Micropropagation and pharmacological evaluation of *Boophone disticha*

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COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCES

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

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Thesis title: **Micropropagation and pharmacological evaluation of *Boophone disticha***

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

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ABSTRACT

Boophone disticha (L.f.) Herb is one of the most widely distributed bulbous species in southern Africa. Of Africa's many bulbous plants, it is widely known for its poisonous and medicinal properties. It is of considerable ethnobotanical interest in traditional medicine because of its hallucinogenic alkaloids and it has great potential as an ornamental due to its fan-shaped foliage and large umbel of bright pink to deep red flowers.

In South Africa, many bulbous plants are used in traditional medicine which are collected from wild populations. The high demand for trade and use of such plants, that are destructively harvested, places an enormous pressure on natural populations. According to the Red List of South African Plants, the conservation status of *B. disticha* has been listed as 'declining'. It is, therefore, important to develop conservation strategies for these medicinal plants, such as the development of alternative propagation methods.

Micropropagation is a useful technique for rapid clonal multiplication of plant material which could alleviate the pressure on the wild plant populations, as well as potentially producing useful secondary metabolites. The *in vitro* induction of storage organs is especially beneficial as it can limit the loss of plants during acclimatization since bulblets are generally hardier than shoots or plantlets. Thus, the main aim of this research was to establish a micropropagation protocol which could be a valuable tool for conservation of this plant species. In addition, *B. disticha* plants were assessed in various ethnopharmacological assays to evaluate their medicinal properties, and a preliminary study on the population genetics was also conducted.

As part of the development of a suitable micropropagation protocol, the effect of environmental and physiological factors on the initiation and growth of bulblets were investigated. These factors included the effect of various plant growth regulators, carbohydrates, temperature, photoperiod and liquid culture. Different explants (i.e. ovaries, anthers, filaments, pedicels, embryos, seeds and bulb twin-scales) were tested to determine which explants were the most suitable for subsequent experiments. Although success was limited, twin-scales proved to be the most suitable explant and it was demonstrated that activated charcoal, ascorbic acid and N^6-benzyladenine were required as media supplements.

Antimicrobial activity was tested between different plant parts and seasons. The plant parts (roots, leaves, outer and inner bulb scales) were extracted with a range of differing polarity solvents. These were screened for antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*, and for antifungal activity against *Candida albicans*. Extracts from roots of plants collected in spring and summer showed the best antimicrobial activity against *B. subtilis, E. coli* and *K. pneumoniae,* indicating that plant part and collection time do affect activity. *In vitro* grown bulblets also showed antimicrobial activity, demonstrating that antibacterial properties were maintained in cultured plantlets.

Extracts from plants collected in summer were tested for mutagenicity using the Ames test (*Salmonella*/microsome assay; plate incorporation method, with or without metabolic activation). None of the extracts tested were found to induce mutations and also did not modify the effect of the mutagenic compounds (2AA with S9 and 4NQO without S9). Although the results do not indicate a mutagenic response, this does not necessarily confirm that it is not mutagenic nor carcinogenic to other bacterial strains, however, *B. disticha* must be used with caution, especially considering the levels of alkaloids in the plant.

The two major constituent alkaloids of *B. disticha* were identified as buphanidrine and distichamine. In the antibacterial assay, both compounds exhibited broad-spectrum micromolar-level activity against the two Gram-positive and two Gram-negative bacteria tested. The best MIC value, of 0.063 mg/ml, was found for bupanidrine/distichamine against *S. aureus*, *E. coli* and *K. pneumonia.* The isolated compounds were tested and found to be neither mutagenic nor toxic at the concentrations tested. Thus, buphanidrine and distichamine are thought to be the constituents likely responsible for the medicinal properties of the plant.

To determine the level of genetic variation between different populations of *B. disticha*, plants were collected from six wild populations in KwaZulu-Natal, South Africa. DNA was isolated and tested for genetic variation using ten Inter Simple Sequence Repeat (ISSR) primers. The level of inter-population polymorphism ranged between 23% and 39%, showing that the populations had low genetic polymorphism. From the genetic distance results, it was found that the Midmar and Umgeni Valley populations are closely related, and these populations are similar to two sister populations. The Amatikulu and Lions River populations were similar but slightly different to the other populations. Antimicrobial assays showed minor difference in activity from the six wild populations.

Although the micropropagation of *B. disticha* had limited success, this study did develop a successful decontamination protocol as well as determine the most useful explant and supplements. This information provides an important starting point for the development of a successful micropropagation protocol for the conservation of *B. disticha*. Since, *B. disticha* is an important medicinal plant in South Africa, this study has also deepened our understanding of the constituents that could be responsible for the medicinal properties of *B. disticha* and, in so doing, confirmed the value of this plant for use in traditional medicine in South Africa.

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LIST OF ABBREVIATIONS

2AA	2-aminoanthracene
ABA	abscisic acid
AFLPs.....	amplified fragment length polymorphisms
AIDS.....	acquired immunodeficiency syndrome
ANOVA	analysis of variance
BA	N^6-benzyladenine; 6-benzylaminopurine
[3G]BA.....	3-glycosylbenzyladenine
[7G]BA.....	7-glycosylbenzyladenine
[9G]BA.....	9-glycosylbenzyladenine
2,4-D.....	2,4-dichlorophenoxy acetic acid
DCM.....	Dichloromethane
DMRT	Duncan's Multiple Range Test
DMSO	dimethyl sulfoxide
EtOH.....	Ethanol
GAs.....	Gibberellins
$HgCl_2$.....	mercuric chloride
HIV.....	Human immunodeficiency virus
IAA.....	indole-3-acetic acid
IBA	indole-3-butyric acid
INT	*p*-iodonitrotetrazolium chloride
iP.....	N^6-isopentenyladenine
ISSRs.....	inter-simple sequence repeats
MeJa	methyl jasmonate
MFC	minimal fungicidal concentration

MH broth	Mueller-Hinton broth
MIC	minimal inhibitory concentration
MS	Murashige and Skoog (1962) medium
*m*T	*meta*-topolin
NAA	napthaleneacetic acid
NaOH	sodium hydroxide
4NQO	4-Nitroquinoline-N-oxide
PAA	phenylacetic acid
PAC	Paclobutrazol
PE	petroleum ether
PCR	polymerase chain reaction
PGR	plant growth regulator
PVP	Polyvinylpyrrolidone
RAPDs	random amplified polymorphic DNAs
RFLPs	restriction fragment length polymorphisms
rpm	revolutions per minute
SERT	serotonin transporter
SSRs	simple sequence repeats
UKZN	University of KwaZulu-Natal
YM broth	yeast malt broth

Chapter One

LITERATURE REVIEW

1.1 MEDICINAL PLANTS IN SOUTH AFRICA

South Africa has an extremely rich diversity of plants and boasts approximately 30 000 plant species of which approximately 2 700 are geophytes (LOUW *et al.*, 2002). The indigenous peoples of southern Africa have a long history of traditional plant usage for medicinal purposes, with approximately 4 000 species of plants being used (MULHOLLAND & DREWES, 2004; NIELSEN *et al.,* 2004). The World Health Organization has estimated that more than 80% of the world's population in developing countries depends mainly on herbal medicine for basic healthcare use (CANTER *et al.*, 2005). This is a result of the high cost of drugs, treatment failure due to drug resistance and the prolonged and costly treatment of some diseases which the general population cannot afford (AFOLAYAN & ADEBOLA, 2004). In China, India and Europe, plants are grown on a large scale to meet the growing demand for traditional herbal medicine, however in Africa the most common practice is to collect medicinal plants from the wild (ZSCHOCKE *et al*., 2000).

The trade in medicinal plants is an important part of the regional economy in South Africa with over 700 plant species being traded (DOLD & COCKS, 2002). The collection of medicinal plants has become a form of rural self-employment which generates a lot of income for the rural poor. It is estimated that there are 27 million indigenous people who consume medicinal plants in South Africa (MULHOLLAND & DREWES, 2004), and demand, therefore, exceeds supply. Up until the late 1980's, little attention was paid in southern Africa to the conservation of medicinal plants. However, due to destructive harvesting and the rapid increase in the informal trade of medicinal plants, the management of these natural plant resources has become a matter of urgency (ZSCHOCKE *et al*., 2000). This is evident in the local extinction of species such as *Siphonochilus aethiopicus* (wild ginger) and *Ocotea bullata* (black stinkwood). Approximately two-thirds of the medicinal plant species in use are collected from the wild and this harvesting from the wild is resulting in diminishing populations, loss of genetic diversity, local extinction and habitat degradation. Based on this, CANTER *et al.* (2005), reported that between 4 000 and

10 000 medicinal species around the world might now be endangered. DOLD and COCKS (2002), carried out a study on the trade of medicinal plants in the Eastern Cape Province which revealed a minimum of 166 plant species providing 525 tons of plant material valued at R27 million annually. This case study gives credence to the claim of CANTER *et al.* (2005).

The most commonly used medicinal plants in southern Africa are slow-growing trees, bulbous and tuberous plants. In KwaZulu-Natal, 1 032 plant species from 147 families are reportedly used, of which, bark and roots (54%) and bulbs and whole plants (28%) are the most important ingredients of Zulu herbal medicine sold in street markets (MANDER, 1997; LIGHT *et al*., 2005). The indigenous bulbous plants that are of most importance to traditional healers mainly belong to the Amaryllidaceae and Hyacinthaceae families (LOUW *et al*., 2002). *Boophone disticha* (L.f.) Herbert (= *Boophane disticha*) (Amaryllidaceae), also known as Century bulb, Sore-eye flower, Tumbleweed (Eng.); Gifbol, Perdeskop (Afr.); Incotho, Incwadi and Ibhade (Xhosa, Zulu), is one of the most widely distributed bulbous species in southern Africa. Of Africa's many bulbous plants, *B. disticha* is generally well known for its poisonous and medicinal properties (COCKS & DOLD, 2000; MANNING *et al*., 2002).

Boophone disticha is of considerable ethnobotanical interest as a hallucinogen in traditional medicine and is in high demand with the indigenous people and traditional healers (GOLDING, 2002). In 2010, the conservation status of *B. disticha* was listed as 'declining' (http://redlist.sanbi.org) and because of this it needs to be cultivated. As it is a perennial that requires several years to establish and become harvestable, a system has to be developed whereby many plants can be grown in a shorter period of time. This will ensure the production of bulbs that can be used by traditional healers and in so doing, reduce large scale collection from the wild.

1.2 *BOOPHONE DISTICHA*

1.2.1 Distribution and morphology

Boophone is a genus that consists of two species, namely *B. disticha* and *B. haemanthoides* (F.M.) Leight. Both species are distributed throughout southern Africa, extending into southern tropical Africa. *Boophone disticha* is the most widespread and variable member, occurring mainly in the summer rainfall regions, whereas *B. haemanthoides* is found mainly in Namaqualand and the Western Cape of South Africa, in areas with winter rainfall (DU PLESSIS & DUNCAN, 1989) (Figure 1.1).

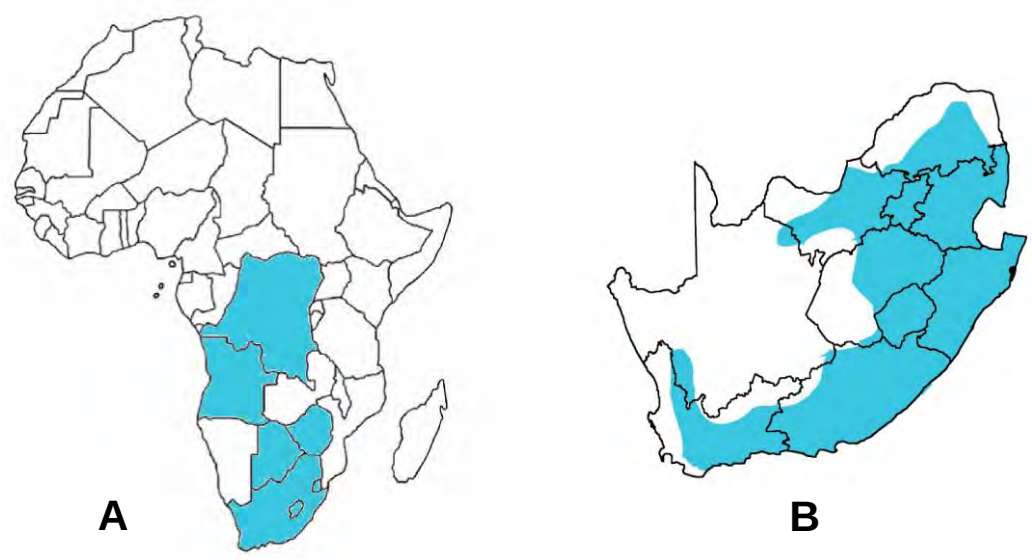


Figure 1.1: Map of (A) Africa showing the distribution of both *Boophone* species; and (B) South Africa showing the distribution of *B. disticha*.

Boophone disticha is a bulbous geophyte with a large proportion of the ovoid bulb growing above the ground. A normal bulb is usually about 22 cm long, 15 cm thick and 1.4 kg in weight. The dry outer scales are papery and wine-red to brownish in colour, while the inner scales are white and fleshy (NEUWINGER, 1994). The foliage of between 12 and 20 leaves is usually grey-green after flowering, spreading into an erect fan, approximately 450 x 50 mm. The margins are flat or wavy (Figure 1.2).

Figure 1.2: Photographs of *B. disticha* showing the fan-like leaves, ovoid bulb and the dense umbel of flowers.

The stem is short and the inflorescence is a dense umbel, 300 mm in diameter, of numerous small to medium star-shaped flowers, 50 mm in diameter (Figure 1.2). *Boophone disticha* is hysteranthous, the inflorescence is usually produced before the leaves (DU PLESSIS & DUNCAN, 1989). The flowers are sweetly scented and vary in colour from bright pink to deep red. The flower stalks elongate as the seeds ripen and the whole umbel dries and breaks off to roll around and disperse the seeds. The seeds are fleshy, subglobose, 8-11 mm in diameter, pale green with a thin corky covering (MANNING *et al.,* 2002).

Boophone haemanthoides is proteranthous in which the foliage dies before the inflorescence is produced. The bulb is normally 18 cm in diameter and the leaves are in an upright fan, 2.5-8 cm wide, bluish-grey and the margins are wavy with obtuse tips. There are 100 or more, creamy-pink flowers in a compact, brush-like cluster, surrounded by two large red bracts. The fruit is a capsule containing a few rounded fleshy seeds (DU PLESSIS & DUNCAN, 1989; MANNING & GOLDBLATT, 1996; MANNING *et al.*, 2002).

