number of stomata was obtained at 80 μmol m^{-2} s^{-1} (60 ± 15.7 mm^2) and 120 μmol m^{-2} s^{-1} (60.0 ± 6.4 mm^2) **(Table 2.4, Fig. 2.7)**. Continuous light was the photoperiod with the highest number of stomata (40.80 ± 6.4 mm^2) **(Fig. 2.9 A)**. There was, however, no significant difference between all the treatments **(Figs. 2.6 – 2.9)**. Stomata are more numerous on the abaxial surface than the adaxial surface **(DRISCOLL et al., 2006)**. The low number of stomata in the study is due to the use of the adaxial surface for the experiment. There may not have been a significant difference in the stomatal density between the treatments, but differences in the structure of the stomata in different treatments were noticed. The open state of the stomata seemed to increase with an increase in the light intensity. For instance, stomata from the treatment 120 μmol m^{-2} s^{-1} were open the widest of all the treatments, giving them a round shape, with those from 40 μmol m^{-2} s^{-1} closely resembling those of the control **(Figs. 2.5 – 2.8)**. Stomata from

continuous light were elliptical, while the guard cells of stomata from continuous dark **(Fig. 2.9 B)** were not as distinct as those from continuous light **(Fig. 2.9 A)** and alternating light **(Fig. 2.9 C)**. Stomata with a circular or an elliptical shape were considered abnormal by **MIGUENS et al. (1993)**.

Table 2.4: Effects of light intensity and photoperiod on stomatal density of *T. violacea* leaves. Values with different letters in a column indicate significant differences between treatments ($P \leq 0.05$) according to DMRT.

Treatment	**No. of stomata (mm^2)**
Light intensity (µmol m^{-2} s^{-1})	
Control	40.8 ± 11.0a
40	31.2 ± 2.4a
80	60.0 ± 15.7a
120	60.0 ± 6.4a
Photoperiod	
Control	40.8 ± 11.0a
16/8 light	36.0 ± 8.3a
24-h light	40.8 ± 6.4a
Continuous dark	26.4 ± 10.5a

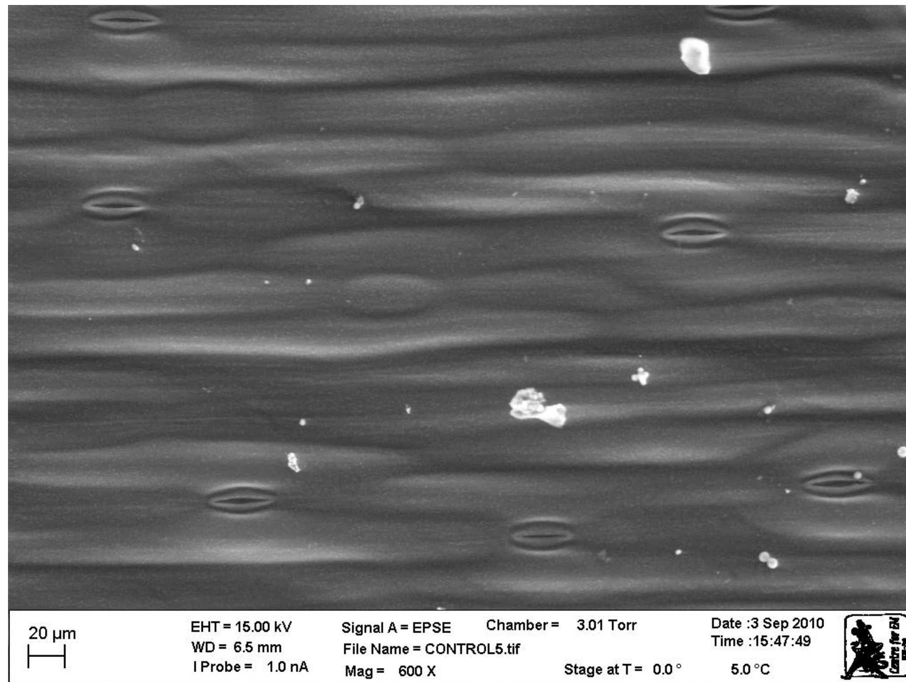

Figure 2.5: Structure of the adaxial side of the control leaf of *T. violacea* showing stomata.

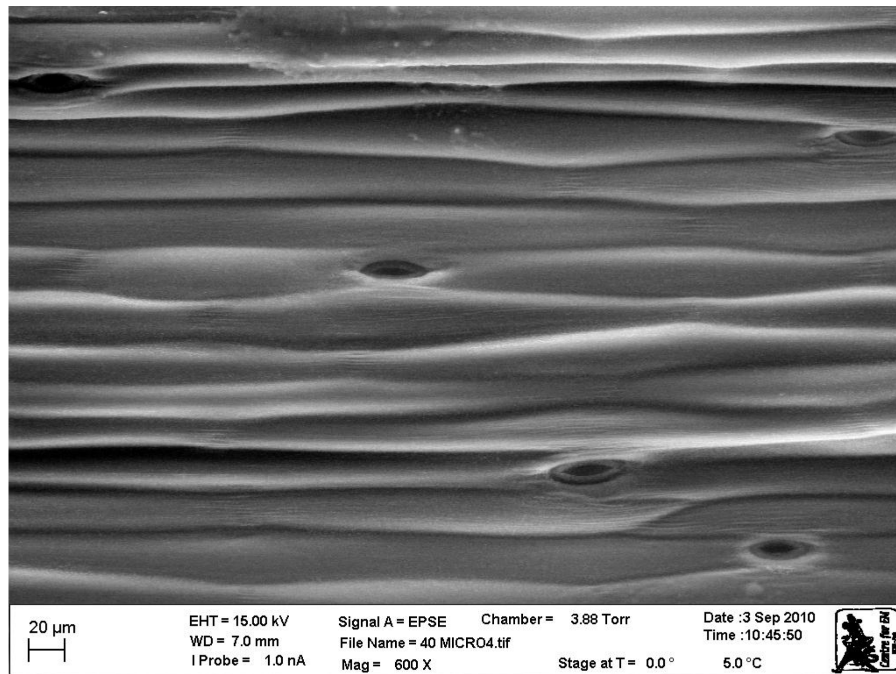

Figure 2.6: Structure of the adaxial side of *T. violacea* leaf grown at a light intensity of 40 µmol m^{-2} s^{-1} showing stomata.

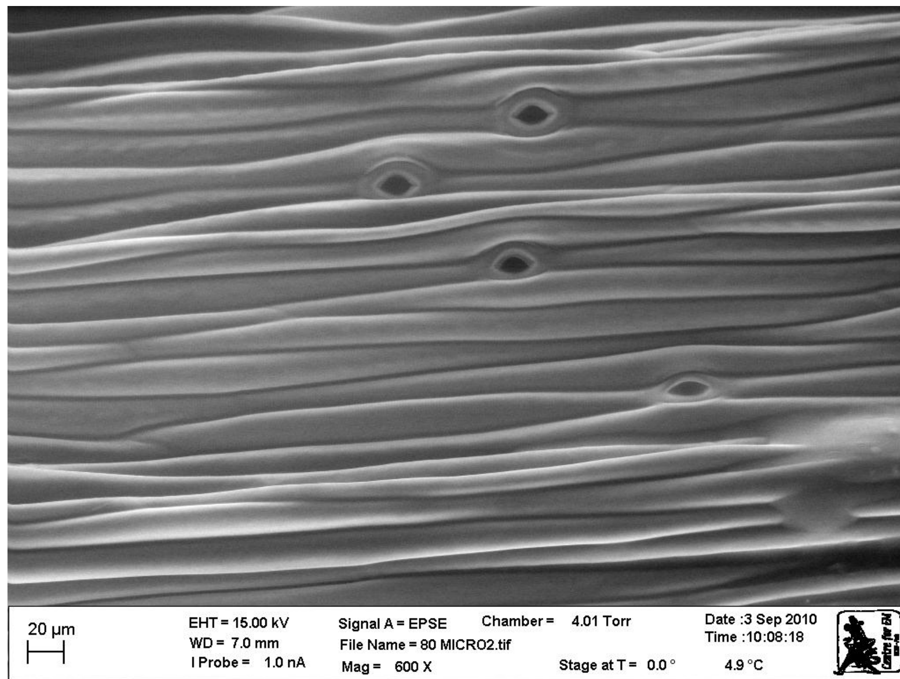

Figure 2.7: Structure of the adaxial side of *T. violacea* leaf grown at a light intensity of 80 µmol m^{-2} s^{-1} showing stomata.

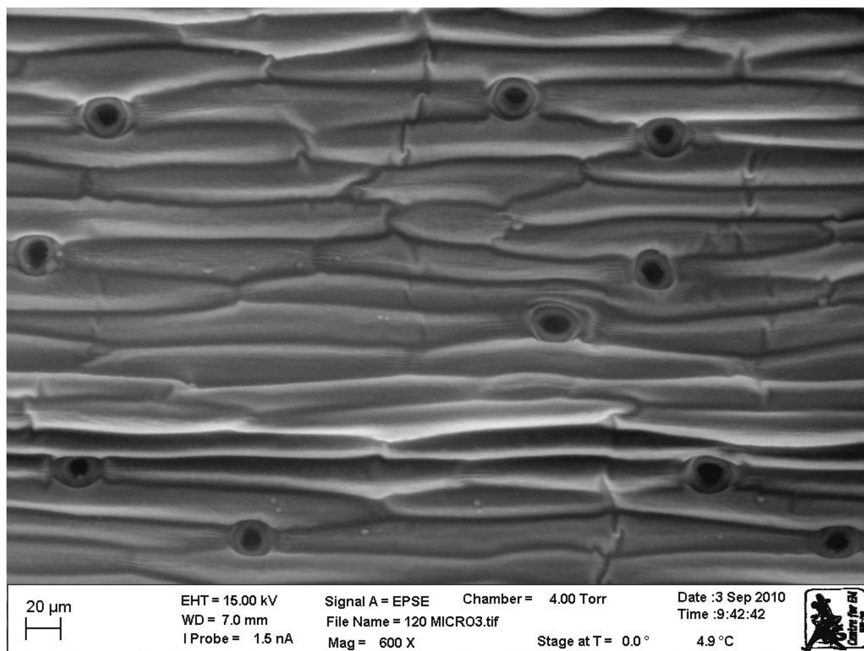

Figure 2.8: Structure of the adaxial side of *T. violacea* leaf grown at a light intensity of 120 µmol m^{-2} s^{-1} showing stomata.