1.2.2 Horticultural use

According to BRYAN (2002), both *Boophone* species have, in recent times, been introduced to cultivation. *B. haemanthoides* is highly ornamental, while, *B. disticha*, the most common species, deserves consideration because of its fan-shaped foliage and large umbel of flowers. *Boophone disticha* is highly ornamental and can be grown in the garden or planted in large containers on a sunny veranda.

1.3 MEDICINAL AND OTHER USES

1.3.1 Use in traditional medicine

Boophone disticha has been cited much in the literature with regards to its widespread use in treating various ailments. The bulbs of the plants are the richest source of alkaloids, especially the epidermis of the scales (FROHNE & PFÄNDER, 2004). Mucilage-filled raphide cells, which occur in all parts of the plant, are also said to contain a large amount of alkaloids (FROHNE & PFÄNDER, 2004), which is why, *B. disticha* is used in a number of countries to treat ailments.

Bulbs of *B. disticha* are used extensively for medicinal purposes by many tribes in South Africa, Namibia, Zimbabwe and Mozambique. The Xhosa people use the dry scales of the bulb as an antiseptic and pain-relieving dressing after circumcision. The scales are also used to treat painful joints, swelling, bruises, sores, rashes, burns, abscesses and boils (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS *et al*., 1996; VAN WYK & GERICKE, 2000; DIEDERICHS, 2006). In Mozambique, the macerated bulb is used to increase sexual potency, whereas, in cases of paralysis, powder obtained from pieces of burnt bulb is rubbed into scratches in the skin over the area of paralysis (NEUWINGER, 1994).

Bulb decoctions are given to adults suffering from headaches, sharp chest pains, weakness and persistent bladder pains. A weak decoction is often administered as a sedative to violent, psychotic patients and sometimes to hysterical adolescent females (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS *et al*, 1996; VAN WYK *et al*., 1997; VAN WYK & GERICKE, 2000; NIELSEN *et al.,* 2004).

According to HUTCHINGS *et al*. (1996), mattresses filled with teased bulbs, are used for hysteria and sleeplessness. Dried bulb scales are often placed in a pillow, as it is believed to provide relief from asthma. Bulbs have also been used as narcotics by the Sotho and Xhosa peoples, and are used in medicines taken to "arouse spirits" (POOLEY, 1998).

Fresh leaves are used in the treatment of various skin ailments and to stop bleeding. In Malawi, flower infusions are taken in porridge for dizziness (HUTCHINGS *et al*., 1996).

1.3.2 Other uses

The concentrated bulb juice of *B. disticha* was used as an arrow poison by the Khoi and San people during hunting. The poison is a fast-acting cardiac glycoside and is used primarily for hunting small game (LEWIS & ELVIN-LEWIS, 1977; DU PLESSIS & DUNCAN, 1989; HUTCHINGS *et al*., 1996; VAN WYK & GERICKE, 2000). Bulbs are used as drenches for constipation in ruminants in Zimbabwe and the Xhosa people use it as a remedy for red-water disease in cattle (WATT & BREYER-BRANDWIJK, 1962).

Leaves are stripped to make fringes and are worn as decorative body ornaments. In Mozambique, the bulb is used for magical purposes such as "helping the soccer team win". The Manyika people grow *B. disticha* outside their hut as a charm to ward off evil dreams, to bring good luck and to bring rain (NEUWINGER, 1994).

The plant is sometimes used as a hallucinogen in male adolescent initiation rites and in the initiation of diviners. At the beginning of the secret initiation ceremony, the South African preadolescent Basuto are given food mixed with the crushed bulb and other ingredients. The remedy is believed to allow the boys to "contact their ancestors" and instil them with the qualities of their ancestors and hasten adulthood (DE SMET, 1996; RATSCH, 2005). The Sotho people use the "alcoholising" properties of *B. disticha* during the preliminaries of initiation. A powder which is made from the bulb is mixed with other plants and heated and the smoke is inhaled. The smoke makes the initiates "drunk" and when the signs of intoxication appear, this is taken as a sign that the spirit of manliness has entered into the youths (RATSCH, 2005).

It is clear that *B. disticha* has many uses; from the bulb being used as a means to commit suicide, arrow poison and traditional medicine, to the flowers being used to treat dizziness.

1.4 ALKALOIDS

1.4.1 Amaryllidaceae alkaloids

Many members of the Amaryllidaceae are regarded as toxic. The toxic constituents that occur in the whole family are referred to as the Amaryllidaceae alkaloids because of their limited taxonomic distribution. More than 100 structurally different Amaryllidaceae alkaloids, comprising 12 distinct ring types, have been isolated from 150 species belonging to 36 genera of this plant family (GHOSAL *et al*., 1985). WALLER and NOWACKI (1978) reported that only the Amaryllidaceae have alkaloids of the norbelladine type. Interestingly, alkaloids found in other plant families have not been found in Amaryllidaceae species (NEUWINGER, 1994; ELGORASHI *et al.,* 2003a).

The Amaryllidaceae alkaloids are not only known for their toxicity but they also demonstrate narcotic, hypotensive, vasodilatory and analgesic activity. Consequently, plants belonging to this family have become the focus of many pharmacological investigations (VAN WYK *et al*., 1997; ELGORASHI *et al.,* 2003a). There are three major structurally distinct classes of Amaryllidaceae alkaloids: lycorane, galanthamine and crinane. Several other less common structural variants are also known (McNULTY *et al*., 2009). Galanthamine is the first of the Amaryllidaceae alkaloids to have been approved as a pharmaceutical in the treatment of a human disease. Due to galanthamine's reversible anticholinesterase activity, which is of importance in the treatment of Alzheimer's and other neuro-degenerative diseases, a prescription drug under the generic name Reminyl has been clinically approved (McNULTY *et al*., 2009). Another pharmacological action of galanthamine is its ability to amplify the nerve-muscle transfer by reversing non-depolarizing neuromuscular blockers and restoring synaptic transmission (GHOSAL *et al*., 1985; MARTIN, 1987). Besides this, galanthamine acts as a mild analeptic, shows analgesic power as strong as morphine and is used in Eastern Europe as a reversal agent in anaesthetic practice (EICHHORN *et al*., 1998).

Alkaloids of the crinane class have been shown to display a range of biological activities. Crinamine is known to be cytotoxic to the malaria parasite and to a number of tumour cell lines (LIKHITWITAYAWUID *et al*., 1993), while NAIR *et al.* (1998) reported the activity of 6-hydroxycrinamine against mouse melanoma cells. Haemanthamine inhibits protein synthesis by preventing peptide bond formation as well as having anti-proliferative action and hypotensive

activity (WILDMAN, 1960; JIMENEZ *et al*., 1976; GHOSAL *et al.,* 1985; HOHMANN *et al*., 2002). McNULTY *et al*. (2007) showed through biological screening that haemanthamine is a potent inducer of apoptosis in tumour cells at micromolar concentrations.

In the lycorane class, much attention has focused on pancratistatin and its analogues such as narciclasine, due to their powerful and selective anticancer properties (KORNIENKO & EVIDENTE, 2008). McLACHLAN *et al.* (2005) showed that pancratistatin induces apoptosis selectively in cancer cells with minimal effect on normal cells. This provides an encouraging incentive for the development of possible tumour cell-line targeted chemotherapeutics. Narciclasine inhibits protein synthesis in eukaryotic cells, has antileukemic properties, and is an antimitotic substance that shows promise as an anti-cancer agent (SPOERKE & SMOLINSKE, 1990; HARBORNE & BAXTER, 1993).

According to LOUW *et al.* (2002) the Amaryllidaceae alkaloids have shown antitumour potential and also showed *in vivo* activity against a number of different human viruses. Lycorine is the most common alkaloid in the Amaryllidaceae family and shows a great variety of biological properties. It has shown outstanding antiviral activity against poliomyelitis, coxsackie, vaccinia smallpox, SARS-associated coronavirus and herpes type 1 viruses (HARBORNE & BAXTER, 1993; LI & TAN, 2005; DENG *et al.,* 2007). Lycorine was reported to inhibit the synthesis of DNA and proteins in murine cells as well as to inhibit ascorbic acid synthesis (WILDMAN, 1960; GHOSAL *et al.,* 1985; LEWIS, 1990). *In vivo* growth of a murine ascite tumour was inhibited and the viability of *in vitro* grown tumour cells reduced (GHOSAL *et al.,* 1985). LIKHITWITAYAWUID *et al.* (1993) reported that as a potential chemotherapeutic, lycorine showed most promise as an antiproliferative agent of a number of cancer cell lines. During an investigation into the *in vitro* mode of action in a leukemia cell line, it was discovered that lycorine suppressed tumour cell growth and reduced cell survival via cell cycle arrest and induction of apoptosis (LIU *et al.*, 2004).

Palmilycorine, a derivative of lycorine, was shown to have an inhibitory effect on the viability of ascites tumour cells (GHOSAL *et al.*, 1985), while McNULTY *et al.* (2009) showed that only lycorine and pseudolycorine, also a derivative of lycorine, were able to induce apoptosis in human leukemia cells. With regards to antibacterial and antifungal activity, lycorine and pseudolycorine, inhibit protein synthesis in eukaryotic cells by blocking peptide bond formation, while lycorine has demonstrated antifungal activity against *Phymatotrichum omnivorum* and

Saccharomyces cerevisiae (WALLER & NOWACKI, 1978; DEL GIUDICE *et al.*, 2005). Lycorine is also fatal to the protozoan parasite *Trypanosoma brucei*, is a potent anti-inflammatory agent, has insect antifeedant activity and possesses plant growth inhibitory properties as it suppresses cell division and elongation (EVIDENTE *et al.*, 1986; HARBORNE & BAXTER, 1993; CITOGLU *et al.*, 1998; MACKEY *et al.*, 2006). Lastly, lycorine inhibits the enzyme acetylcholinesterase, which is of great importance in motor neuron diseases (LOPEZ *et al.*, 2002).

Plants from the Amaryllidaceae still continue to yield novel compounds which have interesting biological activity. It is the isolation of these alkaloids and further *in vitro* and *in vivo* testing that may lead to the discovery of new pharmaceuticals.

1.4.2 *Boophone disticha* alkaloids

Boophone disticha is used to treat a large variety of ailments, as discussed in Section 1.3.1, because of the medicinal activity which probably can be attributed to the alkaloids in the plant. Eleven alkaloids have been isolated from *B. disticha* and eight of them are known to occur in other Amaryllidaceae plants, namely; buphanidrine, undulatine, buphanisine, buphanamine, nerbowdine, crinine, crinamidine, and lycorine (Figure 1.3). The three alkaloids unique to *B. disticha* are distichamine, acetylnerbowdine and buphacetine (WATT & BREYER-BRANDWIJK, 1962; NEUWINGER, 1994; DE SMET, 1996; SANDAGER *et al.,* 2005). The *B. disticha* alkaloids are mainly neurotoxins and significant analgesic activity has been demonstrated in some of them, such as buphanidrine and crinine (NEUWINGER, 1994; VAN WYK & GERICKE, 2000). Buphanidrine is the main toxin found in *B. disticha* and is a powerful analgesic, hallucinogen and neurotoxin.

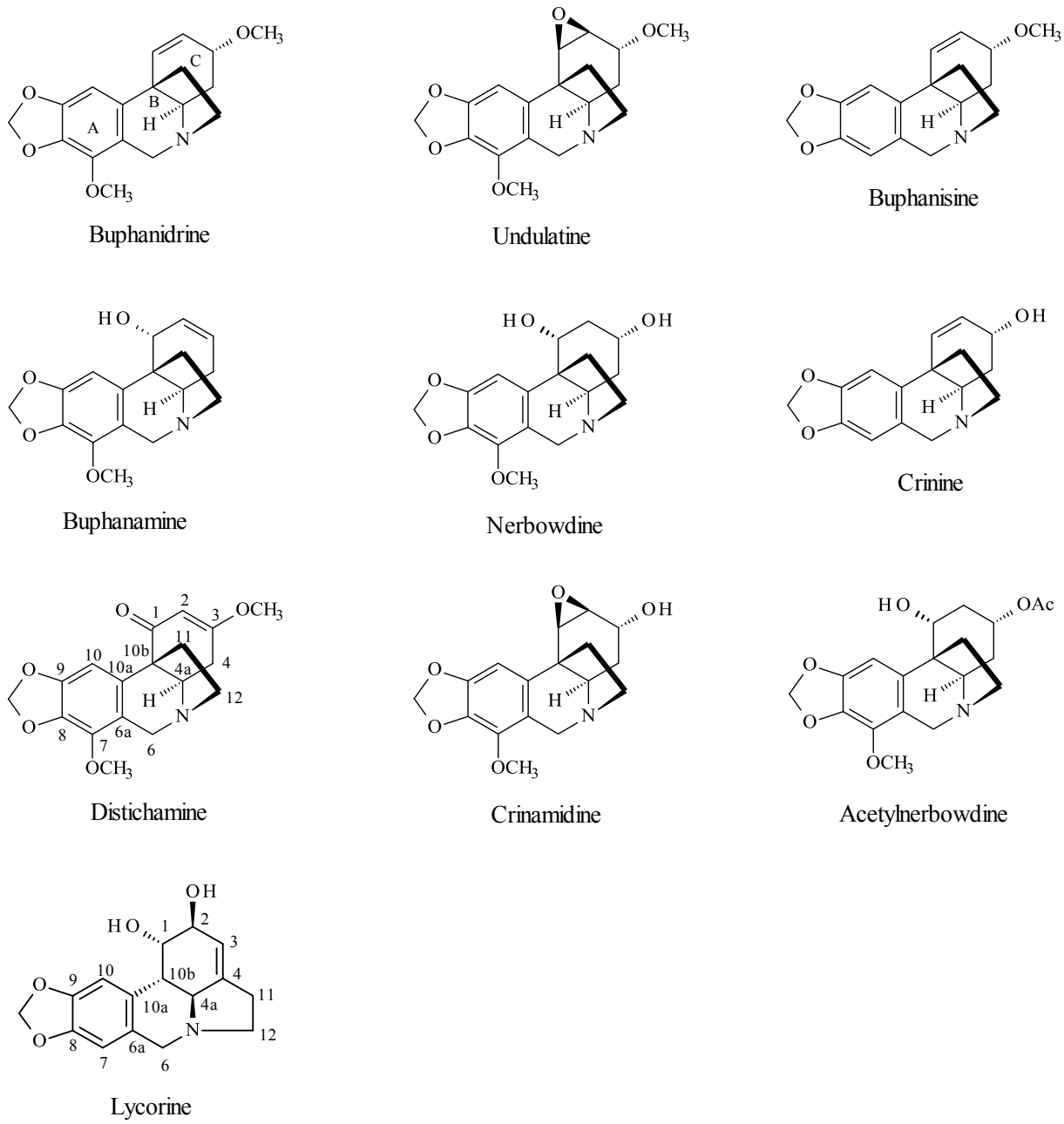


Figure 1.3: Ten of the eleven alkaloids found in *Boophone disticha*.