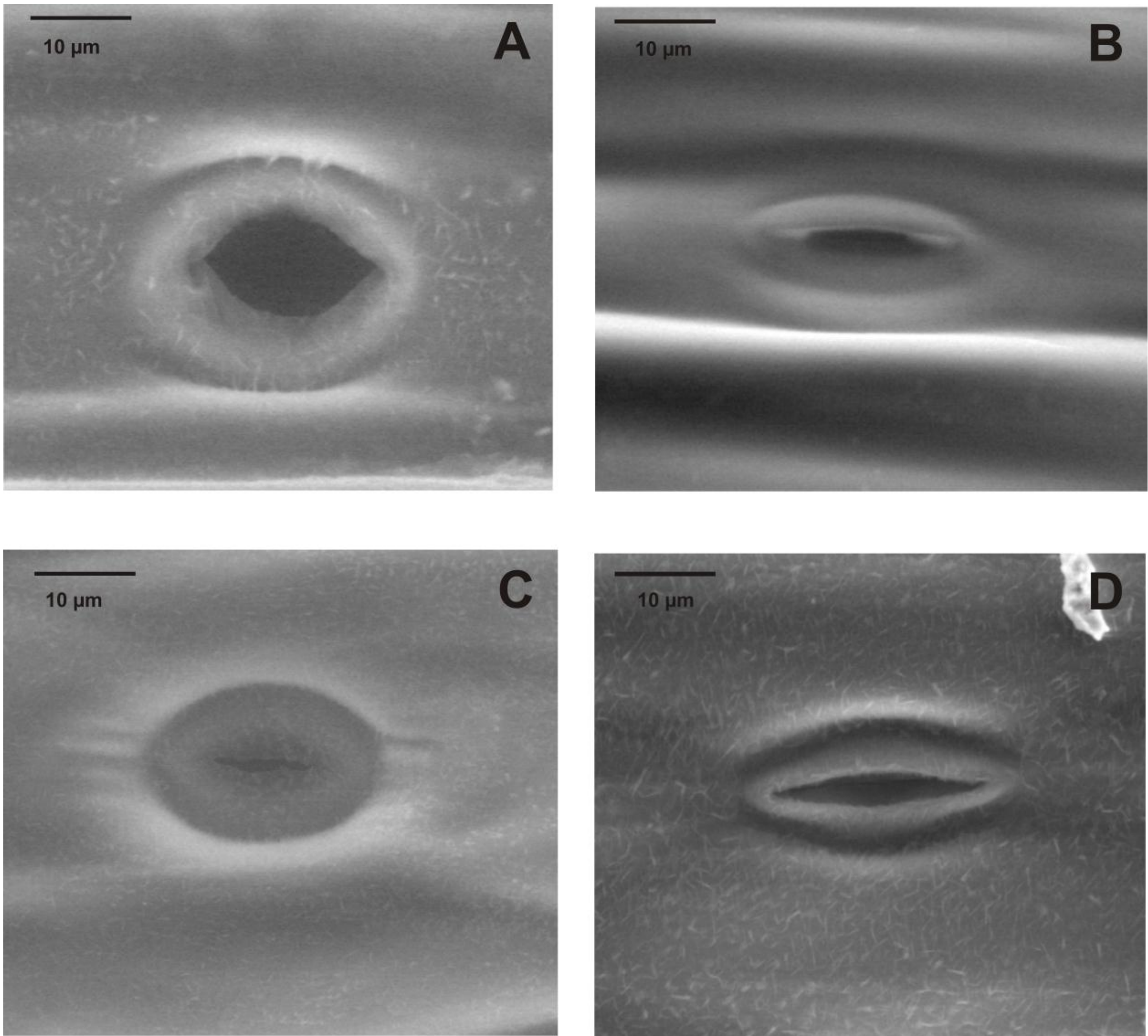

Figure 2.9: Scanning electron micrographs of the adaxial side of *T. violacea* leaves showing individual stomata under different photoperiods **(A)** continuous light **(B)** continuous dark **(C)** alternating light **(D)** control.

2.5 CONCLUSIONS

The results of this study clearly indicate that temperature plays a significant role in both seed germination and seedling growth of *T. ludwigiana* and *T. violacea*. The optimum temperature for *T. ludwigiana* and *T. violacea* was 22.8 °C and 19.9 °C, respectively. Light does not play a significant role in seed germination of both species. Continuous light is favoured by *T. ludwigiana* for seedling growth in terms of number of leaves, root length and fresh weight. Low light intensity is preferred for seed germination while seedling growth is favoured by a high light intensity in both species. Light did not play a significant role in the stomatal density of *T. violacea* seedlings, but structural differences were observed in the stomata between the different treatments. The *in vitro* germination of seeds under optimum conditions ensured the production of "sterile" seedlings which can be used as sources of explants in micropropagation.

CHAPTER 3
Micropropagation of *Tulbaghia* species

3.1 INTRODUCTION

Various types of explants have been used in the micropropagation of members of the family Alliaceae **(**see **Table 1.2)**, with bulbs and inflorescences having been the most common choice of explants, while seedlings have been used the least. A disadvantage of using bulbs as a source of explants is the fact that the parent plant is destroyed in order for the bulb to be used. Another disadvantage is that mentioned by **FENNELL & VAN STADEN (2004)** which is the contamination of plantlets produced often associated with the use of soil-borne organs as sources of explants, resulting in plant loss. The success of micropropagation does not only rest with the type of explant used, but with the correct combination and concentration of auxin and cytokinin as well **(HARTMANN et al., 1997)**. The application of exogenous plant growth regulators has an effect on the endogenous levels of plant growth regulators, thus modifying the growth and development of plants to a desired extent **(NICKELL, 1982)**.

The transfer of plants from an *in vitro* to an *ex vitro* environment is an important stage **(KANE et al., 2008)**. Shoots require to be rooted, so as to prepare them for successful transfer to the soil. Shoots are transferred to a rooting medium that usually has little or no cytokinin and increased auxin **(HARTMANN et al., 1997)**. Auxin is known to play a key role in the determination of rooting capacity **(FOGAÇA & FETT-NETO, 2005)**. Some shoots do not require a rooting stage as they will have rooted spontaneously during the shoot induction stage. The aim of this study was to develop an efficient protocol for the micropropagation of *T. ludwigiana* and *T. violacea* by determining the most suitable explants as well as a suitable combination of auxin and cytokinin for their growth and development. This study led to the initiation of an endogenous cytokinin analysis in order to serve as a possible explanation for the behaviour of hypocotyls in culture. The most suitable concentrations of IBA in root formation were also investigated.

3.2 MATERIALS AND METHODS

3.2.1 Explant decontamination and selection

Root, leaf and hypocotyl sections of *T. ludwigiana* and *T. violacea* derived from four-week-old *in vitro* germinated seedlings were surface decontaminated using 70% ethanol for 1 min and 2% NaOCl with a few drops of Tween 20 for 5 min, after which they were rinsed with sterile distilled water. The explants were cultured individually in test tubes (100 mm x 25 mm, 40 ml volume) containing 10 ml of full strength MS medium supplemented with sucrose (30 g l^{-1}) and myo-inositol (0.1 g l^{-1}). Strips of Parafilm® were used to seal the culture tubes. Leaf explants were cultured with the abaxial side on the growth medium. A 4 x 4 factorial experiment with 0.0, 0.5, 1.0 and 1.5 µM concentrations of naphthalene acetic acid (NAA) and 0.0, 5.0, 10.0 and 15.0 µM concentrations of benzyladenine (BA) for roots and leaves was conducted. The concentrations for the hypocotyls were 0.0, 0.5, 1.0 and 1.5 µM for NAA and 0.0, 4.0, 8.0 and 12.0 µM for BA. Each treatment consisted of 20 explants. The pH of the medium was adjusted to 5.8 with 1 N NaOH or 1 N HCl and agar (8 g l^{-1}) was added to solidify the medium. The medium was sterilized by autoclaving at 121 ºC for 20 min. Cultures were grown under Osram® 75 W cool white fluorescent tubes at continuous light (24h light), with a light intensity of 50 µmol m^{-2} sec^{-1} at 25 ± 2 ºC. After eight weeks of culture, the number of shoots produced per explant, the length of shoots (cm), the fresh weight of shoots (g), and the frequency (%) of callus and root initiation were recorded.

3.2.2 Effects of NAA and *m*TR on shoot multiplication

Based on the results obtained from the experiment above, only hypocotyl explants were used for subsequent experiments and these were cultured as outlined above, the only difference being the type and concentration of plant growth regulators used as well as the photoperiod used in the growth room. Concentrations of NAA (0.0, 1.0 and 2.0 µM) were combined in a 3 x 6 factorial experiment with *m*TR concentrations (2.5, 5.0, 10.0, 15.0, 25.0 and 30.0 µM) for *T. ludwigiana.* Concentrations of NAA (0.0, 1.0 and 2.0 µM) were combined in a 3 x 5 factorial experiment with *m*TR concentrations (2.5, 5.0, 10.0, 20.0 and 30.0 µM) for *T. violacea.* Each treatment consisted of 15 explants. A control with no plant growth regulators was included for both species. Cultures were grown under alternating

light (16/8 h) conditions. After eight weeks of culture, the number of shoots produced per explant, the length of shoots (cm), the fresh weight of shoots (g), and the frequency (%) of callus and roots produced were recorded.

3.2.3 Effects of different auxin and cytokinin combinations on shoot multiplication of *T. violacea*

An experiment was conducted for *T. violacea* where one or more cytokinins were used in a treatment in the presence or absence of an auxin. The total concentration of cytokinin used per treatment was 10 µM divided equally as per the number of cytokinins used. The concentration used for an auxin was 0.53 µM. Nine treatments were used, each with 20 explants, in the following combinations: the control (no plant growth regulators); an isoprenoid cytokinin (*t*Z) with no auxin; an isoprenoid cytokinin (*t*Z) with NAA; an isoprenoid cytokinin (*t*Z) with IAA; an aromatic cytokinin (BA) with NAA; an aromatic cytokinin (BA) with IAA; an isoprenoid cytokinin (*t*Z) combined with an aromatic cytokinin (BA) and no auxin; an isoprenoid cytokinin (*t*Z) combined with an aromatic cytokinin (BA) and NAA; an isoprenoid cytokinin (*t*Z) combined with an aromatic cytokinin (BA) and IAA; two isoprenoid cytokinins (*t*Z and Kinetin) with an aromatic cytokinin (BA) and no auxin; two isoprenoid cytokinins (*t*Z and Kinetin) with an aromatic cytokinin (BA) and NAA; two isoprenoid cytokinins (*t*Z and Kinetin) with an aromatic cytokinin (BA) and IAA. Each treatment consisted of 20 explants. Cultures were grown under Osram® 75 W cool white fluorescent tubes at alternating light (16/8 h), with a light intensity of 50 µmol m^{-2} sec^{-1} at 25 ± 2 ºC. After eight weeks of culture, the number of shoots produced per explant, the length of shoots (cm), the fresh weight of shoots (g) and the frequency (%) of callus and roots produced were recorded.

3.2.4 Endogenous cytokinin analysis of *T. violacea*

Endogenous cytokinins were identified and quantified in *T. violacea* seedlings. Seedlings obtained from four-week-old *in vitro* germinated seeds (approximately 6 cm long) were cut into three different sections i.e. roots, hypocotyls and leaves **(Fig. 3.1)**. These sections were separately frozen in liquid nitrogen (-196 ºC) and ground into fine powders in a mortar. They were freeze-dried completely, their dry weight was recorded and they were kept at -20 ºC until analysis.

A modified protocol as described by **NOVÁK et al. (2003)** was used to extract samples in duplicate using 70% ice-cold ethanol. Deuterium labeled cytokinin standards, each at 5 pmol per sample, were added to measure recovery during purification as well as to validate the cytokinin determination. The standards were [$^{2}H_{5}$]*t*Z, [$^{2}H_{5}$]*t*ZOG, [$^{2}H_{5}$]*t*ZR, [$^{2}H_{5}$]*t*ZROG, [$^{2}H_{5}$]*t*Z9G, [$^{2}H_{5}$]*t*ZR5MP, [$^{13}C_{5}$]*c*Z, [$^{2}H_{3}$]DHZ, [$^{2}H_{7}$]DHZOG, [$^{2}H_{3}$]DHZR, [$^{2}H_{7}$]DHZROG, [$^{2}H_{3}$]DHZ9G, [$^{2}H_{3}$]DHZR5MP, [$^{2}H_{6}$]iP, [$^{2}H_{6}$]iPR, [$^{2}H_{6}$]iP9G, [$^{2}H_{6}$]iPR5MP, [$^{2}H_{7}$]BAP, [$^{2}H_{7}$]BAPR, [$^{2}H_{7}$]BAP9G, [$^{2}H_{7}$]BAR5MP, [$^{15}H_{4}$]*m*T, [$^{15}H_{4}$]*o*T and [$^{15}N_{4}$] Kin (Olchemim Ltd., Czech Republic). After 3 h the homogenate was centrifuged and the same procedure was used for the re-extraction of pellets. The combined supernatants were concentrated to approximately 1.0 ml *in vacuo* at 35 ºC, and diluted to 20 ml with ammonium acetate buffer (40 mM, pH 6.5). Extracts were purified using a combined DEAE-Sephadex (1.0 x 5.0 cm)-octadecylsilica (0.5 x 1.5 cm) column and immunoaffinity chromatography (IAC) based on generic monoclonal cytokinin antibody **(FAISS et al., 1997)**. This resulted in three fractions: (1) the free bases, ribosides and N-glucosides (2) a ribotide fraction and (3) an O-glucoside fraction. The second and the third fractions were also purified using IAC, where the second fraction was treated with alkaline phosphatase (Roche diagnostics, Mannheim, Germany) and the third fraction was treated with β-glucosidase.

The fractions from the IAC columns were evaporated to dryness and dissolved in 50 µl of the mobile phase used for high performance liquid chromatography (HPLC–MS) analysis. The samples were analyzed by HPLC (Waters Alliance 2690) linked to a Micromass ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface [LC(+)ES-MS] and diode-array detector (Waters PDA 996). Using a post-column split of 1:1, the effluent was introduced into an electrospray source (source block temperature 100 ºC, desolvation temperature 250 ºC, capillary voltage + 3.0 V, cone voltage 20 V) and PDA (scanning range 210-300 nm with 1.2 nm resolution) and quantitative analysis of the different cytokinins performed in selective ion recording mode. Quantification was performed by Masslynx software using a standard isotope dilution method. The ratio of endogenous cytokinin to appropriate labelled standard was determined and further used to quantify the level of endogenous compounds in the

original extract, according to the known quantity of added internal standard **(NOVÁK et al., 2003)**.

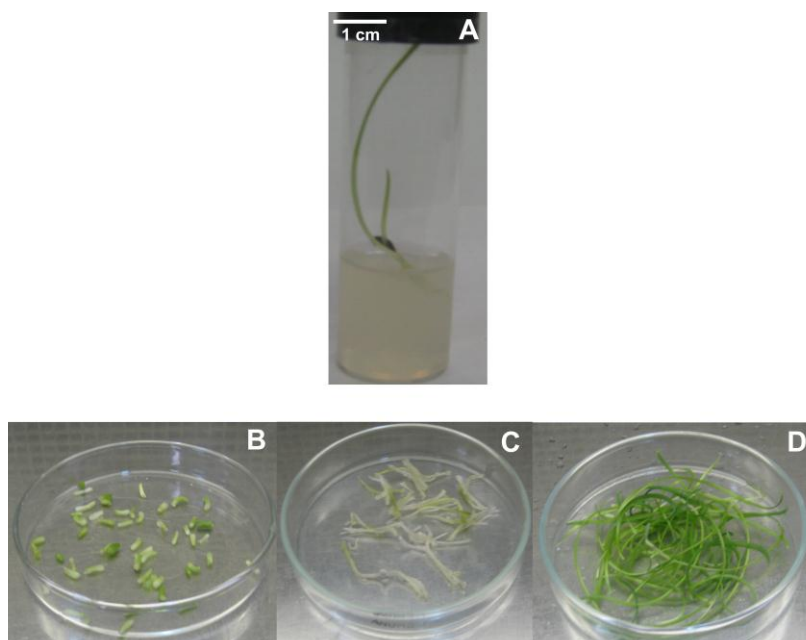

Figure 3.1: Sample preparation for endogenous cytokinin analysis from *in vitro* grown seedlings of *T. violacea*. **(A)** seedling **(B)** hypocotyls **(C)** roots **(D)** leaves.

3.2.5 Rooting and acclimatization

Regenerated shoots of *T. ludwigiana* and *T. violacea* obtained from combinations of NAA and *m*TR were cultured on full strength MS medium supplemented with differing IBA concentrations (0.0, 2.5 & 5.0 µM). Each treatment consisted of 15 explants. Cultures were grown in alternating light (16/8 h) conditions and a temperature of 25 ºC. The number of rooted shoots and number of roots per shoot were recorded after four and eight weeks of culture, while the root length was recorded only after eight weeks of culture.

Shoots which had rooted during shoot induction were acclimatized. The medium was washed from 20 plantlets using distilled water. They were transferred to a

mixture of potting soil and vermiculite (1:1, v/v) and placed in the mist house for two weeks **(Fig. 3.5)**, after which they were transferred to the greenhouse, where they were watered once a day. The survival percentage was recorded after four weeks.

3.3 DATA ANALYSIS

Data were subjected to one-way analysis of variance (ANOVA). Means were separated by Duncan's Multiple Range Test (DMRT) using SPSS statistical package (version 10.0) at the 5% level of significance. Graphs were drawn using SigmaPlot software (version 8.0).

3.4 RESULTS AND DISCUSSION

3.4.1 Shoot induction from seedling sections

Hypocotyls were the explants which produced the greatest number of shoots in both species, even though the number of shoots produced in *T. ludwigiana* was much lower than that for *T. violacea* **(Table 3.1)**. **ASCOUGH (2008)** attributed the responsiveness of hypocotyls in culture to the meristematic region being located in them, hence their competency to divide, grow and differentiate. No shoots were produced from the leaves of both species. No shoots were produced by the roots of *T. ludwigiana*, while 3% of *T. violacea* roots produced shoots. **ASCOUGH (2008)** obtained a similar result where leaf explants of three *Watsonia* species were unresponsive and a small percentage of shoots were produced from leaf explants of one species out of the four that were cultured on various concentrations of NAA and BA.

Table 3.1: Shoot induction (%) from *Tulbaghia* seedling explants.

Species	Hypocotyls	Roots	Leaves
T. ludwigiana	3	0	0
T. violacea	84	3	0

3.4.2 Effects of NAA and *m*TR on shoot multiplication

No report has been found on the use of topolins in the shoot multiplication of members of the Alliaceae. Topolins were found to be better than BA in the micropropagation of *Musa* species **(BAIRU et al., 2008)**. Most of the treatments of *T. ludwigiana* had the highest percentage of explants producing shoots in the absence of NAA, hence the highest frequency of explants producing shoots (67%) was obtained in the absence of NAA where *m*TR was 15 µM **(Fig. 3.2 A)**. The lowest frequency of explants producing shoots (18%) was found in the treatment where NAA and *m*TR were highest. The presence of 2 µM NAA and 5 µM *m*TR inhibited shoot production in *T. ludwigiana*. There were more explants producing shoots in *T. violacea* than there were in *T. ludwigiana*. The highest frequency of explants producing shoots (100%) in *T. violacea* was obtained at 5 µM *m*TR, in the absence of NAA and where NAA was highest. The absence of NAA with 10 µM *m*TR inhibited shoot production, while at the highest concentration of NAA, the fewer explants (36%) produced shoots. Generally, more than 60% of explants produced shoots. It was observed that, unlike *T. ludwigiana*, in the absence of NAA, the percentage of explants producing shoots was lower. The highest concentration of NAA generated a higher production of shoots than the absence of NAA.

The highest frequency of explants producing roots (31%) in *T. ludwigiana* was obtained in the treatment 1 µM NAA and 2.5 µM *m*TR. Roots, like shoots, were produced mostly in the absence of NAA, where the frequency of explants producing roots was the same (17%). This was the only instance where the control had the lowest frequency of explants producing either shoots or roots. This was the same for root production of *T. violacea*. The highest frequency of explants

producing roots (42%) in *T. violacea* was obtained in the treatment 2 µM NAA and 5 µM *m*TR. Unlike *T. ludwigiana*, roots were mostly produced in the presence of NAA, with the lowest percentage of explants producing roots obtained in the absence of NAA where *m*TR was highest.

Generally, the production of callus is favoured by a high auxin to cytokinin ratio and in some instances auxin alone at high levels may produce callus in monocotyledons **(VAN STADEN et al., 2008)**. This held true for both these species, but more prominently so in *T. violacea* than *T. ludwigiana*. No callus was produced in the control in *T. ludwigiana*. In all treatments where callus was produced in the absence of NAA, this treatment had the lowest percentage of explants producing callus. No callus was produced in the absence of NAA including the control in *T. violacea*. An increase in the concentration of *m*TR at 2 µM NAA yielded a decrease in the percentage of explants producing callus until no callus was produced at 20 and 30 µM *m*TR. The highest percentage of explants producing callus (58%) was found with the highest concentration of NAA and *m*TR.

In *T. ludwigiana*, the least mean number of shoots produced was 0.18 ± 0.12 in the treatment 2 µM NAA and 30 µM *m*TR and the highest mean number of shoots produced was 0.67 ± 0.14 in the absence of NAA and 15 µM *m*TR **(Fig. 3.3 A)**. There was, however, no significant difference in all treatments for the mean number of shoots. Shoot production was inhibited in the treatment 2 µM NAA and 5 µM *m*TR. The mean number of shoots was higher for *T. violacea* than it was for *T. ludwigiana*. In *T. violacea*, the lowest mean number of shoots produced was 1.00 ± 0.00, which was produced in most of the treatments, including the control. The highest mean number of shoots produced was 1.33 ± 0.24 in the treatment 2 µM NAA and 20 µM *m*TR. The reason for the low mean number of shoots in both species could be that *Tulbaghia* is one of the genera that multiplies slowly **(PHELAN et al., 2007)**, which therefore could mean that it requires a longer period to generate multiple shoots. **HUSSEY (1975)** reported *Ipheion uniflorum,* also a bulbous plant of the family Alliaceae, as a species slow to react, when he cultured its bulbs, leaves, inflorescence stems and ovaries on MS medium supplemented with various concentrations of NAA, IAA, 2,4-D. As with *T. ludwigiana*, there was

no significant difference in all treatments for the mean number of shoots in *T. violacea*. Shoot production was inhibited in the absence of NAA and 10 µM *m*TR **(Fig. 3.3 D)**.

The longest mean shoot length (2.77 ± 1.15 cm) in *T. ludwigiana* was obtained in the treatment 1 µM NAA and 2.5 µM *m*TR. The shortest mean shoot length (0.55 ± 0.25 cm) was obtained in the absence of NAA and 25 µM *m*TR. However, no significant difference was observed in all treatments for the mean shoot length. The mean shoot length of *T. violacea* was higher in the presence of NAA, hence the longest mean shoot length (5.19 ± 0.90 cm) was obtained in 1 µM NAA and 10 µM *m*TR. This was significantly higher than two treatments where NAA was absent. One of these treatments had the shortest mean shoot length (1.57 ± 0.32 cm).