In general, the alkaloids are of high to medium toxicity. The alkaloids belonging to the crinane class are more toxic than alkaloids from the other two classes. For buphanidrine, the LD_{50} is 8.9 mg/kg for mice s.c. while 10 mg/kg i.v. of buphanidrine or crinine is fatal for dogs (WILDMAN, 1960). For lycorine, on the other hand, the LD_{50} for mice is reported to be 117 mg/kg i.p., 123 mg/kg i.v. and 230 mg/kg orally, while 41 mg/kg i.v. was found to be lethal for dogs (WILDMAN, 1960). Many cases of both acute and fatal poisoning in humans with *B. disticha*

have been reported (WATT & BREYER-BRANDWIJK, 1962). These cases are usually accidental as a result of the underestimation of the toxicity of the particular bulb used to prepare the medicine or due to the administration of too large a dose of the decoction (WATT & BREYER-BRANDWIJK, 1962). Mild poisoning will produce temporary symptoms such as an unsteady gait, nausea, vomiting, giddiness, impaired vision and variable emotional reactions followed by stupor and sleep. Acute poisoning, however, results in severe vomiting, quickening and weakening of the heart, coma and death (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS *et al*., 1996).

According to BOTHA *et al*. (2005), due to the suggested therapeutic properties of the extracts from the bulb of *B. disticha* on the immune system and inflammatory response, as well as its ability to treat anxiety and other central nervous system diseases in traditional medicine (NEERGAARD *et al.,* 2009), *B. disticha* alkaloids are of possible pharmacological importance.

Boophone disticha is used as a remedy for depression and anxiety, and was, therefore, investigated for its affinity to the serotonin reuptake transport protein, using an *in vitro* serotonin reuptake transport protein binding assay (NIELSEN *et al.*, 2004). NEERGAARD *et al.* (2009) also tested *B. disticha* alkaloids for their affinity to the serotonin transporter (SERT). Of the 34 plant species tested by NIELSEN *et al.* (2004), *B. disticha* showed high affinity (more than 50% inhibition at 5, 1 and 0.5 mg/ml) to the serotonin reuptake transport protein in both aqueous and ethanolic extracts of leaves and bulbs. NEERGAARD *et al.* (2009) isolated 5 alkaloids from *B. disticha*; crinine, buphanamine, buphanidrine, distichamine and buphanisine, which were tested in a binding assay using [^{3}H]-citalopram as a ligand and a functional SERT inhibition assay. Buphanamine, buphanidrine and distichamine were the most active in the SERT binding assay with IC_{50} values of 55 µM, 62 µM and 65 µM, while buphanisine showed a lower potency with an IC_{50} value of 199 µM. In a previous study by SANDAGER *et al.* (2005), buphanidrine and buphanamine were also shown to have affinity to SERT. Crinine showed weak activity in the SERT assay and this corresponded to earlier findings by ELGORASHI *et al.* (2006). Of the 5 compounds isolated, only buphanidrine and distichamine were tested in the functional SERT inhibition assay and both showed activity with IC_{50} values of 513 µM and 646 µM. ELGORASHI *et al.* (2006) suggested that some of these alkaloids could be responsible for a dual effect in the treatment of depression and Alzheimer's disease. A problem with this, however, is that the crinine-type alkaloids show affinity for SERT but have weak acetylcholinesterase inhibitory effects, while lycorine-type alkaloids have shown activity as

acetylcholinesterase inhibitors but have no or weak affinity for SERT (ELGORASHI *et al.,* 2004; 2006).

1.5 PROPAGATION

1.5.1 Conventional propagation

Boophone species require full sun and an extremely well-drained, sandy medium. The plants thrive in dry grassland and also in rocky areas where the roots can become firmly established. Both members of the genus do not like being moved and require permanent positions.

Offset bulbs, which form slowly, are produced in small numbers and should be removed without disturbing the roots of the parent bulbs (BRYAN, 2002). Propagation is, therefore, quickest by seeds that are freely produced. Seeds should be collected and planted as soon as they are ripe, into a well-drained mixture of soil and compost. The seeds usually germinate within two weeks and the germinated seeds should be carefully inserted into a soil medium that is kept moist. Once the first leaves appear, the young seedlings can be potted up into deep permanent containers (DIEDERICHS, 2006). Plants grown from seed can be expected to flower and set seed within three to four years. According to DU PLESSIS and DUNCAN (1989), *B. disticha* may take seven years to flower from seed.

The genus requires protection from severe frost and amaryllis caterpillars. The caterpillars can be a serious pest, as they are known to bore into the leaves and bulbs of plants which they destroy (DU PLESSIS & DUNCAN, 1989; DIEDERICHS, 2006).

1.5.2 Value of *in vitro* culture methods

Although traditional propagation methods for geophytes have been adequate for centuries, tissue culture is a valuable alternative and has several advantages. Conventional propagation methods include natural division, scaling, chipping and other methods. There are, however, two main disadvantages with these methods. The first is that it is difficult to produce a large number of plants in a short period of time and the second is that diseases can be easily spread from infected plants (KIM & DE HERTOGH, 1997).

Some of the advantages for tissue culturing geophytes is that new cultivars, species and recalcitrant species can be rapidly multiplied, virus-free plants can be produced and the technique allows for crop improvement (HUSSEY, 1982a; KIM & DE HERTOGH, 1997; CHANG *et al.,* 2000). Tissue culture can also aid with the conservation of valuable biodiversity and the micropropagation of whole plants can play a part in the establishment of breeding material from wild populations and in mass-producing material for selection or genetic engineering. Tissue culture often leads to genetic disturbances, which results in somaclonal variation, which can extend the range of variation for the plant breeder to possibly use for plant improvement (KRIKORIAN & KANN, 1986; DEBERGH, 1994; CHANDLER & LU, 2005; CANTER *et al*., 2005).

Micropropagation also allows for storage of plantlets in a small space, year round multiplication, the avoidance of long dormant seasons and easy international exchange of plant material (HUSSEY, 1980; KIM & DE HERTOGH, 1997). Lastly, controlled culture conditions, both chemical and environmental, makes tissue culture very advantageous. By controlling the conditions, optimal conditions for the production of secondary metabolites, plantlet regeneration and growth can be established. Therefore, tissue culture techniques can provide plant breeders with a large volume of high quality material that will limit the need for large-scale collection from the field.

1.5.3 Tissue culture of the Amaryllidaceae

The Amaryllidaceae is one of the most horticulturally important families of monocotyledons. The family includes a number of plants that are an important source of pharmaceuticals, as well as important ornamentals. Consequently, many species of Amaryllidaceae have been successfully cultured *in vitro* (Table 1.1).

Table 1.1: Successful micropropagation protocols that have been developed for various genera of the Amaryllidaceae.

Genus	Explant	Growth response	Reference
Amaryllis belladonna	Bulb-scales	Bulblets and plantlets	MURASHIGE, 1974 DE BRUYN *et al*., 1992
Clivia spp.	Inflorescences and seedlings	Shoots	RAN & SIMPSON, 2005
Crinum 'Ellen Bosanquet'	Bulb-scales and shoots	Bulblets	ULRICH *et al*., 1999
Crinum macowanii	Bulb-scales	Bulblets	SLABBERT *et al*., 1993
Crinum moorei	Bulb-scales	Bulblets	FENNELL, 2002
Crinum variabile	Bulb-scales	Bulblets and shoots	FENNELL *et al.*, 2001
Cyrtanthus clavatus	Bulb-scales	Bulblets and shoots	MORÁN *et al.*, 2003
Cyrtanthus loddigesianus	Bulb-scales	Bulblets and shoots	ANGULO *et al*., 2003
Cyrtanthus speciosus	Bulb-scales	Bulblets and shoots	ANGULO *et al*., 2003
Cyrtanthus spiralis	Bulb-scales	Bulblets and shoots	MORÁN *et al.*, 2003
Eucharis grandiflora	Bulb-scales	Bulblets and plantlets	PIERIK *et al*., 1983
Eucrosia radiate	Peduncle	Bulblets	ZIV & LILIEN-KIPNIS, 2000
Eucrosia stricklandii	Bulb-scales	Bulblets and shoots	COLQUE *et al*., 2002
Galanthus ikariae	Bulb-scales	Bulblets	TIPIRDAMAZ, 2003
Galanthus elwesi	Bulb-scales	Bulblets	STAIKIDOU *et al*., 2006
Galanthus nivalis	Bulb-scales, leaves and stems	Bulblets and shoots	HUSSEY, 1977 STAIKIDOU *et al*., 2006
Gethyllis spp.	Bulb-scales	Plantlets	NIEDERWIESER *et al.*, 2002
Gethyllis linearis	Bubl-scales	Bulblets	DREWES & VAN STADEN, 1994

Genus	Explant	Growth response	Reference
Haemanthus spp.	Bulb-scales and leaves	Bulblets and shoots	KROMER, 1985 RABE & VAN STADEN, 1999
Haemanthus coccineus	Peduncle	Bulblets	ZIV & LILIEN-KIPNIS, 2000
Hippeastrum spp.	Bulb-scales, leaves and stems	Shoots and plantlets	HUSSEY, 1977 SMITH *et al*., 1999
Hippeastrum spp. Hybrids	Bulb-scales, scapes, peduncles, ovaries and leaves	Plantlets	SEABROOK & CUMMING, 1977
Hippeastrum hybridum	Bulb-scales, inflorescences and ovaries	Bulblets and plantlets	HUANG *et al.,* 1990a HUANG *et al.,* 1990b HUANG *et al*., 2005 HUSSEY, 1975 OKUBO *et al.,* 1990 STANCATO *et al.,* 1995 YANAGAWA & OSAKI, 1996
Hymenocallis spp.	Bulb-scales	Bulblets	YANAGAWA & SAKANISHI, 1980a YANAGAWA & SAKANISHI, 1980b
Hymenocallis littoralis	Bulb-scales	Shoots and bulblets	BACKHAUS *et al*., 1992
Leucojum aestivum	Bulb-scales and leaves	Shoots and bulblets	KOHUT *et al*., 2007
Leucojum vernum	Bulb-scales	Bulblets	KROMER, 1985
Lycoris aurea	Bulb-scales and shoot tips	Bulblets, shoots, buds and plantlets	YANAGAWA & SAKANISHI, 1980a YANAGAWA & SAKANISHI, 1980b HUANG & LIU, 1989
Narcissus spp.	Bulb-scales, inflorescences, stems, leaves and ovaries	Callus, bulblets, shoots and plantlets	HOL & VAN DER LINDE, 1992 HANKS & REES, 1977 HANKS *et al*., 1986 HANKS, 1986 HANKS, 1987 HUSSEY, 1975 HUSSEY, 1977 HUSSEY, 1982b HUSSEY & HILTON, 1977 LANGENS-GERRITS & NASHIMOTO, 1997

Genus	Explant	Growth response	Reference
Narcissus asturiensis	Bulb-scales	Bulblets and shoots	SANTOS *et al*., 2002
Narcissus bulbocodium	Bulb-scales	Bulblets	SANTOS *et al.*, 1998
Narcissus confuses	Bulb-scales, embryos and buds	Callus, buds, bulblets, shoots and plantlets	BERGOÑÓN *et al.*, 1996 SELLÉS *et al.*, 1997 SELLÉS *et al*., 1999
Narcissus tazetta	Bulb-scales, buds, leaves and anthers	Callus, buds, bulblets and shoots	CHEN & ZIV, 2001 CHEN & ZIV, 2006 CHEN *et al*., 2005 STEINITZ & YAHEL, 1982
Nerine spp.	Bulb-scales	Bulblets and plantlets	McDONALD, 1985 PIERIK & IPPEL, 1977
Nerine angustifolia	Bulb-scales	Bulblets	CUSTERS & BERGERVOET, 1992
Nerine bowdenii	Bulb-scales, leaves and stems	Callus, bulblets and shoots	HUSSEY, 1977 GROOTAARTS *et al.*, 1981 JACOBS *et al.*, 1992 MOCHTAK, 1989
Nerine sarniensis	Bulb-scales, peduncle, leaves and stems	Callus, bulblets and shoots	HUSSEY, 1977 CUSTERS & BERGERVOET, 1992 ZIV & LILIEN-KIPNIS, 2000
Pancratium maritimum	Bulb-scales	Bulblets	DRAGASSAKI *et al.*, 2003
Paramongaia weberbaueri	Bulb-scales	Bulblets	DINKELMAN *et al*., 1989
Rhodophiala bagnoldii	Bulb-scales	Bulblets	OLATE & BRIDGEN, 2005
Scadoxus spp.	Bulb-scales	Plantlets	NIEDERWIESER *et al*., 2002
Sternbergia clusiana	Bulb-scales	Bulblets	ORAN & FATTASH, 2005
Sternbergia fischeriana	Immature embryos and bulb-scales	Bulblets	MIRICI *et al*., 2005
Sternbergia sicula	Bulb-scales	Bulblets	ANTONIDAKI-GIATROMANOLAKI *et al*., 2008

Genus	Explant	Growth response	Reference
Vallota purpurea	Bulb-scales	Bulblets	KUKUŁCZANKA & KROMER, 1988
Vallota speciosa	Bulb-scales, leaves and stems	Shoots	HUSSEY, 1977
Zephyranthes spp.	Seedlings and bulb-scales	Callus and bulblets	SMITH *et al*., 1999
Zephyranthes robusta	Bulb-scales, ovules and ovaries	Bulblets and seedlings	SACHAR & KAPOOR, 1959 FURMANOWA & OLEDZKA, 1981

The ornamental flower bulbs that are important to the horticultural industry, are the plants that have been micropropagated the most. These Amaryllidaceae genera are *Amaryllis, Hippeastrum, Narcissus* and *Nerine*. Twin scales, leaf bases and stem explants from bulbs of the above genera will give rise to shoots and/or callus over a wide range of combinations of auxin and cytokinin (HUSSEY, 1980).

1.5.3.1 The effect of explant type

The choice of explant is an important factor when it comes to the desired outcome, and will vary with each species. For *in vitro* regeneration and growth of bulbous plants, scales from the basal regions where they are joined to the basal plate, stems and buds that have already differentiated, have been found to be the most successful explants. However, the choice may depend on the family to which the bulb belongs (FENNELL & VAN STADEN, 2004). With regards to the Amaryllidaceae family, the most successful cultures have been established *in vitro* from twin-scales, tri-scales or chips (Table 1.1). Twin-scaling is when two scales attached to a piece of the basal plate is placed on medium in culture, and chipping is a mechanical variation of twin scaling. Examples of the regeneration of shoots from twin-scales have been conducted with *Crinum variabile*, *Cyrtanthus clavatus* and *Cyrtanthus spiralis* (KIM & DE HERTOGH, 1997; FENNELL *et al.,* 2001 MORÁN *et al.,* 2003). ULRICH *et al.* (1999) successfully micropropagated *Crinum* lilies using tri-scales. A disadvantage of using the bulb as an explant is that the parent plant is destroyed. This is a problem in terms of conserving vulnerable species.