The greatest mean fresh weight (81.60 ± 37.89 mg) in *T. ludwigiana* was found in the treatment 2 µM NAA and 2.5 µM *m*TR, which was significantly greater than the treatments 1 µM NAA and 30 µM *m*TR and 2 µM NAA and 30 µM *m*TR, the former being the treatment with the lowest mean fresh weight (13.91 ± 13.03 mg). The absence of NAA resulted in a low mean fresh weight in *T. violacea.* This can be seen in the greatest mean fresh weight (266.50 ± 199.38 mg) obtained in 1 µM NAA and 20 µM *m*TR, which was significantly greater than the lowest mean fresh weight (27.86 ± 10.83 mg) in the absence of NAA but same concentration of *m*TR.

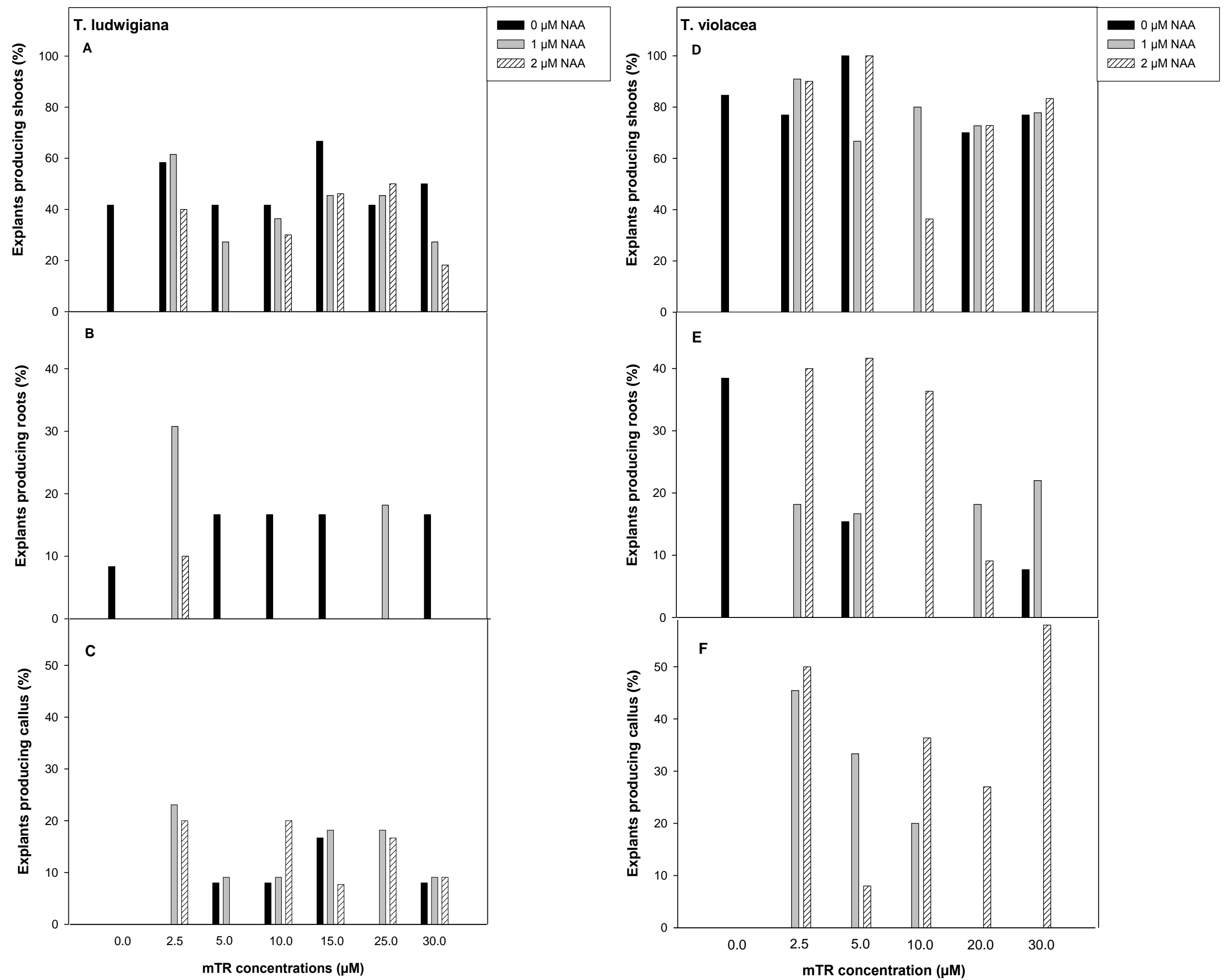

Figure 3.2: Effects of NAA and mTR on shoot multiplication of *T. ludwigiana* and *T. violacea*. **(A)** Percentage of explants producing shoots, **(B)** percentage of explants producing roots, **(C)** percentage of explants producing callus, **(D)** percentage of explants producing shoots, **(E)** percentage of explants producing roots, **(F)** percentage of explants producing callus. **(A - C)** *T. ludwigiana* **(D - F)** *T. violacea*.

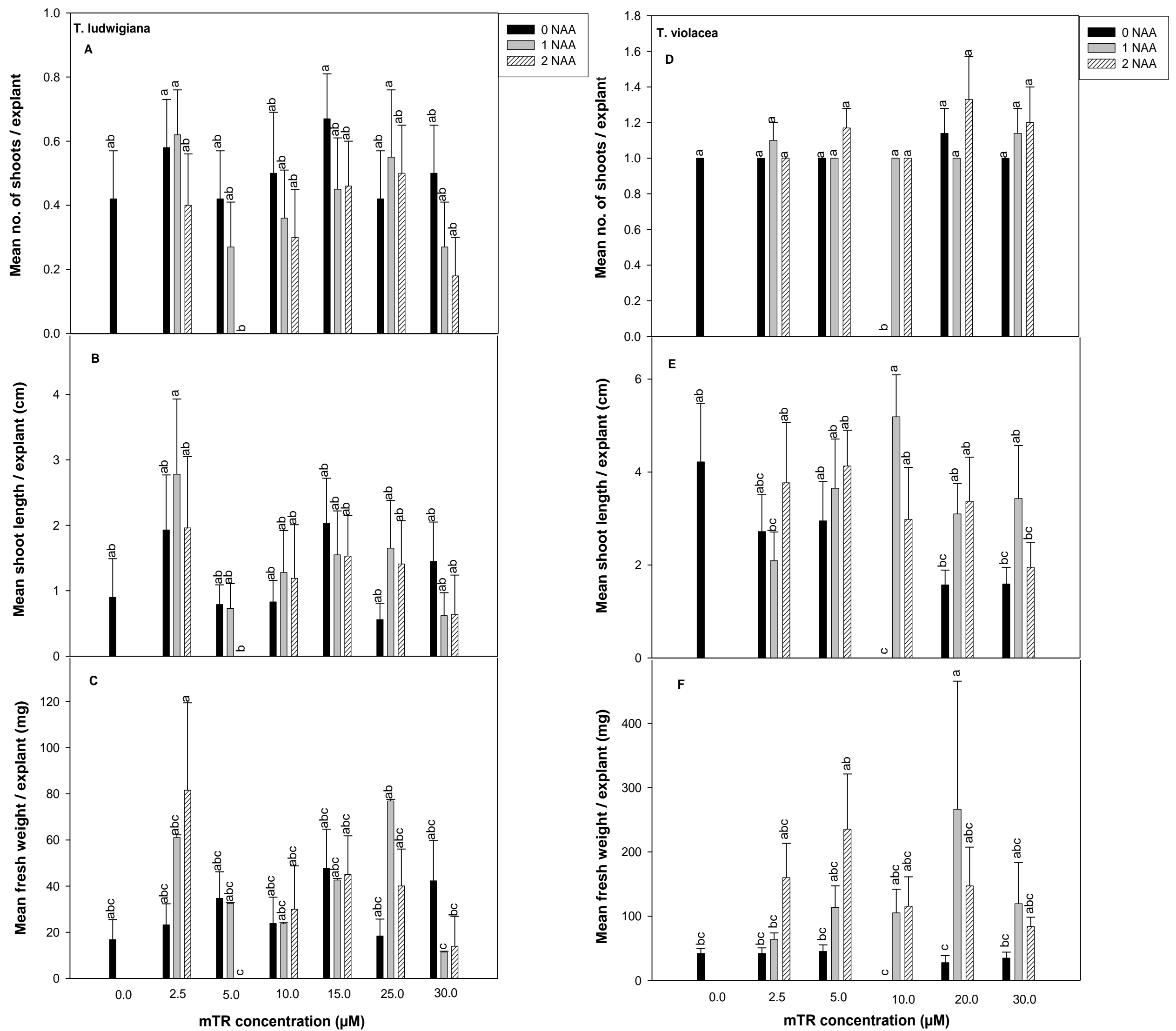

Figure 3.3: Effects of NAA and *m*TR on shoot multiplication of *T. ludwigiana* and *T. violacea*. **(A)** Mean number of shoots per explant, **(B)** mean shoot length per explant, **(C)** mean fresh weight per explant, **(D)** mean number of shoots per explant, **(E)** mean shoot length per explant, **(F)** mean fresh weight per explant, **(A - C)** *T. ludwigiana* **(D - F)** *T. violacea*. Bars with different letters indicate significant differences between treatments (P ≤ 0.05) according to DMRT.

3.4.3 Effects of different auxins and cytokinins on shoot multiplication of *T. violacea*

The addition of more than one cytokinin in a medium has mostly been reported in woody species. **SRIVASTAVA & JOSHI (2009)** reported multiple shoot formation to have been better in the presence of kinetin and BA in the micropropagation of the herbaceous *Portulaca grandiflora*. This was later confirmed by **NEGI & SAXENA (2011)** who reported stimulation in the growth and multiplication of bamboo shoots when cultured in a combination of BA and kinetin. Shoots were produced in all treatments of *T. violacea* **(Table 3.2)**. Most explants produced shoots in the absence of auxin. In both cases where 100% of explants produced shoots, auxin was absent. The lowest frequency of explants producing shoots (74%) was obtained in the presence of IAA combined with BA and *t*Z. Only one treatment did not produce roots and that is the one with *t*Z alone. The BA and *t*Z treatments yielded the least frequency of explants producing roots (5%), while the BA and NAA yielded the highest (65%). Treatments with NAA or IAA either did not produce callus or NAA was the one where a higher percentage of plants producing callus was obtained. The highest frequency of plants producing callus was obtained in the treatment with BA in combination with *t*Z and NAA (30%), while the lowest (5%) was obtained in the control. In general, less callus was produced because of the high cytokinin to auxin ratio or the absence of auxin altogether.

In all cases, the mean number of shoots was highest in the presence of NAA, followed by the treatment with no auxin, with the treatment with IAA having the lowest mean number of shoots. The exception to this was the treatment with kinetin, *t*Z and BA, where the highest mean number of shoots (1.47 ± 0.32) was in the absence of auxin, followed by the treatment with NAA. None of these treatments were significantly different from each other, except for the BA, *t*Z and NAA treatment which was significantly higher than the BA, *t*Z and IAA treatment with a 2.12 ± 0.67 mean number of shoots compared to a 1.00 ± 0.00 mean number of shoots demonstrated by the BA, *t*Z and IAA treatment. The BA, *t*Z and IAA treatment as well as the BA and IAA treatment were the treatments with the lowest mean number of shoots (1.00 ± 0.00). The longest mean shoot length (5.62 ± 0.98 cm) was obtained in the presence of BA, *t*Z and IAA. There was, however,

no significant difference between this treatment and the one where IAA was substituted by NAA. Benzyladenine alone with IAA yielded the shortest mean shoot length (2.23 ± 0.37 cm). The greatest mean fresh weight (179. 57 ± 38.43 mg) was obtained in the presence of BA, *tZ* and IAA and this was significantly higher than the treatment where IAA was substituted with NAA. In three out of four treatments, the greatest mean fresh weight was obtained in the presence of IAA, followed by the treatment where NAA substituted IAA, while the absence of an auxin yielded the lowest mean fresh weight.