Microbial contamination of plantlets produced *in vitro* is often associated with the use of the bulb as explants (ZIV & LILIEN-KIPNIS, 2000; FENNELL & VAN STADEN, 2004).

Inflorescence stalks, as well as various types of inflorescences such as the umbel or spike, are useful alternatives for overcoming contamination problems when working with underground organs (ZIV & LILIEN-KIPNIS, 1997; 2000). *Narcissus* and *Nerine* cultures have been established *in vitro* from inflorescence stalks. In both *Narcissus* and *Nerine*, buds were regenerated. According to ZIV and LILIEN-KIPNIS (1997), if the inflorescence is isolated at an early stage, before the differentiation of reproductive organs, both the peduncle and pedicel tissue can be induced to form buds, plantlets or bulbs *in vitro*.

Existing shoot meristems, in the form of axillary buds may be dissected out of bulbs and cultured directly. Other organs such as leaves and stems can also be used and are reported to be useful for the induction of adventitious shoot meristems (HUSSEY, 1980; HUSSEY, 1982a). Leaf material is a more suitable source of explant material than bulbs as harvesting is non-destructive to the parent plant (DREWES & VAN STADEN, 1994; ZIV & LILIEN-KIPNIS, 2000). A limiting factor when using leaf material is seasonal availability, as young leaves are only available in spring and leaf explants of many species do not respond in culture (ASCOUGH *et al.,* 2008). HUSSEY (1980) and HUSSEY (1982a) reported that callus formation can also be used to obtain shoots.

Explant orientation also plays a role in bulblet regeneration. LESHEM *et al.* (1982) observed that explant orientation in *Lilium longiflorum* greatly affected regeneration percentage. Scale sections that were placed adaxial side down regenerated fewer and smaller bulbs, less roots but produced more callus than scales that were abaxial side down on the medium. JACOBS *et al.* (1992) also examined the effect of orientation on *Nerine bowdenii*. Explants that were orientated abaxial side down formed bulblets more readily than adaxially orientated explants. Similarly, TAYLOR and VAN STADEN (2001) found fewer shoots initiated on explants of *Eucomis* species when the adaxial side of the leaf explant was placed in contact with the culture medium.

1.5.3.2 The effect of plant growth regulators

Responses of explants to external additives vary considerably. These responses may also be affected by the type, the physiological or ontogenetic age, size and overall quality of the explant (MURASHIGE, 1974). The vigour and type of response depends on the presence and concentrations of plant growth regulators in the media. Auxins and cytokinins are the two most important plant growth regulators used in tissue culture. Generally, low levels of auxin produce

swelling of the explant and very few shoots are produced. However, FENNELL and VAN STADEN (2004) claim that with regards to bulbous plants, low concentrations of auxin increase the induction of cell division leading to shoot production. High auxin concentrations are often used to induce callus and shoots, which may form on explants weeks later (HUSSEY, 1977). The type of auxin and cytokinin used is important with regards to the production of callus type.

Media containing both cytokinin and auxin result in the formation of multiple shoots on the explant and most monocotyledons require both these hormones for maximum shoot production. Cytokinins enhance axillary and adventitious shoot formation. When bulbs are used as the explant source, cytokinins may not be essential (FENNELL & VAN STADEN, 2004). A study on *Hyacinth* showed that cytokinins had hardly to any effect on bulblet regeneration and bulblet weight (PIERIK & STEEGMAN, 1975). HUSSEY (1982a) reported that plantlets can be produced in the absence of plant hormones.

1.5.3.3 Media and culture conditions

Nutrient media for plant tissue culture were developed to allow explants to grow in artificial environments. They were formulated to contain the essential elements for plant growth and development that would usually be available in the soil. The essential elements are divided into two groups: the macro-elements and the micro-elements. The macro-elements, are elements required by the plant in large quantities, including calcium, magnesium, nitrogen, phosphorous, potassium and sulfur. The micro-elements, required in small quantities, include boron, cobalt, copper, iodine, iron, manganese, molybdenum and zinc. MURASHIGE and SKOOG (1962) (MS) is the most common basal medium used in the tissue culture of the Amaryllidaceae (GEORGE, 1993). Along with the macro- and micro-elements, the MS basal medium contains vitamins, plant growth regulators and a carbohydrate source.

TAKAYAMA and MISAWA (1979) studied the effect of the MS medium on bulblet formation on *Lilium auratum* and *Lilium speciosum*. Formation and growth of bulbs and roots were affected by the strength of the MS medium. The number of bulbs and the bulb mass increased with an increase in MS medium strength. Root number and mass were not influenced by the MS medium strength, although root length was inhibited by an increased MS strength.

BONNIER and VAN TUYL (1997) reported that bulblet and sprout growth of *Lilium* sp. at 25 °C was significantly reduced by using ¼-strength MS medium, while VAN DER LINDE *et al.* (1988) reported that the optimum growth condition for *Iris hollandica* is 20 °C in the dark on ½-strength MS.

Carbohydrates serve as the energy source for plant tissue cultures and are essential for plantlet growth and adventitious root formation. Although plant tissues have internal reserves of carbohydrates, an external supply is provided by either adding sucrose to the media or through photosynthesis in green tissues (ECONOMOU & READ, 1987). Sucrose is considered to be the best source of carbon for *in vitro* tissue culture with optimum concentrations generally being between 30 and 60 g/l. This, however, may vary among plant species with some plants needing higher or lower concentrations.

Plant morphogenesis is affected by environmental factors such as temperature, carbon dioxide concentration, nutrients and light (DA SILVA & DEBERGH, 1997). Light can have an effect on the morphology of the cultured plants and a plant's response to light will vary depending on the light intensity, duration and quality. Light quality has an important effect on morphological characteristics such as plant elongation, axillary shoots and leaf anatomy. Light intensity regulates leaf and stem size and morphogenic pathways. There are great differences with regards to the effect of illumination on *in vitro* bulblet formation for different bulbous plants. STEINITZ and YAHEL (1982) found that continuous darkness increased bulblet size and number during the micropropagation of *Narcissus*. In contrast to this, continuous light promoted bulblet production of *Lilium japonicum* (MAESATO *et al*., 1994), whereas LESHEM *et al.* (1982) and SLABBERT and NIEDERWIESER (1999) found no difference in the effect of light or dark on bulblet formation in *Lilium longiflorum* and *Lachenalia,* respectively. This emphasizes the effect of explant material (variety, age, time of culture) on regeneration and *in vitro* growth of bulbous plants (SLABBERT & NIEDERWIESER, 1999).

Temperature plays a pivotal role in the tissue culture environment influencing many responses of the cultured plants. Temperature is a natural regulator of plant growth and morphogenesis. It not only regulates growth rates but also the transition between various vegetative and reproductive phases during development (ASCOUGH *et al*., 2008). Cultures are generally maintained in an environment where the temperature is kept constant and is usually kept at 25 ± 2 °C. The problem with this is that the explant is not exposed to the diurnal and seasonal temperature

fluctuations under which a plant would normally develop (MURASHIGE, 1974). Although constant temperatures may be adequate for the tissue culture of certain plants (mainly annuals and tropical species which grow in relatively uniform temperature conditions) more research is needed to investigate the influence on temperature of plants that are adapted to more temperate and desert climates. MURASHIGE (1974) suggested that maximum success from tissue culture may only be achieved when the precise temperature needs of a plant are fulfilled.

Activated charcoal is incorporated into tissue culture media for several reasons, namely; absorption of inhibitory or undesirable substances in the medium or those produced by the explants, provision of a dark environment in the medium, adsorption and stabilization of organic compounds and plant growth regulators as well as the release of substances that are naturally present in or adsorbed by the activated charcoal (PAN & VAN STADEN, 1998; FENNELL & VAN STADEN, 2004; ASCOUGH *et al.*, 2008). The effects of activated charcoal on the explants response in *in vitro* culture depends on the type of charcoal and its degree of activation and also on the plant species cultured (PAN & VAN STADEN, 1998).

STEINITZ and YAHEL (1982) found that the addition of activated charcoal to the basal medium increased bulblet production in *Narcissus tazetta* while FENNELL *et al.* (2001) reported that the addition of activated charcoal more than doubled bulblet production in *Crinum variabile*. However, there was no influence on bulblet induction in *Crinum moorei* (FENNELL, 2002). NHUT *et al.* (2001) showed that the addition of activated charcoal without the addition of plant growth regulators had a positive effect on shoot regeneration of *Lilium longiflorum*. NHUT *et al.* (2001) believe the results could be due to the adsorption of inhibitory substances. It is well known that in plant tissue culture, no two genotypes give the same responses under a given set of culture conditions. Similarly, the addition of activated charcoal to the tissue culture medium may have either a beneficial or an adverse effect on growth and development, depending on a number of factors. As PAN and VAN STADEN (1998) stated, further research into the adsorption properties of charcoal and the identification of substances that are adsorbed and released by the charcoal will help in the understanding of how activated charcoal acts in plant growth and development.

1.5.3.4 Methods of multiplication

Multiplication *in vitro* can be the result of either organogenesis or embryogenesis and both these processes may occur directly from the explant or indirectly from callus tissue. Multiplication of higher plants through callus is very difficult and the cultures are frequently genetically unstable (PIERIK, 1991). Therefore, the most common and the most successful multiplication method, with regards to the multiplication of Amaryllidaceae bulbs, is through organogenesis.

In vitro regeneration and growth of bulbs has been reported using bulb scales (Table 1.1). Bulblet growth of *Hyacinthus orientalis* was stimulated by transferring into liquid medium and shake-culture (TAKAYAMA *et al*., 1991). Liquid culture systems are becoming more popular with regards to mass propagation as well as offering many other benefits. Liquid culture is more cost effective as a gelling agent is not required and liquid cultures exhibit higher multiplication and proliferation rates (VARSHNEY *et al.,* 2000; ASCOUGH & FENNELL, 2004). The growth and multiplication of bulbous plants in liquid cultures occurs more rapidly, with an increase in the number of buds produced and better bulblet growth (ZIV, 1997; FENNELL & VAN STADEN, 2004). According to MORÁN *et al.* (2003), the use of liquid medium supplemented with sucrose accelerated biomass gain and the bulbing of small shoots of *Cyrtanthus clavatus* and *Cyrtanthus spiralis*. Therefore, the use of liquid culture in order to obtain a high number of bulbs is a good alternative to the conventional methods.

1.5.3.5 Bulb induction and growth

Once vigorously growing shoots are obtained, it has been reported that bulblets form spontaneously. Bulbs can, however, be induced by varying a number of factors. This includes the transfer of shoots to a medium containing a high level of sucrose, high temperatures, darkness, low pH and low concentrations of auxins (ZIV, 1991).

NIIMI *et al.* (1999) studied the effects of temperature and light conditions on excised scales of bulbs from several *Lilium* species. The study showed that temperature and light do affect *in vitro* regeneration and that more bulblets were regenerated at a higher temperature of 25 °C than at 15 °C. With regards to the effect of light, different species require different light regimes for bulb induction.

Some plants, such as *Lilium* species, may become dormant depending on the culture conditions. LANGENS-GERRITS *et al.* (2003) reported that dormancy can be broken by storage for a few weeks at a low temperature (0 to 10 °C). The effect of low temperature on sprouting, time of leaf emergence and further bulb growth was studied. Not only did the low temperature affect the number of sprouted bulblets but also the time of emergence. Generally, bulblets grew faster after a low temperature treatment for six weeks.

1.5.4 Future prospects

Although many of the Amaryllidaceae have been cultured *in vitro*, techniques for other members of the family still need to be developed while the existing techniques need to be improved before they are put into practice in commercial tissue culture laboratories. Traditional uses of some bulbous species belonging mainly to the Amaryllidaceae could lead to new pharmaceutical developments. Therefore, future research of species that are not well known could be very useful with regards to the discovery of new compounds.

1.6 GENETIC MARKERS

Genetic markers were originally used in gene mapping to determine the order of genes along chromosomes. Today, however, genetic markers are used in plant research and plant breeding to characterize plant germplasm, for gene isolation, cultivar identification and to determine genetic diversity (ANDERSEN & LÜBBERSTEDT, 2003). A variety of molecular markers have been developed and these include; random amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs) and more recently simple sequence repeats (SSRs) otherwise known as microsatellites (STAUB *et al.,* 1996; VARSHNEY *et al.,* 2005). The major limitations of these markers are low reproducibility of RAPDs, high cost of AFLPs and the requirement for sequence information from flanking regions to design species specific primers for SSR polymorphism (LIU & WENDEL, 2001; REDDY *et al*., 2002). However, a recently developed modification of SSR-based marker systems, inter simple sequence repeats (ISSRs) overcomes most of these problems.

Inter-simple sequence repeat (ISSR) techniques involve polymerase chain reaction (PCR) amplification of genomic DNA at an amplifiable distance in between two identical microsatellite repeat regions orientated in opposite directions (REDDY *et al*., 2002). The technique uses

microsatellites which are usually 16 to 25 base pairs (bp) long as primers in a single primer PCR reaction, targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes (REDDY *et al*., 2002). ISSR-PCR is a simple, quick and efficient technique which has high reproducibility and the use of radioactivity is not essential (REDDY *et al*., 2002; PHARMAWATI *et al.*, 2005). The amplified products are usually 200 to 2000 bp long and amenable to detection by agarose and polyacrylamide gel electrophoresis. ISSR markers have been used extensively to identify and determine relationships at the species and cultivar levels (PHARMAWATI *et al.*, 2005).

BUSH *et al.* (2010) used ISSR fingerprinting to determine the phylogeny of *Hymenocallis* (Amaryllidaceae). This was the first phylogenetic analysis on *Hymenocallis* and several novel relationships were reported and were supported by both molecular and morphological data. SHI *et al.* (2006) investigated the interspecific relationships of *Lycoris* (Amaryllidaceae) using ISSR markers. Spontaneous interspecific hybridization occurs with high frequency in *Lycoris* in the wild and cultivated genotypes. KURITA and HSU, (1996) reported that about two-thirds of the recognized taxa are of hybrid origin, and it is therefore, difficult to identify species of *Lycoris* from morphological traits. More attractive hybrids are wanted to be bred for markets, although this is difficult as there is limited information on genetic variation and interspecific relationships among *Lycoris*. In the study carried out by SHI *et al.* (2006), ISSR markers identified the parents of some sterile species and showed that the ISSR markers could provide an essential basis for screening the genetic variation and inferring interspecific relationships in *Lycoris*. This will be beneficial for future hybrid breeding programs of this horticulturally important genus.