Table 3.2: Effects of different combinations of cytokinins with and without auxin on shoot multiplication of *T. violacea*. Values with different letters in a column indicate significant differences between treatments ($P \leq 0.05$) according to DMRT.

PGRs used (µM)				**Explants producing shoots (%)**	**Explants producing roots (%)**	**Explants producing callus (%)**	**Mean no. of shoots / explant**	**Mean shoot length / explant (cm)**	**Mean fresh weight / explant (mg)**
Cytokinin		**Auxin**							
Isoprenoid	**Aromatic**	**NAA**	**IAA**						
-	**-**	**-**	**-**	90	40	5	1.17 ± 0.17ab	3.38 ± 0.58bc	37.67 ± 6.42de
10 *tZ*	**-**	**-**	**-**	100	0	26	1.30 ± 0.25ab	3.03 ± 0.43bc	31.95 ± 6.47de
10 tZ	**-**	**0.53**	**-**	79	10	0	1.73 ± 0.34ab	2.30 ± 0.40c	56.13 ± 13.48cde
10 tZ	**-**	**-**	**0.53**	90	40	0	1.28 ± 0.13ab	3.54 ± 0.64bc	155.94 ± 45.83ab
-	**10 BA**	**-**	**-**	95	35	16	1.06 ± 0.06b	3.01 ± 0.61bc	24.22 ± 2.92e
-	**10 BA**	**0.53**		95	65	10	1.21 ± 0.12ab	4.38 ± 0.73ab	76.58 ± 15.05cde
-	**10 BA**	**-**	**0.53**	90	15	0	1.00 ± 0.00b	2.23 ± 0.37c	18.17 ± 2.10e
5 *tZ*	**5 BA**	**-**	**-**	100	5	10	1.40 ± 0.35ab	2.46 ± 0.37bc	29.40 ± 5.06e
5 *tZ*	**5 BA**	**0.53**		85	30	30	2.12 ± 0.67a	4.38 ± 0.80ab	104.06 ± 25.84bc
5 tZ	**5 BA**		**0.53**	74	30	0	1.00 ± 0.00b	5.62 ± 0.98a	179.57 ± 38.43a
3.3 *tZ* + 3.3 Kin	**3.3 BA**	**-**	**-**	84	20	15	1.47 ± 0.32ab	2.81 ± 0.50bc	39.12 ± 8.15de
3.3 tZ + 3.3 Kin	**3.3 BA**	**0.53**		80	20	0	1.38 ± 0.18ab	4.34 ± 0.72ab	97.31 ± 24.22bcd
3.3 tZ + 3.3 Kin	**3.3 BA**		**0.53**	68	37	0	1.23 ± 0.17ab	4.23 ± 0.87abc	120. 38 ± 30.27abc

3.4.4 Endogenous cytokinin analysis of *T. violacea*

Results of the endogenous cytokinin analysis are presented in **Table 3.3**. A total of 34 different cytokinins were found in the seedlings of *T. violacea*. Cytokinins which were not detected in the seedlings include *o*T, *o*TOG, *o*TR, *o*TROG, *o*T9G, *o*TR5′MP, *m*T9G, *m*TR5′MP and *p*TR5′MP. Cytokinins which were only detected in roots were BAP9G, *m*TROG, *p*T, *p*TOG and *p*TROG.

The total cytokinin content was greatest in the roots, with the hypocotyls having the second greatest cytokinin content, while the leaves contained the least cytokinin **(Fig. 3.4 A)**. Generally, cytokinins are abundant in proliferating tissues such as immature seeds, roots, apical meristems and young leaves. The site of their biosynthesis is, however, not clearly established. It has therefore been accepted that the tissues with high levels of cytokinins are considered to be the site of cytokinin biosynthesis. Roots are therefore thought to be the major sites of cytokinin biosynthesis because they contain high levels of cytokinins **(MIYAWAKI et al., 2004)**. The results obtained for *T. violacea* are consistent with this view.

Cytokinin content may be discussed in terms of the cytokinin class i.e. aromatic and isoprenoid as well as the functional type i.e. free bases, O-glucosides, RO-glucosides, 9-glucosides, ribotides and ribosides. The isoprenoid cytokinins were more abundant than the aromatic cytokinins in each of the plant parts analysed **(Fig. 3.4 B-C)**. This pattern of abundance is consistent with the statement by **KAMADA-NOBUSADA & SAKAKIBARA (2009)** that isoprenoid cytokinins are more abundant than aromatic cytokinins in higher plants. *Trans-zeatin* was the most abundant and iP the least abundant among the isoprenoid cytokinins. This result concurs with the report by **STIRK et al. (2009)**, which states that *t*Z with its metabolites and DHZ are the most prevalent cytokinins in vascular plants, while iP-type cytokinins are minor components. Among the aromatic cytokinins, BA was the predominant, followed by *p*T, with *m*T being the least prominent. Ribotides were the most abundant cytokinin forms especially *t*ZR5′MP **(Fig. 3.4 D; Table 3.3)**. This could be due to the fact that ribotides are the first cytokinins formed in the biosynthetic pathway. They are also

thought to play a key role in regulating cytokinin levels by being readily converted to ribosides and free bases **(STIRK et al., 2005; STIRK et al., 2009)**. The presence of free bases in low concentrations can be attributed to their active utilization in various growth processes, seeing that they, together with ribosides, are active forms of cytokinins **(STIRK et al., 2009)**.

Table 3.3: Cytokinin content in seedling sections of *T. violacea* (pmol/g dry weight). <LOD – below limit of detection.

Cytokinin	Hypocotyls	Roots	Leaves
*t*Z	2.23 ± 0.13	1.48 ± 0.30	0.78 ± 0.04
*t*ZOG	6.71 ± 1.28	15.03 ± 3.47	2.30 ± 0.52
*t*ZR	8.32 ± 0.06	5.7 ± 0.57	5.31 ± 0.14
*t*ZROG	3.83 ± 0.46	21.59 ± 3.94	0.88 ± 0.13
*t*Z9G	0.50 ± 0.07	1.23 ± 0.11	1.32 ± 0.29
*t*ZR5′MP	2015.08 ± 395.34	2829.62 ± 57.07	206.25 ± 29.42
*c*Z	12.82 ± 0.64	5.08 ± 0.06	10.55 ± 0.74
*c*ZOG	4.07 ± 0.67	13.33 ± 0.49	2.52 ± 0.27
*c*ZR	582.96 ± 4.84	172.23 ± 14.20	611.36 ± 27.93
*c*ZROG	7.10 ± 0.97	12.68 ± 0.16	11.99 ± 1.43
*c*Z9G	0.09 ± 0.02	0.09 ± 0.00	0.12 ± 0.02
*c*ZR5′MP	183.09 ± 39.31	1113.56 ± 177.94	180.33 ± 17.88
DHZ	0.59 ± 0.01	0.39 ± 0.03	0.29 ± 0.05
DHZOG	1.98 ± 0.37	4.30 ± 1.02	3.78 ± 0.02
DHZR	14.7 ± 0.54	4.12 ± 0.17	13.63 ± 0.35
DHZROG	1.26 ± 0.05	6.52 ± 1.09	1.13 ± 0.14
DHZ9G	0.42 ± 0.08	0.15 ± 0.03	0.22 ± 0.03
DHZR5′MP	18.56 ± 1.54	28.91 ± 3.01	7.17 ± 1.66
iP	0.89 ± 0.18	1.04 ± 0.23	3.57 ± 0.24
iPR	21.18 ± 0.38	16.62 ± 1.47	307.05 ± 4.96
iP9G	0.21 ± 0.02	1.27 ± 0.27	0.59 ± 0.12
iPR5′MP	1.03 ± 0.19	23.73 ± 3.45	22.30 ± 4.61
BAP	1.42 ± 0.03	7.48 ± 0.69	0.58 ± 0.05
BAPR	5.38 ± 0.10	3.58 ± 0.12	0.56 ± 0.05
BAP9G	<LOD	0.21 ± 0.05	<LOD
BAPR5′MP	1.16 ± 0.29	1.17 ± 0.14	0.45 ± 0.09
*m*T	0.33 ± 0.02	1.69 ± 0.12	1.15 ± 0.18

*m*TOG	1.95 ± 0.04	0.65 ± 0.12	1.71 ± 0.06
*m*TR	1.56 ± 0.36	1.85 ± 0.02	1.10 ± 0.07
*m*TROG	<LOD	1.36 ± 0.10	<LOD
*p*T	<LOD	3.78 ± 0.54	<LOD
*p*TOG	<LOD	3.78 ± 0.54	<LOD
*p*TR	1.48 ± 0.12	1.08 ± 0.25	0.39 ± 0.05
*p*TROG	<LOD	5.92 ± 1.34	<LOD

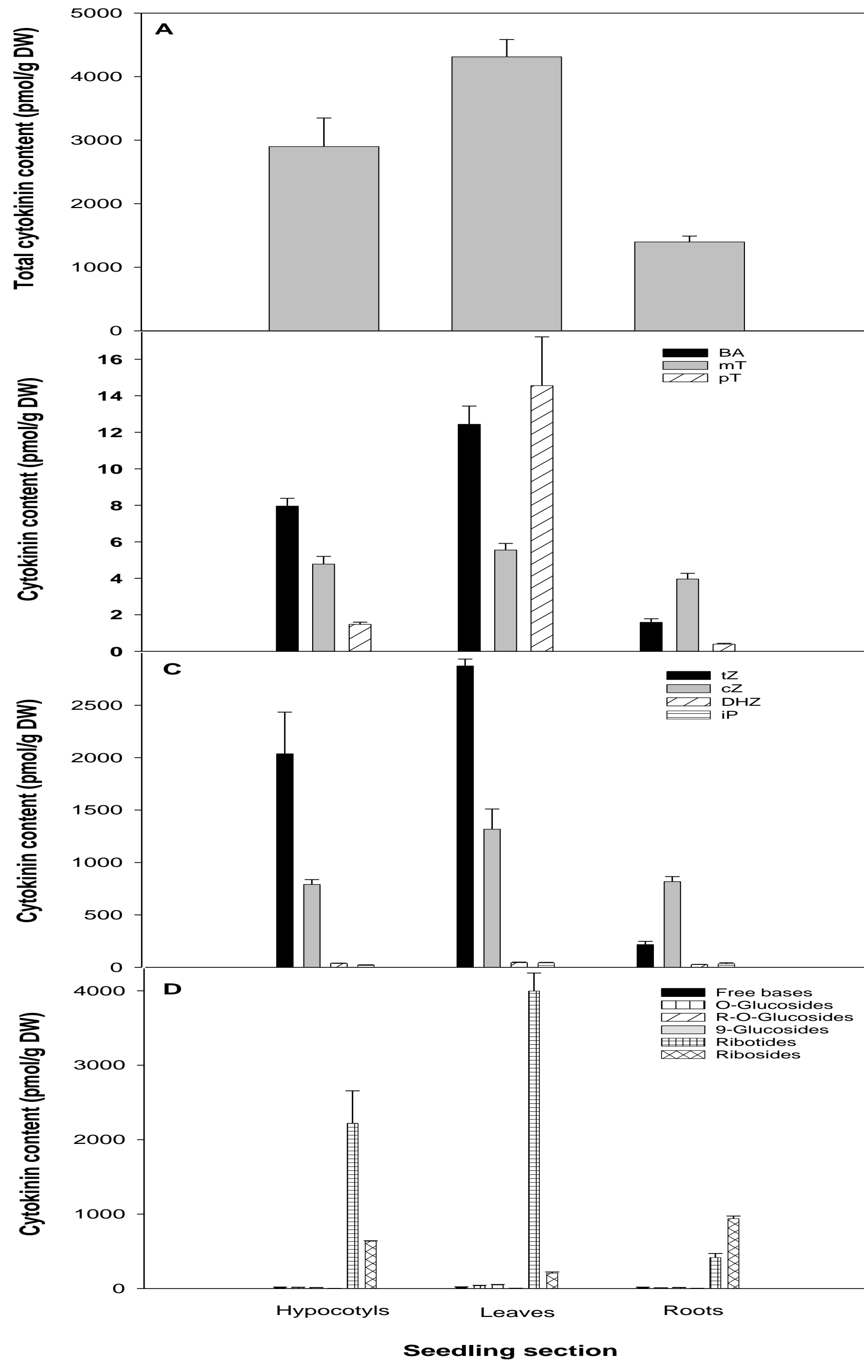

Figure 3.4: Cytokinin content in seedling sections of *T. violacea*. **(A)** Total cytokinin content **(B)** content of aromatic cytokinins **(C)** content of isoprenoid cytokinins **(D)** content of functional cytokinins.

3.4.5 Rooting and acclimatization

Studies on *Tulbaghia simmleri* indicated deformed roots with the use of NAA for the rooting of shoots **(ZSCHOCKE & VAN STADEN, 2000)**. When IAA and IBA were used for rooting, IBA yielded a higher percentage of explants that rooted than IAA **(WAWROSCH et al., 2001)**, hence IBA was selected for the rooting of *T. ludwigiana* and *T. violacea*. Rooting was recorded in all treatments for both species. No significant difference was observed in the percentage of rooting explants at four and eight weeks in *T. ludwigiana* **(Table 3.4)**. The highest percentage of rooting explants (84.62 ± 10.42) at eight weeks was obtained in the presence of 2.5 µM IBA. There was no increase from four to eight weeks in the percentage of rooting explants (66.67 ± 14.21) in the treatment where there was an absence of IBA. The treatment with a significantly high mean number of roots was 2.5 µM IBA, whose mean number of roots was 3.54 ± 1.25 at four weeks and 5.25 ± 1.25 at eight weeks. The mean root length was significantly high (10.07 ± 2.21 cm) in the absence of IBA and decreased with an increase in IBA concentration.

The rooting of *T. violacea* was quite different from that of *T. ludwigiana*. There was no significant difference between all treatments of *T. violacea*. There was, however, a clear pattern. At four weeks, the percentage of rooting explants and the mean number of roots per explant was the same in the absence and where the concentration of IBA was lower. At a higher concentration of IBA, both these growth parameters were lower. The growth parameters at eight weeks were higher at the highest concentration of IBA. Most roots of *T. violacea* were green in colour, which is explained by their growth in the presence of light **(FLORES et al., 1993)**. Roots of *T. ludwigiana* were all white. Acclimatization for *T. violacea* was 100% successful, while *T. ludwigiana* failed to acclimatize. The plants were attacked by slugs while still in the mist house **(Fig. 3.5)**.

Table 3.4: Effect of IBA on rooting of *T. ludwigiana* and *T. violacea.* Values with different letters in a column indicate significant differences between treatments ($P \leq 0.05$) according to DMRT.

Species	Conc. (µM)	Time (weeks)				
		4		8		
		% of rooting explants	Mean no. of roots/explant	% of rooting explants	Mean no. of roots/explant	Mean root length (cm)
T. ludwigiana	0	66.67 ± 14.21a	1.08 ± 0.34b	66.67 ± 14.21a	2.00 ± 0.49b	10.07 ± 2.21a
	2.5	61.54 ± 14.04a	3.54 ± 1.25a	84.62 ± 10.42a	5.25 ± 1.25a	5.77 ± 0.90b
	5	41.67 ± 14.86a	1.17 ± 0.44b	50.00 ± 15.08a	2.09 ± 0.85b	2.83 ± 1.03b
T. violacea	0	33.33 ± 16.67a	0.67 ± 0.44a	33.33 ± 16.67a	1.22 ± 0.64a	3.83 ± 2.08a
	2.5	33.33 ± 21.08a	0.67 ± 0.49a	33.33 ± 21.08a	1.17 ± 0.98a	3.92 ± 2.53a
	5	28.57 ± 18.44a	0.57 ± 0.43a	57.14 ± 20.20a	2.71 ± 1.34a	5.34 ± 2.28a

Figure 3.5: Acclimatizing plants of *T. ludwigiana* in the mist house.

3.5 CONCLUSIONS

A micropropagation protocol was established for both species. The addition of NAA and BA to the growth medium revealed hypocotyls to be the most responsive seedling sections. The addition of *m*TR and NAA in the culture medium yielded a low number of shoots in both species but more so in *T. ludwigiana* than *T. violacea*. The best response of *T. violacea* in culture was in the presence of a combination of BA, *t*Z and NAA, where this treatment resulted in the highest mean number of shoots, with a fairly high mean shoot length and mean fresh weight. *Tulbaghia ludwigiana* did not respond as well as *T. violacea* in culture. Cytokinins are present in all regions of *T. violacea* seedlings, with a high distribution in roots and a low distribution in leaves. There were more isoprenoid cytokinins than there were aromatic cytokinins in each of the seedling sections analysed, with *t*Z being the predominant isoprenoid cytokinin, while BA was the predominant aromatic cytokinin. *Tulbaghia ludwigiana* rooted well at 2.5 μM IBA, while there was no significant difference between the rooting treatments of *T. violacea*. The latter acclimatized successfully in soil and vermiculite, while this was not so for *T. ludwigiana*.

CHAPTER 4
Antimicrobial and phytochemical properties of micropropagated *Tulbaghia violacea* plants

4.1 INTRODUCTION

Tulbaghia violacea is used for a number of medicinal properties as outlined in Chapter 1. Due to the devastating effects and the high prevalence rate of HIV/AIDS in South Africa, the search for anti-HIV agents has stimulated the screening of medicinal plants based on their ethnobotanical data **(WORLD HEALTH ORGANISATION, 1989; MOTSEI et al., 2003; KLOS et al., 2009)**. *Tulbaghia violacea* is one of these plants and has shown promising antimicrobial activity against some medically important pathogenic bacteria and fungi that cause opportunistic infections in HIV/AIDS patients **(McGAW et al., 2000; GAIDAMASHVILI & VAN STADEN, 2002; MOTSEI et al., 2003)**. The plant has undoubtedly found a use among HIV/AIDS patients. There has not been any comparison on the evaluation of micropropagated *T. violacea* for their medicinal properties as has been obtained for outdoor-grown plants. It was, thus, of interest to evaluate the micropropagated plants for medicinal properties in order to supplement the use of outdoor-growing plants with micropropagated ones. This is due to the many advantages offered by using micropropagated plants. In order for this to be done, a comparative study between the antimicrobial and phytochemical properties of outdoor-grown *T. violacea* and those of micropropagated *T. violacea* was carried out.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Due to limited plant material, only *T. violacea* was evaluated for antimicrobial and phytochemical properties. Plantlets obtained as indicated in Chapter 3, were subcultured onto a medium containing 1.5 µM NAA and 12 µM BA for a period of two

months before being taken for analysis. Outdoor-grown *T. violacea* plants (12 years old) were collected in summer (December) from the University of KwaZulu-Natal Botanical Garden, Pietermaritzburg, South Africa and a voucher specimen (NCUBE 04 NU) deposited in the University of KwaZulu-Natal Herbarium (NU), Pietermaritzburg. Whole plant samples (bulbs and leaves), including those of *in vitro* grown plants were then dried at a constant temperature of 50 °C in an incubator and then ground into a fine powder.

4.2.2 Preparation of plant extracts

The ground samples were sequentially extracted with 20 ml/g of petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water in a sonication bath containing ice for 1 h. The crude extracts were then filtered under vacuum through Whatman No. 1 filter paper and the organic extracts concentrated *in vacuo* at 35 °C using a rotary evaporator. The concentrated extracts were subsequently dried at room temperature under a stream of cold air. Water extracts were freeze dried and kept in airtight containers.

4.2.3 Antibacterial activity

Minimum inhibitory concentrations (MIC) of extracts for antibacterial activity were determined using the microdilution bioassay as described by **ELOFF (1998)**. Overnight cultures (incubated at 37 °C in a water bath with an orbital shaker) of two Gram-positive (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) bacterial strains were diluted with sterile Mueller-Hinton (MH) broth to give final inocula of approximately CFU/ml (colony forming units). The dried crude organic plant extracts were resuspended in 70% ethanol to a concentration of 50 mg/ml while water extracts were dissolved in distilled water to the same concentration. One hundred microlitres of each extract were serially diluted two-fold with sterile distilled water in a 96-well microtitre plate for each of the four bacterial strains. A similar two-fold serial dilution of neomycin (Sigma-Aldrich, Germany) (0.1 mg/ml) was used as a positive control against each bacterium. One

hundred microlitres of each bacterial culture were added to each well. Water and 70% ethanol were included as negative and solvent controls, respectively.

The plates were covered with parafilm and incubated at 37 °C for 24 h. Bacterial growth was indicated by adding 50 µl of 0.2 mg/ml p-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) and a further incubation at 37 °C for 2 h. Since the colourless tetrazolium salt is biologically reduced to a red product due to the presence of active organisms, the MIC values were recorded as the concentrations in the last wells in which no colour change was observed after adding the INT indicator. Bacterial growth in the wells was indicated by a reddish-pink colour. The assay was repeated twice with two replicates per assay.

4.2.4 Antifungal activity

A microdilution method as described by **ELOFF (1998)** and modified for fungi **(MASOKO et al., 2007)** was used to determine the antifungal activity of the extracts against *Candida albicans* (ATCC 10231). An overnight fungal culture was prepared in yeast malt (YM) broth. Four hundred microlitres of the overnight culture were added to 4 ml of sterile saline and absorbance was read at 530 nm. The absorbance was adjusted with sterile saline to match that of a 0.5 M McFarland standard solution. From this standardised fungal stock, a 1:1000 dilution with sterile YM broth was prepared giving a final inoculum of approximately 10^6 CFU/ml. Dried organic extracts were resuspended in 70% ethanol to a concentration of 50 mg/ml and water extracts were dissolved in water to the same concentration. One hundred microlitres of each extract were serially diluted two-fold with sterile water in a 96-well microtitre plate. A similar two-fold dilution of amphotericin B (Sigma-Aldrich, Germany) (2.5 mg/ml) was used as the positive control while water and 70% ethanol were used as negative and solvent controls, respectively. One hundred microlitres of the dilute fungal culture were added to each well.