Phaedranassa tunguraguae (Amaryllidaceae) is an endangered species endemic to Ecuador. OLEAS *et al.* (2005) used microsatellites to evaluate polymorphism levels in individuals from a single population. The observed heterozygosity ranged from 0.387 to 0.903. The high levels of polymorphism of these loci make them useful for investigation of genetic variation in *P. tunguraguae* and in the future will be useful for refining conservation efforts of this species (OLEAS *et al.,* 2005). Therefore, there is enormous potential for integrating ISSR markers into studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology.

1.7 AIMS

The aim of this research was to study the medicinally useful, yet toxic plant, *Boophone disticha,* and in so doing, provide a micropropagation protocol which will aid in the conservation of this plant as well as provide answers about the biological activity of the alkaloids present in the plant. Therefore, the specific objectives of this research were the following:

- Develop a protocol for the tissue culture of *B. disticha*;
- Optimise bulblet production;
- Investigate the antimicrobial activity of tissue cultured plants;
- Investigate biological activity (antibacterial and antifungal activity) of alkaloids and test for genotoxic effects; and
- Investigate genetic variation between different wild populations of *B. disticha*.

Chapter Two

MICROPROPAGATION

2.1 CONSERVATION AND CULTIVATION THROUGH MICROPROPAGATION

2.1.1 Introduction

Micropropagation can be defined as "the growing of plants from meristematic tissue or somatic cells of plants on suitable nutrient media under controlled aseptic physical conditions" (KYTE & KLEYN, 1996). This technique is performed in a carefully controlled environment and the media that the plants are grown on contains a carbohydrate source, a range of micro- and macro-nutrients and a gelling agent. Often plant growth regulators and vitamins are added to encourage growth.

Micropropagation has a number of advantages over traditional plant propagation techniques. Some of these include: the high production rate (producing thousands of propagules), the production of disease-free plants, and its usefulness in multiplying plants which produce seeds in small amounts, or when plants are sterile and do not produce viable seeds or when seed can't be stored (such as recalcitrant seeds). Micropropagation can also aid with the conservation of valuable biodiversity and play a part in the establishment of breeding material from wild populations and in mass-producing material for selection or genetic engineering (KIM & DE HERTOGH, 1997; CHANG *et al.,* 2000).

The Amaryllidaceae is one of the most horticulturally important families of monocotyledons as it includes a number of plants that are medicinally and ornamentally important. It is for these reasons that tissue culture has been carried out on various members of the Amaryllidaceae (AFOLAYAN & ADEBOLA, 2004).

The Amaryllidaceae family is known as a rich source of Amaryllidaceae alkaloids. They have been some of the first plants to be studied *in vitro* in order to determine whether the naturally produced alkaloids are also produced in culture. Two genera of the family used for the production of alkaloids *in vitro* are *Hippeastrum* and *Narcissus*. The production of alkaloids are

currently being tested in other *Narcissus* species. However, according to KRIKORIAN and KANN (1986), the production and maintenance of callus containing secondary metabolites is difficult.

Since *Boophone disticha* is a medicinally important species that is widely used and threatened, this study was undertaken to establish a micropropagation protocol that will aid in conserving this plant. This micropropagation study investigated various factors such as: explant type, plant growth regulators, carbohydrates, temperature, photoperiod and liquid culture.

2.2 EXPLANT SELECTION

2.2.1 Introduction

The choice of explant is an important factor when it comes to the desired outcome and the choice will vary depending on the species. PAN and VAN STADEN (1998) reported that the growth and development of an explant *in vitro* depends on four factors. These factors are: the genetic "make-up" of the plant; the nutrients available to the explant (macro- and micro-elements); environmental factors (temperature, light, pH, O_2 and CO_2 concentrations); and organic substances available to the explant (plant growth regulators and vitamins).

Bulbs are mainly used for the establishment of Amaryllidaceae species *in vitro*. A disadvantage with using a bulb as an explant is that it destroys the parent plant, which is a particular problem in terms of conserving vulnerable species. Another disadvantage is contamination. When establishing cultures from soil-borne explants, such as bulb explants, contamination of the plantlets produced *in vitro* is the largest problem faced (ZIV & LILIEN-KIPNIS, 1997; ZIV & LILIEN-KIPNIS, 2000; FENNELL & VAN STADEN, 2004). Bulb and root tissues come into contact with contaminants and pathogens in the soil, therefore making bulbs very difficult to decontaminate (HUSSEY, 1975). HOL and VAN DER LINDE (1992) reported that contamination rates are often high in plant tissue cultures which have been initiated from bulbs, rhizomes and stolons. This can be due to bacteria, fungi, mites and thrips which are found in the soil. Therefore, the use of a strong sterilant is necessary when preparing bulbous explants for tissue culture.

In order to overcome contamination problems, other organs such as leaves, stems and parts of the inflorescence can also be used and are reported to be useful for the induction of adventitious shoot meristems. Callus formation can be used to obtain shoots (HUSSEY, 1980; HUSSEY, 1982a). ZIV and LILIEN-KIPNIS (1997) reported that the inflorescence stalk is a very good source for regeneration of explants and ZIV and LILIEN-KIPNIS (2000) showed that buds can be regenerated *in vitro* from various types of inflorescence such as the umbel or spike.

Boophone disticha produce large, fleshy green seeds in capsules which are found at the ends of the flower stalk. The seeds of *B. disticha*, like other members of the Amaryllidaceae family, are recalcitrant. Recalcitrant seeds have the ability to germinate and establish themselves quickly after they have fallen from the plant (COPELAND & McDONALD, 2001). However, recalcitrant seeds do not become dormant, but rather continue to develop and germinate (BERJAK *et al.*, 1990). The seeds have a high moisture content of between 50 % to 70 % at maturity (COPELAND & McDONALD, 2001) and will germinate without any additional moisture. This allows for the seeds to germinate readily without water and in very dry conditions (VERDOORN, 1973). An alternative for obtaining uncontaminated explants is to take explants from seedlings which are aseptically grown from surface-sterilized seeds. Since seeds are sealed entities, the hard surface of a seed is less permeable to penetration of harsh surface sterilizing agents, thereby making them a useful explant.

2.2.2 Materials and methods

Flower cultures

Flowers were collected in October 2009 and decontaminated with 2.5 % JIK® bleach (NaOCl) and a few drops of Tween 20 for 3 min. The flowers were then rinsed three times with sterile water to remove all traces of NaOCl. Immature and mature floral parts [ovaries, anthers, filaments and pedicels (Figure 2.1)] were placed on solid MURASHIGE and SKOOG (1962) (MS) media. The MS media contained 8 g/l agar (Agar Bacteriological-Agar No. 1, Oxoid Ltd.), 30 g/l sucrose, 100 mg/l *myo*-inositol and was supplemented with various concentrations of plant growth regulators (Table 2.1). The pH of the media was adjusted to 5.8 with diluted NaOH before autoclaving at 121 °C and 103 kPa for 20 min. The glass culture tubes (2.5 x 15 cm) were sealed with metal caps and a single layer of stretched Parafilm®. The flower cultures were grown

at 25 ± 2 °C under a 16 h photoperiod. The growth room was fitted with Osram L58W/640 cool white florescence light bulbs which provided an average light intensity of 74.4 µmol/m^2/s.

Table 2.1: Various concentrations of napthaleneacetic acid (NAA), N^6-benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), kinetin and *meta*-topolin (*m*T) used to supplement the MS media.

Hormones	Concentration (µM)
NAA : BA	5 : 10
NAA : 2,4-D	5 : 10
IAA : Kinetin	2.85 : 0.46
IAA : Kinetin	2.85 : 2.32
IAA : Kinetin	0.57 : 2.32
NAA : *m*T	10 : 10
NAA : *m*T	10 : 15
NAA : *m*T	10 : 22.2

Embryo cultures

Seeds were collected from wild growing *B. disticha* at Midmar (29° 31′ 56″ S, 30° 12′ 13″ E) once the capsules were about to burst. Since recalcitrant seeds cannot be stored, they were used for embryo culture or were germinated *in vitro* (Figure 2.1). The seeds were washed with water to remove any excess dirt and then decontaminated with 0.2 % mercuric chloride ($HgCl_2$) with a few drops of Tween 20 for 5 min. The seeds were then rinsed three times with sterile distilled water to remove all the $HgCl_2$.

The embryos were removed and placed on half-strength MS media with either no plant growth regulators, 4.52 µM 2,4-D, 500 mg/l casein hydrolysate or both 4.52 µM 2,4-D and 500 mg/l casein hydrolysate.

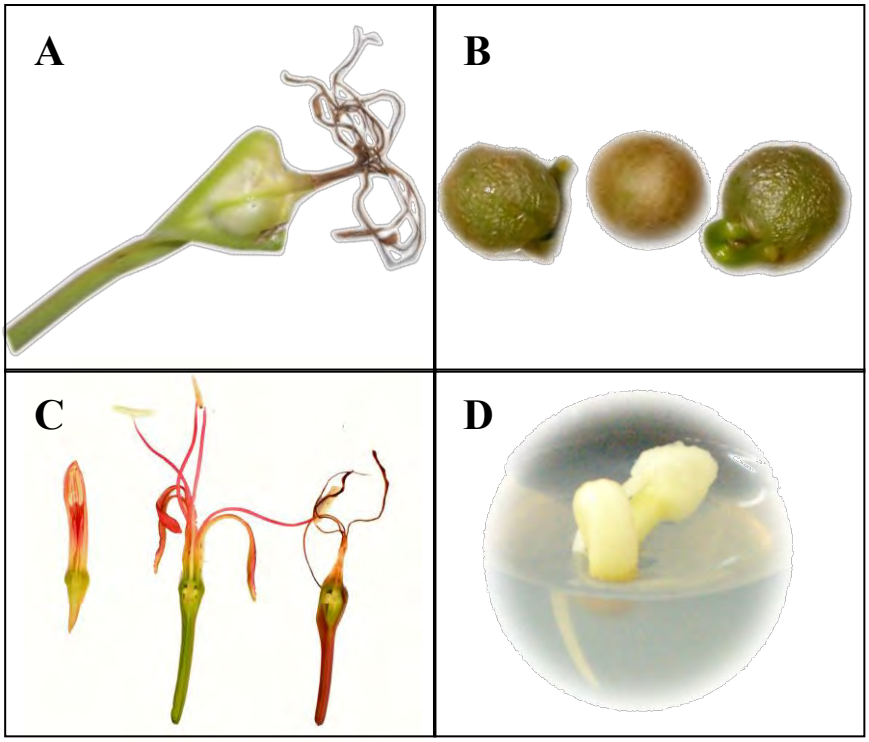


Figure 2.1: Different explants of *B. disticha* used *in vitro*. A - seeds used for embryo culture, B - germinating seeds used for seedling culture, C - floral parts used in culture and D - embryo removed from a seed and used in seedling culture.

Seedling cultures

Seedlings that developed from embryos and *in vitro* germinated seeds were used in further experiments to induce bulblets. Seedlings grown aseptically from decontaminated seeds are a good source of shoot-tips and young organ explants for further cultures (HUSSEY, 1986). This is because these explants do not have any damage from sterilants as only the seed coat comes into contact with the sterilant during decontamination (HUSSEY, 1986).

Figure 2.2: ***B. disticha*** **seedling grown** ***in vitro*** **from a germinated seed showing 10 sections which were used in further experiments.**

In vitro grown seedlings were cut into 10 segments (Figure 2.2) and placed on MS media supplemented with various concentrations of NAA (0, 2.69, 5.37 and 13.43 µM) and BA (0, 2.22, 4.44 and 11.1 µM). Sections 1, 2 and 3 were cut from the primary roots of the seedlings. Section 4 was the junction between the root and shoot. Section 5 was the section of coleoptile before the leaves. Section 6 was the base of the leaves and top of the coleoptile. Sections 7, 8, 9 and 10 were cut from the leaves of the seedlings.

Twin-scale cultures

Twin-scales were first used in tissue culture of *Narcissus* (BRUNT, 1985), but now they are widely used for the micropropagation of members of the Amaryllidaceae, Liliaceae, Hyacinthaceae and Iridaceae (VAN AARTRIJK & VAN DER LINDE, 1986), since meristematic tissue is present where the leaves join the basal plate. Twin-scales are removed from bulbs and comprise two adjacent scales connected by a small piece of basal plate tissue. GEORGE (1993) reported that it is necessary to include the basal plate tissue in the explant when excising twin-scales. With regards to the Amaryllidaceae, FENNELL (2002) and FENNELL and VAN STADEN (2004) showed no bulblets formed without the presence of basal plate tissue.

Boophone disticha bulbs were collected in Spring 2009 from a wild population in the Mpomphomeni area, (29° 33’ 29” S, 30° 11’ 46” E) of KwaZulu-Natal (South Africa) and a voucher specimen (Cheesman 01 NU) retained at the University of KwaZulu-Natal (UKZN) Herbarium. Leaves and roots were removed from the bulb and the outer scales were peeled off and discarded. Whole bulbs were decontaminated in 0.2 % $HgCl_2$ for 5 min. The bulbs were then cut longitudinally in half and further decontaminated in 0.1 % $HgCl_2$ for 5 min. The bulb halves were rinsed three times with sterile distilled water. Twin-scales were cut from bulb segments and

placed on 15 ml solid MS media. The MS media contained 8 g/l agar (Agar Bacteriological-Agar No. 1, Oxoid Ltd.), 30 g/l sucrose, 100 mg/l *myo*-inositol and supplemented with various concentrations of NAA (0, 5.37, 10.74, 26.85 and 53.7 µM) and BA (0, 4.44, 8.88, 22.2 and 44.4 µM). The pH of the media was adjusted to 5.8 with diluted NaOH before autoclaving at 121 °C and 103 kPa for 20 min. Twin-scale explants were placed on the media and the glass culture tubes (2.5 x 15 cm) were sealed with metal caps and Parafilm®. The cultures were grown at 25 ± 2 °C under a 16 h photoperiod. The growth room was fitted with Osram L58W/640 cool white florescence light bulbs which provided an average light intensity of 74.4 µmol/m^2/s.

2.2.3 Results and discussion

Flower cultures

For the cultures prepared from flower tissues, the immature and mature ovaries became swollen while the anthers, filaments and pedicels showed no response. HUSSEY (1975) studied and compared the *in vitro* responses of twelve species of bulbs and corms, and found that plantlets could be induced directly on stem tissue in nine species, on ovary tissue in five species and on leaf tissue in four species. Young, elongating, inflorescence stem proved to be the most consistently reactive tissue. In *Ipheion,* low concentrations of NAA or moderate concentrations of IAA were required for plantlet formation but the responses were erratic. In *Hippeastrum,* occasional plantlets formed on stem pieces on different concentrations of NAA in the media. Ovary wall tissue was also tested and of the three Amaryllidaceous species (*Hippeastrum, Ipheion* and *Narcissus*), only *Ipheion* produced plantlets from this organ (HUSSEY, 1975). Like *Hippeastrum* and *Narcissus*, floriculture was unsuccessful in *B. disticha* cultures. Therefore, it is not possible to predict the reactions of a species, given the family (HUSSEY, 1975).

Embryo cultures

Embryos were used as explants for the production of embryogenic material. In this study, some of the *B. disticha* embryos developed into seedlings and were used in seedling experiments. Other embryos developed roots or formed non regenerative callus. Due to the general recalcitrant natural of Amaryllidaceae embryos *in vitro*, the embryos did not show any signs of embryogenesis and as such the experiment did not achieve its purpose.

Seedling cultures

For the excised seedling sections, sections 1 to 10 did not all undergo morphogenesis in culture. The seedling sections responded differently with some producing non-regenerative callus and others producing roots, shoots or bulblets (Table 2.2). Root sections 1 and 2 swelled when placed on MS media with 2.22 µM BA and 13.42 µM NAA. Non-regenerative callus formed on media supplemented with 11.1 µM BA and 2.69 µM NAA as well as 0 µM BA and 2.69 µM NAA. Root section 3 showed no response. Without the correct plant growth regulators, it is difficult to use root explants to induce other plant structures. FENNELL (2002) also reported that different plant tissues differ in their ability to undergo morphogenesis and form meristematic structures.

Table 2.2: Growth response of seedling sections of *B. disticha* on MS media supplemented with various concentrations of NAA and BA.

	BA:NAA concentration (µM)					
Seedling Section	**0:0**	**2.22:0**	**2.22:13.43**	**4.44:5.37**	**11.1:2.69**	**0:2.69**
1	-	-	Swelled	-	Callus	Callus
2	-	-	Swelled	-	Callus	Callus
3	-	-	-	-	-	-
4	-	Callus, shoots, roots and bulbs	Callus, shoots, roots and bulbs	Callus	Callus, shoots, roots and bulbs	Shoots, roots and bulbs
5	-	-	-	-	Callus	Swelled
6	-	Shoots and roots	Swelled	-	Callus	Callus
7	-	-	-	-	Callus	-
8	-	-	-	-	-	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-

Section 4, which was the area between the root and shoot, was the most successful seedling section as it produced shoots, roots and bulblets. The reason for this success is due to the meristem located between the root and shoot. A pre-existing meristematic region in the explant supersedes the need for cells to become meristematic before morphogenesis can occur. The

combination of 4.44 µM BA: 5.37 µM NAA only produced non-regenerative callus while all the other plant growth regulator combinations produced shoots, roots and bulblets.

The coleoptile, Section 5, either produced non-regenerative callus on media with 11.1 µM BA: 2.69 µM NAA, or the explant swelled when placed on media supplemented with 0 µM BA: 2.69 µM NAA. JÄGER *et al.* (1995) found that hypocotyl segments from *Babiana* species formed meristematic structures at a higher frequency than root and leaf explants. This section, however, did not show any organogenesis.

Section 6, the base of the leaves and top of the coleoptiles, produced roots and shoots when placed on media with 2.22 µM BA: 0 µM NAA. The other plant growth regulator combinations either resulted in the explants swelling or forming non-regenerative callus. Leaf section 7 produced non-regenerative callus only on media supplemented with 11.1 µM BA and 2.69 µM NAA. Leaf sections 8 to 10 did not respond or show any morphogenesis in culture, as expected, because like the root sections, no meristematic cells are present in these sections and this prevents morphogenesis from occurring. This result is in agreement with the work of HUSSEY (1975) who also found that plantlets could not be induced on developing leaves of three Amaryllidaceous species.

Twin-scale cultures

Inhibitory substances such as phenolics are often released by plant materials in culture (PAN & VAN STADEN, 1998) and these can be toxic to explants (WANG & HUANG, 1976). Various factors affect tissue browning and these include the genotype, source of explants and time of year (SATHYANARAYANA & MATHEWS, 2007). The degree of browning and growth inhibition, which occurs in culture, is genotype-dependent. It is particularly severe in genera that naturally contain high levels of tannins or other hydroxyphenols. Differences are also found between species of the same genus and cultivars within a species. Different genotypes not only differ in the amount of phenolic substances produced, but the substances released also vary in their toxicity and plants show different susceptibilities (SATHYANARAYANA & MATHEWS, 2007). With regards to the source of explants, young juvenile tissues are often less prone to browning on excision than older ones. The last factor which can influence the extent of browning can be the time of year at which tissues are explanted. The influence of the time of year varies according to the plant (SATHYANARAYANA & MATHEWS, 2007).

Different approaches have been suggested to overcome the browning of plant tissues. The first approach includes minimizing the damage caused to the explants during excision and decontamination. In some species, the sterilents used to decontaminate explants can be responsible for intensifying the browning of explants. When browning is a severe problem, it is advisable to experiment with replacing one sterilent solution with another (SATHYANARAYANA & MATHEWS, 2007).

SATHYANARAYANA and MATHEWS (2007) suggest another approach is to remove the phenolic compounds produced. In plant species where exudates can be a problem, pre-treatment of plant material can help in obtaining viable explants. During excision, care must be taken not to get exudates from surrounding tissue onto the explants. After that, washing to remove the products released from damaged cells is effective. Explants should be rinsed or left in sterile water for 2 to 3 hours after isolation and before being transferred to culture. Thorough rinsing after sterilization is also necessary to wash away chemicals used for decontamination as these may also provoke the synthesis of phenolics.

Growth of explants can be limited by toxic metabolites even in the absence of obvious browning. If explants do not show any growth after 3 to 4 weeks, their chances of survival may be improved by transfer to fresh media. Rapid transfer is essential if the media around the explants begins to become discoloured or blackened. Browning is often noticeable on solid media where exudates are trapped by the agar and become concentrated in the surrounding area of the explants. In such cases, the most commonly used method of preventing tissue browning is to subculture explants frequently, placing them on fresh media after a few days (SATHYANARAYANA & MATHEWS, 2007). This however, may be an expensive procedure, which would not be ideal in a commercial laboratory.

Liquid culture is another approach which can be used. Explants are often less liable to browning if they are cultured initially on a liquid medium as the phenolics can easily diffuse away (SATHYANARAYANA & MATHEWS, 2007).

One of the most common approaches to minimizing browning of tissues is the addition of activated charcoal to tissue culture media. Activated charcoal has a number of beneficial effects on the culture. Charcoal is made active by treatment with carbon dioxide. This oxidizes the charcoal, giving it adsorbing properties (PAN & VAN STADEN, 1998). The activated charcoal

adsorbs inhibitory substances from the tissue culture media (WEATHERHEAD *et* al., 1978; PECK & CUMMING 1986; PAN & VAN STADEN, 1998; THOMAS, 2008), and inactivates these substances (WANG & HUANG, 1976). This ensures that these inhibitory substances do not have an adverse effect on explants.

Activated charcoal has been used extensively in the induction of bulblets from twin-scales because of its adsorptive properties and its darkening effect. When added to growth media, activated charcoal makes the media black in colour. This darkens the media, thereby creating conditions which more closely resemble soil conditions (WANG & HUANG, 1976; WEATHERHEAD *et al.*, 1978; PAN & VAN STADEN, 1998). Charcoal is often used in the propagation of bulbs as it reduces browning and the decay of twin-scale bases (STEINITZ & YAHEL, 1982) allowing the bases to produce bulblets. It also strongly influences bulblet regeneration size and bulblet regeneration through direct organogenesis (STEINITZ & YAHEL, 1982; PECK & CUMMING, 1986; FENNELL *et al*., 2001).

The inclusion of activated charcoal in the growth media stimulated bulblet formation in *Narcissus* (LAGENS-GERRITS & NASHIMOTO, 1997; ZIV & LILIEN-KIPNIS, 2000), *Eucrosia* (ZIV & LILIEN-KIPNIS, 2000), *Eucrosia stricklandii* (COLQUE *et al*., 2002), *Lilium* (TAKAYAMA & MISAWA, 1979), *Lilium longiflorum* (HAN *et al*., 2004), *Crinum moorei* (FENNELL, 2001) and *Cyrtanthus* species (MORÁN *et al*., 2003). The inclusion of activated charcoal in the growth media doubled regeneration of bulblets in *Narcissus* (STEINITZ & YAHEL, 1982) and increased the size and number of bulblets formed by twin-scales of *Crinum moorei* (FENNELL, 2002).

Several different kinds of polyvinylpyrrolidone (PVP) have also been used to prevent browning of plant tissue cultures, either as rinses for explants or by incorporating into the media. Phenols are absorbed by PVP by hydrogen bonding, preventing their oxidation and polymerization. PVP may also combine with oxidized phenolics, preventing further oxidations by phenolase enzymes (SATHYANARAYANA & MATHEWS, 2007).

The physical environment may be modified so that conditions for the production of phenolics are not optimal. The activity of enzymes concerned with both the biosynthesis and the oxidation of phenols are increased by light (SATHYANARAYANA & MATHEWS, 2007). Tissue browning has been found to be less in explants taken from plants grown in darkness or very low light

intensity. Cultures are often initiated more readily if newly explanted tissues are kept in the dark for several weeks. Browning is usually reduced or prevented by such dark treatments and sometimes subsequent growth is improved. Browning may also be prevented if it is possible to maintain plant material at a slightly lower temperature than normal, while still obtaining growth and/or morphogenesis.

Finally, the last approach which can be used is to modify the redox potential of the plant tissue. The tendency of dissolved compounds to be oxidized or reduced depends on the oxidation-reduction (redox) potential of the solution (SATHYANARAYANA & MATHEWS, 2007). There are several antioxidants which act as reducing agents that lower the redox potential of solutions and are therefore effective in preventing the browning of isolated plant tissues or plant extracts, and it is assumed that they prevent the oxidation of phenols. Explants from the plants whose tissues are susceptible to browning, are often washed in a solution of an antioxidant after sterilization, excised on paper soaked with an antioxidant, and/or submerged in a solution of antioxidant immediately after excision. A solution of ascorbic acid and citric acid is commonly used for rinsing freshly isolated explants to delay the browning process. Antioxidants like ascorbic acid can also be incorporated into the tissue culture media.

In plant systems, ascorbic acid has been shown to have a retarding effect on tissue aging and necrosis (CHINOJ, 1984), as well as on phenolic metabolism (CHINOJ, 1984; MUHITCH & FLETCHER, 1985; TANAKA *et al*., 1985). JOY *et al.* (1988) examined the effect of ascorbic acid on growth and shoot formation in tobacco (*Nicotiana tabacum* L.) callus. It was found that the inclusion of ascorbate in the media enhanced shoot formation as well as speeded up the shoot-forming process in both young and old callus cultures. In addition, ascorbate was shown to reduce the inhibition of shoot formation by gibberellic acid in young callus, but was less effective in old callus (JOY *et al.,* 1988). However, it still remains to be determined whether ascorbate plays a similar role in other species.

In the current study, all the *B. disticha* twin-scales which were placed on MS media turned brown and then black and no growth response was observed. The media itself turned a yellow-brown colour (Figure 2.3), clearly showing that phenolics had been released from the twin-scales. In order to overcome this problem, activated charcoal and ascorbic acid were incorporated into the media for all subsequent experiments. Along with the inclusion of ascorbic acid in the media, after decontamination the *B. disticha* bulbs were rinsed and then soaked in

sterile distilled water containing ascorbic acid. This seemed to delay the browning process while the twin-scales were being excised and placed in culture which is in agreement with what SATHYANARAYANA and MATHEWS (2007) suggested.

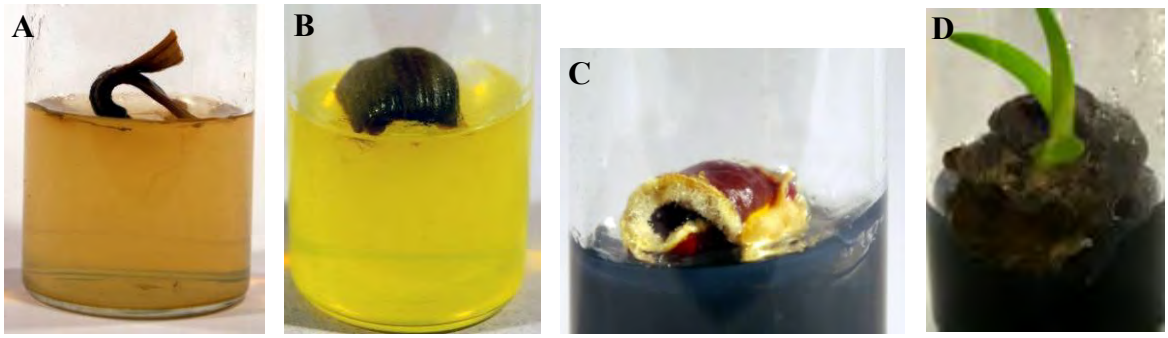


Figure 2.3: Culture of twin-scales of *B. disticha* showing discolouration of the media due to exudation of phenolics.

It was also found that younger *B. disticha* bulbs did not brown as quickly compared to the older/larger bulbs. This again confirms what SATHYANARAYANA and MATHEWS (2007) reported, that the source of explant does affect tissue browning.

In conclusion, the addition of activated charcoal and ascorbic acid to the culture media was very effective. It prevented the *B. disticha* twin-scale explants turning brown which allowed for the induction of bulblets. Although the success rate of bulblet induction was not high, it was still greater than when no charcoal or ascorbic acid was included in the culture media (Figure 2.3).

2.3 PLANT GROWTH REGULATORS

2.3.1 Introduction

Plant growth regulators are naturally occurring organic substances that are needed in low concentrations to facilitate essential plant processes. The processes influenced by plant growth regulators include growth, dormancy, flowering, cell differentiation and fruit ripening (DAVIES, 1987). Environmental factors such as temperature, light and day length interact with plant growth regulators to cause developmental responses in plants.

There were previously five major groups of plant growth regulating compounds; auxins, cytokinins, gibberellins (GAs), ethylene and abscisic acid (ABA) (GASPAR *et al.,* 1996), however, brassinosteroids and strigolactones have recently been recognized as new classes of plant growth regulators (BISHOP & KONCZ, 2002; RUYTER-SPIRA *et al.,* 2012; BREWER *et al.,* 2013). Both auxins and cytokinins are present in all plants at any time and in all the major organs. Auxins and cytokinins are needed for essential developmental processes and no plant can develop in their absence. GAs, ethylene and ABA are widespread in plants and have a number of important roles however, none of these three plant growth regulators are essential for tissue cultured plants although they are important messengers (DAVIES, 1987). Brassinosteroids, a class of polyhydroxysteroids, are involved in a number of plant processes such as promotion of cell expansion and cell elongation; promotion of vascular differentiation and is necessary for pollen elongation and pollen tube formation (BISHOP & KONCZ, 2002). Strigolactones are rhizosphere signaling molecules which trigger germination of parasitic plant seeds and inhibit plant shoot branching. Strigolactone research has quickly revealed that this class of plant growth regulator plays a major role in optimizing plant growth and development, however, a still increasing number of biological functions are being uncovered (RUYTER-SPIRA *et al.,* 2012; BREWER *et al.,* 2013). Plant growth regulators are critical media components in facilitating the developmental processes of plant cells in tissue culture. However, there is considerable difficulty in predicting the effects of these plant growth regulators in micropropagation systems. This is due to the differences in culture response between species, cultivars and even plants of the same cultivar grown under different conditions.