The plates were covered with parafilm and incubated at 37 °C for 24 h, after which 50 µl (0.2 mg/ml) INT were added and incubated for a further 24 h at 37 °C. The wells remained clear where there was inhibition of fungal growth. MIC values were

recorded as the lowest concentrations that inhibited fungal growth after 48 h. To determine the fungicidal activity, 50 µl of sterile YM broth were added to all the clear wells and further incubated at 37 °C for 24 h after which the minimum fungicidal concentrations (MFC) were recorded as the last clear wells. The assay was repeated twice with two replicates per assay.

4.2.5 Phenolic content determination

4.2.5.1 Preparation of extracts

Phenolic compounds were extracted from plant material as described by **MAKKAR (1999)**. Dried plant samples (2 g) were extracted with 10 ml of 50% aqueous methanol by sonication on ice for 20 min. The extracts were then filtered under vacuum through Whatman No. 1 filter paper.

4.2.5.2 Determination of total phenolic compounds

The amounts of total phenolics in plant samples were determined using the Folin Ciocalteu (Folin C) assay for total phenolics as described by **MAKKAR (1999)** with slight modification **(NDHLALA et al., 2007)**. Fifty microlitres of each extract from the plant samples were transferred into test tubes into which 950 µl of distilled water were added followed by 1 N Folin C phenol reagent (500 µl) and 2% sodium carbonate (2.5 ml). A blank that contained aqueous methanol instead of plant extracts was also prepared. The test mixtures were incubated for 40 min at room temperature and the absorbance was read at 725 nm using a UV-vis spectrophotometer (Varian Cary 50, Australia). Each extract had three replicates. Total phenolic concentrations were expressed as gallic acid equivalents (GAE).

4.2.5.3 The butanol-HCl assay for condensed tannins (proanthocyanidins)

Three millilitres of butanol-HCl reagent (95:5, v/v) were added to 500 µl of each extract, followed by 100 µl ferric reagent (2% ferric ammonium sulphate in 2 N HCl). The test combinations were mixed by vortexing and then placed in a boiling water bath for 60 min. Absorbance was then read at 550 nm using a UV-vis spectrophotometer against a blank prepared by mixing the extract (500 µl) with

butanol-HCl reagent (3 ml) and ferric reagent (100 µl), but without heating. Each extract had three replicates. Condensed tannins (%) were calculated as leucocyanidin equivalents using the formula developed by **PORTER et al. (1986)**.

4.2.5.4 Vanillin assay for flavonoids

Plant extracts (50 µl), were made up to 1 ml with methanol in test tubes before adding 2.5 ml methanolic-HCl (95:5, v/v) and 2.5 ml vanillin reagent (1 g/100 ml acetic acid). Similar preparations of a blank that contained methanol instead of plant extracts were made. After 20 min at room temperature, absorbance was read at 500 nm using a UV-vis spectrophotometer. The flavonoid levels were expressed as catechin equivalents (CTE) **(HAGERMAN, 2002)**.

4.2.5.5 Rhodanine assay for gallotannins

Gallotannin contents from plant material were determined as described by **MAKKAR (1999)**. Plant extracts (50 µl) in test tubes were made up to 1ml with distilled water. One hundred microlitres of 0.4 N sulphuric acid and 600 µl of rhodanine were added to the diluted extracts. After 5 min, 200 µl of 0.5 N potassium hydroxide was added followed by 4 ml of distilled water after a further 2.5 min. The mixtures were left for a further 15 min at room temperature, after which the absorbance at 520 nm was read using a UV-vis spectrophotometer against a blank that contained methanol instead of sample. Each extract was evaluated in triplicates and gallotannin concentrations were expressed as gallic acid equivalents (GAE).

4.2.6 Saponin content

4.2.6.1 Qualitative saponin detection

Ten millilitres of distilled water were added to 0.1 g of ground samples in test tubes. The test tubes were corked and vigorously shaken for 2 min. The appearance of stable and persistent foam on the liquid surface for 15 min indicated the presence of saponins **(TADHANI & SUBHASH, 2006)**. The presence of saponins was confirmed by the formation of an emulsion upon addition of ten drops of olive oil to the 2 ml aqueous extract.

4.2.6.2 Preparation of extracts for saponin quantification

Saponins were extracted from the plant material as described by **MAKKAR et al. (2007)**. The dried and ground plant samples were defatted with hexane in a Soxhlet apparatus for 3 h. After airdrying, saponins were extracted twice from the defatted samples (10 g) in 100 ml of 50% aqueous methanol by incubating at room temperature overnight with continuous stirring. The extracts were then centrifuged at 3000 rpm for 10 min and the supernatant collected. The procedure was repeated with the original residue to obtain a second supernatant. The first and second supernatants were combined and filtered under vacuum through Whatman No. 1 filter paper. Methanol from the filtrate was evaporated from the solution under vacuum at 40 °C to remain with the saponin sample in the aqueous phase. The aqueous phase was then centrifuged at 3000 × g for 10 min to remove water insoluble materials. The aqueous phase was then transferred into a separating funnel and extracted three times with an equal volume of chloroform to remove pigments. The concentrated saponins in the aqueous solution were then extracted twice with an equal volume of n-butanol. The n-butanol was evaporated under vacuum at 45 °C. The dried fractions containing saponins were dissolved in 10 ml of distilled water and freeze-dried.

4.2.6.3 Quantitative determination of total saponins

Total saponin content was determined using a spectrophotometric method as described by **HIAI et al. (1976)** with modifications. The crude saponin extracts were dissolved in 50% aqueous methanol to a concentration of 10 mg/ml. From this, aliquots of 250 µl (in triplicate) of each sample were transferred into test tubes into which an equal volume of vanillin reagent (8 g/100 ml ethanol) was added followed by 2.5 ml of 72% (v/v) sulphuric acid. The mixture was mixed by vortexing and placed in a water bath adjusted at 60 °C for 10 min. The tubes were cooled in an ice-cold water bath for 3-4 min and absorbance was measured at 544 nm using a UV-vis spectrophotometer against a blank that contained 50% aqueous methanol instead of sample extract. The saponin concentrations were expressed as diosgenin equivalents (DE) calculated from a standard curve.

4.2.6.4 Quantitative determination of total steroidal saponins

Total steroidal saponins were determined following the method by **BACCOU et al. (1977)**. Crude saponin extracts were dissolved in 50% aqueous methanol (0.1 mg/ml) from which 300 µl aliquots (corresponding to a sapogenin content of between 1 and 40 µg) were transferred into test tubes and placed in a boiling water bath at 100 °C in order to remove methanol. After cooling, 2 ml of ethyl acetate were added followed by 1 ml of anisaldehyde-ethyl acetate reagent (0.5:95.5, v/v) and 1 ml sulphuric acid-ethyl acetate reagent (50:50, v/v). The test combination was vortexed and then incubated in a water bath at 60 °C for 20 min. After cooling for 10 min in a water bath at room temperature, absorbance was measured at 430 nm using a UV-vis spectrophotometer against a blank that contained ethyl acetate instead of sample. Each extract was evaluated in triplicate and steroidal saponin concentrations were expressed as diosgenin equivalents (DE) calculated from a standard curve.

4.3 DATA ANALYSIS

Data on phytochemical evaluation were subjected to one way analysis of variance (ANOVA). Means were separated by least significant difference (LSD) using SPSS statistical package (version 15.0) at the 5% level of significance.

4.4 RESULTS AND DISCUSSION

4.4.1 Antimicrobial activity

The antibacterial MIC values and antifungal MIC and MFC values of *T. violacea* extracts are presented in **Table 4.1**. Only MIC and MFC values less than 1 mg/ml were considered sufficiently active for crude extracts **(GIBBONS, 2005)**. The best antibacterial activity against *B. subtilis* was shown by the PE extracts of micropropagated plants with an MIC value of 0.39 mg/ml compared to 0.78 mg/ml

from the DCM extracts of the outdoor-grown plants. Petroleum ether extracts of micropropagated plants showed good activity (MIC < 1 mg/ml) against *B. subtilis* and *K. pneumoniae* while DCM extracts from the outdoor-grown plants were active against *B. subtilis* and *S. aureus*. Although HIV/AIDS has relatively minimal ethnobotanical treatments, the good MIC values shown by *T. violacea* against these bacterial strains provide prospects for the treatment of such pathogens since the plant is used traditionally among HIV/AIDS patients.

None of the extracts showed good activity against *E. coli*. This is surprising due to the plant's widespread use in traditional medicine in the treatment of gastrointestinal ailments **(HUTCHINGS et al., 1996)** which may be caused by *E. coli*. Petroleum ether extracts of micropropagated plants, however, showed promising activity (1.56 mg/ml) although slightly higher than 1 mg/ml. Manipulation of the culture environment may possibly result in the accumulation of high enough levels of bioactive compound(s) in these plants.

Compared to DCM, PE is a non-polar solvent and accordingly extracts less polar compounds than DCM. The observed activity from the two extracts in different plants indicates that the activity in micropropagated plants is due to non-polar compound(s) compared to the less polar compound(s) in outdoor-grown plants.

Most phytochemical compounds are produced in response to external stimuli **(DERITA et al., 2009)** such as light, moisture stress and temperature amongst others. It is possible therefore, that the consistent exposure to constant temperature and photoperiod, and media compositional balance in micropropagated plants, might have favoured the production of particular compound(s), and hence the increase in the bioactivity. In terms of the antifungal activity, greater than 1 mg/ml MIC and MFC values were recorded in all extracts of micropropagated plants as compared to the outdoor-grown plants **(Table 4.1)**. Only DCM extracts of outdoor-grown plants showed noteworthy fungicidal activity (0.78 mg/ml). The results are, however, consistent with those found in previous studies using outdoor-grown plants against the same fungus **(MOTSEI et al., 2003)**.

C. albicans is more resistant to plant extracts **(HEISEY & GORHAM, 1992; BUWA & VAN STADEN, 2006)**, and the good fungicidal activity demonstrated by *T. violacea* offers promising prospects for the treatment of candidiasis. Particularly interesting were the stable MIC and MFC values (1.56 mg/ml) shown by the PE extracts of micropropagated plants, although they were slightly higher than 1 mg/ml. The concentration of the bioactive compound(s) in these extracts, if identified, can possibly be further increased to effective levels by manipulating the culture environment. Antifungal activities of EtOH and water extracts were the same for both outdoor-grown and micropropagated plants.

The extract yields (%) and total activity of the plants using different extracting solvents are shown in **Table 4.2**. Although the yields of micropropagated plants were lower than the outdoor-grown ones in most of the extracts, their antimicrobial activities were fairly comparable. A comparison of the total activity of the corresponding extracts between the outdoor-grown and micropropagated plants indicated that micropropagated plants had more concentrated active compounds in the PE extracts than the outdoor-grown ones. For example, the yield (1.52%: equivalent to 15.2 mg/g) and MIC value (0.39 mg/ml) of the PE extracts of micropropagated plants against *B. subtilis* gave a total activity of 39 ml/g (15.2 mg/0.39 mg/ml) **(ELOFF, 2004)** compared to the PE extracts of the outdoor-grown plants with a total activity of 11.4 ml/g (17.8 mg/1.56 mg/ml) against the same bacterium.