SKOOG and MILLER (1957), while working on the regeneration of tobacco, discovered that the balance between auxins and cytokinins plays a role in the course of organogenesis and

morphogenesis. When there is a higher level of auxin to cytokinin, then roots develop while with a higher level of cytokinin to auxin, shoots form. When the ratio of cytokinin to auxin is about the same, a callus mass is produced. It is now well established that, by adjusting the auxin: cytokinin ratio, shoots or roots can be induced in tissue culture (HEMPEL, 1979; KRIKORIAN *et al.,* 1987). In some plant species the influence of other growth regulators such as gibberellins and inhibitors are also involved.

Auxins stimulate stem growth and root initiation. They play a role in cell enlargement and elongation and in culture systems they promote cell division (GASPAR *et al.,* 1996). There are a number of naturally occurring auxins, however, most are not available for use other than indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). Synthetic auxins are often used in micropropagation as they are more stable. The most commonly available and used are 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) (DAVIES, 1987; KRIKORIAN *et al.,* 1987).

In tissue culture, cytokinins are necessary for plant cell division (STRNAD, 1997). They are also very effective in promoting direct and indirect shoot initiation. A low concentration of cytokinin is often used in the culture media to induce embryogenic callus. However, there is evidence suggesting that exogenous cytokinins could inhibit embryogenesis in monocotyledons and certain plant genotypes. This could be due to the presence of endogenous cytokinins. Cytokinins are used to stimulate the growth of axillary buds and reduce apical dominance in shoot cultures (GASPAR *et al.,* 1996). High levels of cytokinin have been found to cause small shoots to form which do not elongate, leaves to have an unusual shape, or induce shoots to become hyperhydric. High concentrations of cytokinins also generally inhibit or delay root formation. Natural cytokinins include N^6-isopentenyladenine (iP) and zeatin. Zeatin is about 10 times more potent than any synthetic cytokinin, however, both zeatin and iP are not commonly used as they are very expensive to produce. The synthetic cytokinins most commonly used in tissue culture are kinetin and N^6-benzyladenine (BA), although it is now accepted that BA derivatives occur naturally (GEORGE & SHERRINGTON, 1984; DAVIES, 1987; STRNAD, 1997). The most commonly used cytokinin in the tissue culture of plants is BA, due to its effectiveness and affordability. It does, however, have disadvantages as it can induce genetic alteration and abnormal growth in some plants, and has been found to cause hyperhydricity in many species. A derivative of BA, 9-glycosylbenzyladenine [9G] BA, has been reported to cause heterogeneity in growth and inhibition of rooting during acclimatization (BAIRU *et al.,* 2007).

VAN STADEN and DREWES (1994) investigated the effect of BA and its glucosides on adventitious bud formation on *Lachenalia* leaf sections. The four BA derivatives tested were; BA, 3-glycosylbenzyladenine [3G] BA, 7-glycosylbenzyladenine [7G] BA and 9-glycosylbenzyladenine [9G] BA. Of the four cytokinins tested, BA increased adventitious bud formation in *Lachenalia*. The best results were obtained when the culture media was supplemented with 4 mg/l BA and 2 mg/l NAA. VAN STADEN and DREWES (1994) postulated that the different responses with different cytokinin derivatives is a function of uptake, transport or metabolism of the applied cytokinin.

Media supplemented with BA increased the production of embryogenic structures as well as the maturation of somatic embryos in *Freesia hybrida* (BACH, 1992). ULRICH *et al.* (1999) investigated the micropropagation of *Crinum* 'Ellen Bosanquet'. All BA concentrations (1, 2 and 5 mg/l) enhanced shoot formation, but the best shoot formation was with media containing 5 mg/l BA. NAA had an inhibitory effect on shoot formation as fewer shoots were formed on NAA-containing media, compared to the control. Explants grown on media with NAA became enlarged and swollen, and no organs or callus developed. In another experiment, tri-scales were cultured on media supplemented with 8, 16 or 20 mg/l BA. After four months the explants were transferred to hormone-free media for three months. Optimal shoot and bulblet formation was stimulated when explants were initially grown on media with 8 mg/l BA, and then transferred to hormone-free media.

Rapid propagation of *Hyacinthus orientalis* bulbs was studied by TAKAYAMA *et al.* (1991). Bulb regeneration was stimulated by a combination of 10 mg/l BA and 0.1 mg/l NAA. Low concentrations of BA and either no NAA, or high concentrations of NAA did not stimulate bulblet formation. High concentrations of BA inhibited root development while a high concentration of NAA enhanced the development of regenerative callus. When media was supplemented with only 0.1 mg/l BA, bulblets enlarged in size.

The effect of growth regulators on regeneration of lily bulbs *in vitro* was investigated by DABROWSKI *et al.* (1992). Bulblet formation occurred on explants grown on media containing 0.1 mg/l NAA and low concentrations (0.1 and 0.3 mg/l) of either BA, kinetin or iP. The highest average number of bulblets was produced on media with 0.1 mg/l NAA and 0.1 mg/l iP or BA. Media supplemented with only 0.1 mg/l NAA gave the best results for rooting, while all concentrations of BA and high concentrations of iP and kinetin inhibited root formation. The

results of BA inhibiting root formation are in accordance with data from TAKAYAMA *et al.* (1991) and BAIRU *et al.* (2007).

The induction of roots and bulblets in *Hyacinthus orientalis* grown in culture was investigated by SANIEWSKI *et al.* (1974). Bulblets grown on media with either no plant growth regulators or containing 1 or 10 mg/l BA did not show any growth or bulblet formation. Media supplemented with NAA resulted in the formation of callus which then developed into roots. Equal concentrations of BA and NAA inhibited callus and root formation but new bulb scales developed. New bulblet formation was greatest when media was supplemented with 10 mg/l BA and 1 mg/l NAA.

In vitro bulb formation of *Narcissus asturiensis* was studied by SANTOS *et al.* (2002). Explants were cultured on MS media supplemented with IBA and BA, or NAA and BA. Both media induced shoots but shoot proliferation was the best on the NAA and BA containing media. This data is in agreement with work done with other species of *Narcissus* (HUSSEY, 1982b).

The studies described above illustrate the variability of the effects of different plant growth regulators on *in vitro* cultures. Importantly, the interactions between different classes of plant growth regulators, under different environmental conditions, have an effect on culture growth and development. The effect of endogenous levels of hormones on the effect of exogenous application should not be excluded as different explants at different times may contain different endogenous levels of hormones which may also have an effect on the response *in vitro*.

2.3.2 Materials and methods

Boophone disticha bulbs were collected in Spring 2009 from a wild population in the Mpomphomeni area, 29° 33’ 29” S, 30° 11’ 46” E, of KwaZulu-Natal (South Africa) and a voucher specimen (Cheesman 01 NU) retained at the UKZN Herbarium. Leaves and roots were removed from the bulbs and the outer scales were peeled off and discarded. The whole bulbs were decontaminated in 0.2 % $HgCl_2$ for 5 min. The bulbs were then cut longitudinally in half and further decontaminated in 0.1 % $HgCl_2$ for 5 min. The bulb halves were rinsed three times with sterile distilled water containing ascorbic acid. The bulb halves were then kept in sterile distilled water with ascorbic acid until needed. Twin-scales, which comprise of two adjacent scales connected by a piece of basal plate tissue, were cut from bulb segments and placed on

15 ml solid MS media. The MS media contained 2 g/l charcoal, 150 mg/l ascorbic acid, 8 g/l agar (Agar Bacteriological-Agar No. 1, Oxoid Ltd.), 30 g/l sucrose, 100 mg/l *myo*-inositol and were supplemented with various concentrations of NAA (0, 5.37, 10.74, 26.85 and 53.7 µM) and BA (0, 4.44, 8.88, 22.2 and 44.4 µM). The pH of the media was adjusted to 5.8 with diluted NaOH before autoclaving at 121 °C and 103 kPa for 20 min. Twin-scale explants were excised, placed on the media and glass culture tubes (2.5 x 15 cm) were sealed with metal caps and Parafilm®. Fifteen explants were used per treatment. The cultures were grown at 25 ± 2 °C under a 16 h photoperiod. The growth room was fitted with Osram L58W/640 cool white florescence light bulbs with an average light intensity of 74.4 µmol/m^2/s.

After 4 months, data collected (number of bulblets/per explants, bulblet mass and bulblet diameter) were subjected to one-way analysis of variance (ANOVA). Where there were significant differences ($p<0.05$), the means were separated using Duncan's Multiple Range Test (DMRT). Data analysis was done using SPSS version 15.0.

2.3.3 Results and discussion

The most successful plant growth regulator treatments were 26.85 µM NAA: 4.44 µM BA and 0 µM NAA: 8.88 µM BA, which had the highest bulblet induction of 40 % (Table 2.3). The next highest bulblet induction was 33 % and this was obtained from explants grown on media supplemented with either 5.37 µM NAA: 4.44 µM BA or 53.7 µM NAA: 4.44 µM BA. In terms of bulblet diameter and mass, however, media supplemented with 5.37 µM NAA: 4.44 µM BA gave the best results. The bulblets produced on this medium had a diameter of 9.04 mm and a mass of 626 mg which were significantly different from the other treatments (Figure 2.5). Treatments 26.85 µM NAA: 4.44 µM BA and 0 µM NAA: 8.88 µM BA had bulblet diameter values of 5.84 mm and 4.31 mm, and bulblet mass values of 354 mg and 175 mg, respectively. The dilemma which arises from these results is quantity versus quality. Does one choose treatments that produce more bulblets which are smaller in size or does one choose treatments that produce fewer bulblets which are larger in size and have a better chance of surviving? As all the bulblets that were produced in the micropropagation experiments were dried and used in antimicrobial assays (Chapter 3, Section 3.2), no bulblets were acclimatized. Acclimatization is an important stage in micropropagation, however, many micropropagated plants do not often survive the transfer from *in vitro* conditions to the greenhouse or field environment. If the *in*

vitro grown bulblets had to undergo acclimatization, the larger bulblets would have been selected as there would be a greater chance of survival.

Table 2.3: Effect of different NAA and BA concentrations on percentage bulblet induction, mean number, size and mass of bulblets for *B. disticha* twin-scale cultures.

NAA: BA concentration (µM)	Bulblet induction (%)	No. of bulblets per twin-scale	Bulblet diameter (mm)	Bulblet mass (mg)
0: 0	27	1	5.61 ± 2.54	225.50 ± 122.77
5.37: 0	20	1	7.01 ± 3.43	439.00 ± 443.96
10.74: 0	13	1	4.98 ± 0.85	199.00 ± 76.37
26.85: 0	20	1	4.75 ± 1.28	154.00 ± 78.94
53.7: 0	27	1	7.34 ± 1.44	482.75 ± 133.13
0: 4.44	20	1	8.14 ± 1.03	421.67 ± 206.11
5.37: 4.44	33	1	9.04 ± 1.90	626.20 ± 251.89
10.74: 4.44	27	1	6.10 ± 1.70	227.25 ± 148.97
26.85: 4.44	40	1	5.84 ± 1.63	354.17 ± 144.63
53.7: 4.44	33	1	7.80 ± 1.08	511.00 ± 147.72
0: 8.88	40	1	4.31 ± 1.16	175.17 ± 102.81
5.37: 8.88	13	1	7.48 ± 0.37	611.00 ± 35.36
10.74: 8.88	7	1	7.56	451.00
26.85: 8.88	13	1	4.90 ± 2.74	257.00 ± 244.66
53.7: 8.88	7	1	9.26	498.00
0: 22.19	20	1	5.32 ± 0.42	226.67 ± 109.44
5.37: 22.19	-	-	-	-
10.74: 22.19	13	1	6.25 ± 2.98	290.50 ± 235.47
26.85: 22.19	13	1	6.60 ± 0.74	591.00 ± 403.05
53.7: 22.19	-	-	-	-
0: 44.4	20	1	5.27 ± 0.60	363.00 ± 46.51
5.37: 44.4	7	1	2.54	62.00
10.74: 44.4	7	1	3.68	212.00
26.85: 44.4	13	1	4.62 ± 1.36	218.00 ± 189.50
53.7: 44.4	7	1	4.32	96.00

Figure 2.4: ***B. disticha*** **bulblets grown on media supplemented with various concentrations of NAA: BA. Green cap = 26. 85 µM NAA + 4.44 µM BA, Bronze cap = 5.37 µM NAA + 0 µM BA and Blue cap = 10.74 µM NAA + 4.44 µM BA.**

In general, the results from the plant growth regulator experiments were unsatisfactory as the induction rate of bulblets was very low. Looking at the top seven plant growth regulator treatments, the common concentration is 4.44 µM BA while the concentrations of NAA varied from 5.37 to 53.7 µM (Figure 2.4). FENNELL (2002) showed that BA increased bulb production in *Crinum moorei* and ULRICH *et al*. (1999) reported that pre-treatment by culturing explants on a media with BA followed by culture on hormone-free media increased bulb formation in *Crinum* 'Ellen Boanquet'. A low concentration of cytokinin is often used in the culture media to induce embryogenic callus. However, there is evidence which suggests that exogenous cytokinins could inhibit embryogenesis in monocotyledons and certain plant genotypes. This could be a result of the presence of endogenous cytokinins (GASPAR *et al.,* 1996). This could explain the lack of callus formation on the twin-scale explants of *B. disticha*.

The successful treatments, as stated above, all used 4.44 µM BA other than 0 µM NAA: 8.88 µM BA. These treatments could have been successful in bulblet formation because cytokinins,

as well as wounding the basal plate, are known to reduce apical dominance. The inclusion of cytokinins in the growth media and the cutting of the basal plate, stimulates outgrowth of pre-existing axillary meristems (HUSSEY, 1986; FENNELL, 2002). The involvement of cytokinins in apical dominance has been shown in bulbous plants. Species from the Lilaceae and Amaryllidaceae need higher concentrations of BA to promote branching than the Iridaceae (HUSSEY, 1976). 'Anatomical studies showed that adventitious plantlets from twin-scales developed in a multi-cellular mode at the base of the bulb between the scales' (HUSSEY, 1982a). HUSSEY (1982a) believes this may be a general phenomenon in bulbs. The fact that meristematic tissue is arranged around the bulb centre, between the scales close to the connective tissue between the bulb scales and the basal plate, allows cytokinins in the growth media to be readily available for use by the basal plate of the bulblet (HUSSEY, 1976; HUSSEY & FALAVIGNA, 1980; DE HERTOGH & LE NARD, 1993; FENNELL, 2002).