The same PE extracts of the micropropagated plants showed good activity against *K. pneumoniae* with a total activity of 19.5 ml/g compared to 11.4 ml/g from the outdoor-grown plants, despite the fact that the outdoor-grown plants had higher extract yield than the micropropagated ones. Total activity (ml/g) indicates the degree to which the active compound(s) in one gram of plant material can be diluted and still inhibit the growth of the tested microorganism **(ELOFF, 2000)**.

Table 4.1: Antimicrobial activity of *T. violacea* extracts expressed as MIC (mg/ml) against bacteria and MIC (mg/ml) and MFC (mg/ml) against *Candida albicans*.

Sample	Extract	Yield (%)	Antibacterial MIC (mg/ml)				Antifungal	
			Bacteria				*Candida albicans*	
			Bs	Ec	Kp	Sa	MIC (mg/ml)	MFC (mg/ml)
	PE	1.78	1.56	3.125	1.56	3.125	**0.39**	6.25
T. violacea	DCM	0.93	**0.78**	3.125	1.56	**0.78**	**0.78**	**0.78**
(outdoor-grown)	EtOH	26.1	6.25	3.125	12.5	12.5	3.125	6.25
	Water	17.95	>12.5	12.5	12.5	12.5	12.5	12.5
	PE	1.52	**0.39**	1.56	**0.78**	1.56	1.56	1.56
T. violacea	DCM	1.31	3.125	3.125	3.125	3.125	3.125	6.25
(micropropagated)	EtOH	19.98	12.5	3.125	12.5	3.125	3.125	6.25
	Water	14.05	>12.5	12.5	3.125	3.125	12.5	12.5
Neomycin (µg/ml)[a]			0.098	3.13	1.56	1.56	-	-
Amphotericin B (µg/ml)[b]			-	-	-	-	0.15	9.80

MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration. B.s., *Bacillus subtilis*; S.a., *Staphylococcus aureus*; E.c., *Escherichia coli*; K.p., *Klebsiella pneumonia*; PE, petroleum ether; DCM, dichloromethane; EtOH, 80% ethanol.

Values boldly written are considered very active (1 ≤ mg/ml).

[a] Positive control for the antibacterial assay.

[b] Positive control for the antifungal assay.

The DCM extracts of the outdoor-grown plants on the other hand, were the only active ones compared to those of the micropropagated plants, with total activities of 11.9 ml/g against *B. subtilis* and *S. aureus*, respectively, compared to 4.2 ml/g from the micropropagated plants against the same bacterial strains. The DCM extracts of the outdoor-grown plants were the only extracts that showed good fungicidal activity and had a total activity of 11.9 ml/g compared to 2.1 mg/g from the DCM extracts of micropropagated plants.

Table 4.2: Extract yields (%) and total antibacterial and antifungal activity (ml/g) of *T. violacea* extracts.

Sample	Extract	Yield (%)	Total activity (ml/g)				Total activity	
			Bacteria				*Candida albicans*	
			Bs	Ec	Kp	Sa	(ml/g)	(ml/g)
	PE	1.78	11.4	5.7	11.4	5.7	**45.6**	2.8
T. violacea	DCM	0.93	**11.9**	3.0	6.0	**11.9**	**11.9**	**11.9**
(outdoor-grown)	EtOH	26.1	41.76	83.5	20.9	20.9	83.5	41.76
	Water	17.95	>14.4	14.4	14.4	14.4	14.4	14.4
	PE	1.52	**39.0**	9.7	**19.5**	9.7	9.7	9.7
T. violacea	DCM	1.31	4.2	4.2	4.2	4.2	4.2	2.1
(micropropagated)	EtOH	19.98	16.0	63.9	16.0	63.9	63.9	32.0
	Water	14.05	>11.2	11.2	45.0	45.0	11.2	11.2

B.s., *Bacillus subtilis*; S.a., *Staphylococcus aureus*; E.c., *Escherichia coli*; K.p., *Klebsiella pneumonia*; PE, petroleum ether; DCM, dichloromethane; EtOH, 80% ethanol.

Values boldly written represent total activity of extracts that were active.

This indicates that gram for gram, micropropagated plants had more concentrated active compound(s) of the less polar class (PE extractable) while the outdoor-grown plants have more concentrated active compound(s) extractable by DCM. This, however, suggests some differences in the chemical profile composition of the two plant samples. Results of the antimicrobial and total activity of the two plant samples indicate that the PE extracts of micropropagated plants can supplement outdoor-grown ones in the traditional medicinal use of *T. violacea*, particularly in the treatment of *B. subtilis* and *K. pneumoniae* related ailments.

4.4.2 Phenolic composition

The total phenolic compounds, flavonoid, gallotannin and condensed tannin contents of both outdoor-grown and micropropagated plants are presented in **Table 4.3**. Micropropagated plants showed higher total phenolic, flavonoid and gallotannin concentrations compared to the outdoor-grown plants. Particularly interesting to note was the markedly high levels of the flavonoids in micropropagated plants (15.3 ± 1.5 µg CTE/g) compared to those that are outdoor-grown (0.44 ± 0.03 µg CTE/g dry matter). Flavonoids are reported to have multiple biological effects including antioxidant **(RICE-EVANS et al., 1997; TAPAS et al., 2008)**, hepatoprotective **(TAPAS et al., 2008)** and antimicrobial activities **(TAPIERO et al., 2002; MAKKAR et al., 2007)**.

In medicinal plants, the plant's ability to concentrate bioactive compounds in high amounts within a limited period of time, makes it more preferable for use. The high levels of phenolic compounds in micropropagated plants observed in this study, demonstrate that these plants have the capacity to produce large quantities of these secondary metabolites and thus, have potential to be exploited commercially to accumulate these valuable compounds for medicinal benefits. The biosynthesis and accumulation of plant secondary metabolites depend on exogenous factors but the plant's intrinsic factors, developmental stage and tissue differentiation determine the site of synthesis **(TREUTTER, 2001; MIRDEHGHAN & RAHEMI, 2007)**. Accordingly, based on the results obtained in this study, it therefore, follows that the physiological

and/or environmental conditions are more favourable for phenolic compound production in micropropagated than in outdoor-grown plants. Nevertheless, both types of plants accumulate sufficient quantities of various non polar metabolites to exhibit some good antimicrobial activities.

Table 4.3: Total phenolics, flavonoid, gallotannin, condensed tannin, total saponin and steroidal saponin contents in micropropagated and outdoor-grown *Tulbaghia violacea* extracts. Values represent the means ± standard error (*n* = 3).

	Micropropagated	**outdoor-grown**
Total phenolics (mg GAE/ml)	3.56 ± 0.06a	0.45 ± 0.021b
Flavonoids (µg CTE/ml)	15.3 ± 1.5a	0.44 ± 0.03b
Gallotannins (µg GAE/ml)	4.03 ± 0.24a	2.32 ± 0.02b
Condensed tannins (% LCE/g)	0.72 ± 0.22b	0.84 ± 0.078a
Total saponins (mg DE/ml)	25.14 ± 0.74a	8.94 ± 0.11b
Total steroidal saponins (mg DE/ml)	10.03 ± 0.58a	3.77 ± 0.43b

Values with different letters in a row indicate significant differences between treatments ($P \leq 0.05$). GAE, gallic acid equivalents; CTE, catechin equivalents; LCE, leucocyanidin equivalents; DE, diosgenin equivalent.

4.4.3 Saponin composition

The qualitative froth test for the presence of saponins was positive for both types of plant material. **Table 4.3** represents total saponins and total steroidal saponins in micropropagated and outdoor-grown *T. violacea* plants. As was the trend with the phenolic content, saponins and steroidal saponins were significantly higher in micropropagated plants compared to the outdoor-grown ones.

Saponins, particularly steroidal saponins have been reported in *T. violacea* **(BURTON, 1990)**. The results obtained in this study agree with these previous findings. However, the markedly high saponin content obtained in micropropagated plants compared to the outdoor-grown plants is an indication that *in vitro* culture conditions are favourable for the production of plant secondary metabolites. Saponins are reported to have antimicrobial activity **(BADER et al., 2000)**, anti-inflammatory **(NAVARRO et al., 2001)** and haemolytic **(ODA et al., 2000)** properties. This markedly high saponin composition recorded in micropropagated plants shows their promising pharmacological potential as sources of bioactive molecules. Manipulation of the culture environment may be effective in increasing accumulation of these compounds.

4.5 CONCLUSIONS

The antibacterial results exhibited by micropropagated *T. violacea* plants in comparison with the outdoor-grown ones were fairly comparable. Although micropropagated plants did not show good antifungal activity compared to the outdoor-grown plants, their PE extracts appeared to contain some active antifungal compound(s). However, the concentration(s) of the compound(s) might have been lower than those required to effect good activity. Micropropagated plants of *T. violacea* may be used as alternative sources of treatments of some ailments caused by bacteria in traditional medicine and have a promising potential to produce better activity. The generally high total phenolics, gallotannin, flavonoid and saponin contents recorded in micropropagated plants in contrast to those grown outdoors, makes them potential sources for the production of secondary metabolites for medicinal purposes. The fact that micropropagated plants produce secondary metabolites at an early stage of growth provides an opportunity for rapid production of pharmacological compounds that can be utilised for medicinal purposes. Although the yields of polar extracts (water and EtOH) of the outdoor-grown plants were higher

than those from the micropropagated ones, the latter yielded high levels of the screened polar compounds (phenolics and saponins) in this study. This may, however, indicate the presence of other polar compounds in these extracts, such as carbohydrates. Further synergistic studies to determine the biological activity of all the combined extracts are required to give an overall picture of the true value of this plant.

CHAPTER 5
General Conclusions

An efficient protocol for the micropropagation of *T. ludwigiana* and *T. violacea*, which are known for their ornamental, medicinal and nutritive value, was established, using seedlings as sources of explants. Seeds of *T. ludwigiana* and *T. violacea* were successfully decontaminated using 70% ethanol, 1% Benlate and 3.5% NaOCl. Germinating seeds *in vitro* decreased the chances of contamination, which ensured a "sterile" source of explants for the micropropagation of these plants. Temperature played a significant role in the germination of both species while light did not play a significant role in the germination of both species. Light did not play a major role in the stomatal density of *T. violacea* seedlings.

Hypocotyls were the most regenerative part of the seedlings in both species. A low number of shoots was yielded by the combination of various concentrations of NAA and *m*TR in the growth medium in both species. *Tulbaghia violacea* responded better when cultured in a medium with 5 µM *t*Z, 5 µM BA and 0.53 µM NAA. *Tulbaghia ludwigiana* generally did not respond as well as *T. violacea* in culture. Cytokinins were more abundant in the roots of *T. violacea* seedlings than in the hypocotyls and leaves. Shoots of both species were successfully rooted in a medium with IBA, while some shoots had simultaneously rooted during shoot multiplication. Plantlets of *T. violacea* acclimatized successfully in a mixture of potting soil and vermiculite, while this was not the case for those of *T. ludwigiana*.

The fact that micropropagated *T. violacea* plants were found to have higher concentrations of phytochemical compounds than outdoor-grown plants and displayed good antibacterial activity makes them a good alternative, as a source of medicine, to outdoor-grown plants, thus potentially reducing the pressure on these bulbous medicinal plants. Further work is required to investigate the role of other plant growth regulators on the growth and development of both these plants. Suitable conditions for the successful acclimatization of *T. ludwigiana* also require further

investigation. The results of the study presented here not only provide a better understanding of the micropropagation of these two species, but form a basis for future research.

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