HUSSEY (1976) stated that BA causes branching of shoots in bulbous species. FENNELL (2002) found that divided bulblets of *Crinum moorei* placed on media containing BA enhanced shoot initiation while in *Agapanthus* shoot multiplication was improved (HUSSEY, 1980). Generally it was found that an increase in BA concentration increased multiplication rates and the number of shoots formed by bulblets (HUSSEY, 1976; HUSSEY, 1980; CUSTERS & BERGEVOET, 1992; DE BRUYN *et al*., 1992; FENNELL, 2002). However, in the current study higher concentrations of BA did not increase multiplication rates.

The same composition of growth regulators in the culture media may cause different reactions with different species and even cultivars, or similar reactions may be seen when sections of different organs are placed on the same media. These variations are due to the fact that different species and sections of various organs, differ in their ability to produce endogenous growth substances or they differ in their content at the time of isolation (HEMPEL, 1979). Variations could also be a result of the species or section of organ's ability to recognize and take up exogenous hormones as well as their ability to transport, metabolize and respond to the exogenous hormones. Therefore, the use of plant growth regulators in tissue culture is an art. It is not possible to dictate a particular concentration or plant growth regulator to be used in any single case.

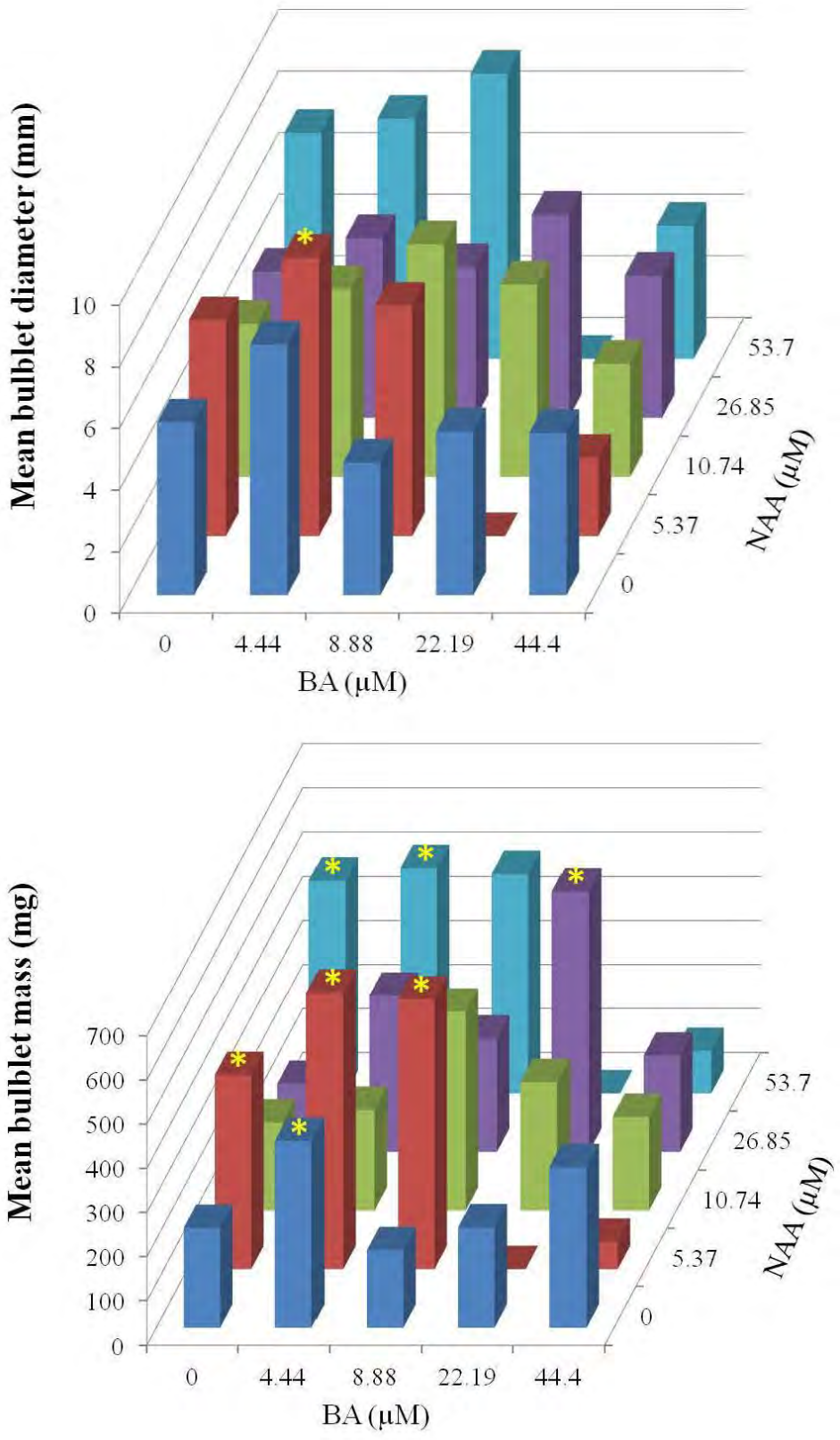


Figure 2.5: **The mean bulblet diameter and mean bulblet mass for the various hormone treatments using *B. disticha* twin-scale explants. Asterix above bars indicate significant differences from the control at the 5% level (ANOVA).**

2.4 TEMPERATURE

2.4.1 Introduction

Temperature and light play a pivotal role in the tissue culture environment influencing many responses of the cultured plants. Temperature is a natural regulator of plant growth and morphogenesis. It not only regulates growth rates but also the transition between various vegetative and reproductive phases during development (ASCOUGH *et al*., 2008). Cultures are generally maintained in an environment where the temperature is kept constant. This temperature is usually 25 ± 2 °C. The problem with this is that the explant does not recognize the diurnal and seasonal temperature fluctuations under which a plant would normally develop (MURASHIGE, 1974). Although constant temperatures may be adequate for the tissue culture of certain plants (mainly annuals and tropical species which grow in relatively uniform temperature conditions) more research is needed to investigate the influence on temperature of plants that are adapted to more temperate and desert climates. MURASHIGE (1974) suggests that maximum success from tissue culture may only be achieved when the precise temperature needs of a plant are fulfilled.

The optimum temperatures for bulblet growth for both *Nerine bowdenii* and *Heloniopsis orientalis* were 21 to 25 °C (PIERIK & IPPEL, 1977; KATO & OZAWA, 1979). Bulblet formation of *N. bowdenii* was strongly promoted by raising the temperature, with an optimum at 21 to 25 °C, for both number of bulbs and bulblet weight (PIERIK & IPPEL, 1977). JACOBS *et al.* (1992) found the optimum temperature for bulblet production of *N. bowdenii* was 17 to 22 °C. The highest fresh weight per bulblet and total fresh weight of bulblets produced per explant was at this temperature. High temperatures were found to reduce the percentage of explants that formed bulblets. These results confirmed the data of PIERIK and IPPEL (1977). Bud formation was best at a temperature of 21 to 25 °C, however, a pre-treatment at 16 °C for 7 to 21 days increased bud formation compared to a constant temperature of 16 and 25 °C. A 30 °C pretreatment decreased the number of buds while a continuous treatment at 30 °C inhibited bud formation. At 16 and 30 °C only a few shoots developed. Lastly, at 30 °C the survival rate was 60 % while at 16, 21 and 25 °C there was a 100 % survival rate (KATO & OZAWA, 1979).

SEABROOK and CUMMING (1982); PIERIK *et al.* (1983); NIIMI *et al.* (1999) and KULKARNI *et al.* (2005) found 25 °C to be the best temperature for shoot and bulblet growth. The largest number of shoots and bulbs of *Narcissus* was formed at 25 °C while the leaf width was greatest at 30 °C but the leaves were abnormal with wrinkled, curled and uneven surfaces. *Narcissus* shoots were significantly greater at a constant temperature of 25 °C compared to other constant temperatures and alternating temperatures tested (SEABROOK and CUMMING, 1982). These findings agree with DE CAPITE (1955). PIERIK *et al.* (1983) looked at the propagation of *Eucharis grandiflora* and discovered that regeneration and bulblet weight were promoted by raising the temperature from 21 to 25 or 29 °C. Rooting and leaf formation were increased by increasing the temperature from 17 to 25 °C.

The temperature affects the type of morphogenesis during *in vitro* culture. It has been found that the optimum temperature for the morphogenetic response varies with species and cultivars (MURASHIGE, 1974).

2.4.2 Materials and methods

The preparation of bulb material and culture media is as described in Section 2.3.2, however, some changes to the media were made. An experiment was carried out to determine which carbohydrate and concentration was optimum for the propagation of *B. disticha* however, success was limited (results not shown). The results indicated that the carbohydrates at the given concentrations outlined in the table below enhanced twin-scale response in culture. The choice of carbohydrates were done in conjunction with the most promising plant growth regulator treatments and included in the media. These included:

Carbohydrate concentration (%)	NAA: BA concentration (µM)
0 sucrose	0: 0
6 sucrose	5.37: 4.44
9 sucrose	0: 4.44
3 glucose	0: 4.44
3 glucose	10.74: 4.44

The pH of the media was adjusted to 5.8 with diluted NaOH before autoclaving at 121 °C and 103 kPa for 20 min. Twin-scale explants were placed on 15 ml of media and glass culture tubes (2.5 x 15 cm) were sealed with metal caps and Parafilm®. Twenty culture tubes per treatment were incubated in plant growth chambers (Conviron E7H) at temperatures of 10, 20, 25 and 30 °C with a 16 h photoperiod. The Convirons contained Osram L58W/640 cool white fluorescent bulbs. The light intensity was measured using a radiation meter and the Conviron E7H had a light intensity range of 70 to 90 µmol/m^2/s.

2.4.3 Results and discussion

MOCHTAK (1989) states that it is a well known fact that cultural conditions of bulbous plants particularly temperature play an important role in their development and growth. The most common temperature used in tissue culture protocols is 25 °C and this is the optimum temperature for many bulbous species. *Lilium* (CHANG *et al*., 2000), *Fritillaria* (PAEK & MURTHY, 2002), *Lycoris* (HUANG & LIU, 1989) and *Crinum* (SLABBERT *et al*., 1993) are a few examples which are all cultured at 25 °C. However, YEOMAN (1986) suggested that the optimum temperature for bulbous species is often lower than 25 °C as they found *Galanthus* and *Narcissus* require temperatures of between 15 and 18 °C.

High temperatures can induce dormancy in bulblets (YAMAGISHI, 1998) while low temperatures can break dormancy in bulblets (ALDERSON *et al*., 1986; VAN DER LINDE *et al*., 1988; TAKAYAMA & MISAWA, 1980; SANTOS *et al*., 1998). Lower temperatures can also induce swelling and development at the base of the bulblet, where meristematic centres are found (ALDERSON *et al*., 1986). Therefore it is vital to establish the optimum temperature for the tissue culture of a specific species and in so doing, the highest bulblet regeneration from the cultures will be achieved.

FENNELL (2002) suggested that the optimum temperature for the tissue culture of a species is related to the temperature of its natural habitat. Thus bulbous species from warmer areas would require warmer culture temperatures for optimum growth. This was seen in the present study. No results were obtained for temperatures 10 and 20 °C (data not shown). *Boophone disticha* is found throughout southern Africa, extending into southern tropical Africa and these areas generally have high temperatures. Therefore, treatments incubated at temperatures 25 and 30 °C are expected to produce better results.

At 25 °C two treatments namely; 6 % sucrose with 5.37 µM NAA: 4.44 µM BA and 3 % glucose with 10.74 µM NAA: 4.44 µM BA, gave results (Table 2.5). The twin-scale on 6 % sucrose only produced one small bulb while the twin-scale on 3 % glucose produced 5 bulblets. These bulblets had a mean diameter of 4.33 mm and a mean mass of 96 mg. At a temperature of 30 °C, only the control media (0 % sucrose with no hormones) produced a result. The twin-scales produced a single bulb which had a mean diameter of 6 mm and a mean mass of 271.50 mg (Table 2.5). Bulblet production for the multiplication of *Narcissus* was highest at 25 °C when compared to cultures maintained at 15, 20 and 30 °C (SEABROOK & CUMMING, 1982). Bulblet development in *Nerine, Crinum moorei* and *Narcissus* was stimulated at 25 °C which was also its optimum temperature for bulblet growth (PIERIK & IPPEL, 1977; STEINITZ & YAHEL, 1982; FENNELL, 2002). These results are in agreement with the present study on *B. disticha*.

Table 2.4: Effect of temperature on percentage bulblet induction, mean number, size and mass of bulblets for *B. disticha* twin-scale cultures.

Temperature (°C)	Carbohydrate concentration (%)	NAA: BA concentration (µM)	Bulblet induction (%)	No. of bulblets per twin-scale	Bulblet diameter (mm)	Bulblet mass (mg)
25	0 Sucrose	0: 0	-	-	-	-
	6 Sucrose	5.37: 4.44	5	1	3.18	53.00
	9 Sucrose	0: 4.44	-	-	-	-
	3 Glucose	0: 4.44	-	-	-	-
	3 Glucose	10.74: 4.44	5	5	4.33 ± 1.08	96.00 ± 33.74
30	0 Sucrose	0: 0	10	1	6.00 ± 2.48	271.50 ± 225.57
	6 Sucrose	5.37: 4.44	-	-	-	-
	9 Sucrose	0: 4.44	-	-	-	-
	3 Glucose	0: 4.44	-	-	-	-
	3 Glucose	10.74: 4.44	-	-	-	-

NIIMI *et al.*, (2000) believe that temperature also affects the uptake and use of sugars and an increase in the uptake of sugars will generate an increase in the growth and development of the explant in culture. FENNELL (2002) also claimed that high temperatures stimulated the use of accumulated carbohydrates, due to respiration at high temperatures. This will supply the explant with more energy and so greater multiplication can take place. Therefore, it makes sense that

bulblet formation in *B. disticha* occurred at temperatures of 25 and 30 °C. The only problem with the above claims by NIIMI *et al.*, (2000) and FENNELL (2002) that growth and multiplication is increased with the increased uptake of sugars at higher temperatures, is that in the current study, the treatment at 30 °C contained 0 % sugar and no plant growth regulators. Therefore, the only explanation for the formation of bulblets in this treatment must be due to the presence of endogenous plant growth regulators.

2.5 PHOTOPERIOD

2.5.1 Introduction

Several environmental factors can affect bulbous plant growth and development, but the major environmental cues are light and temperature. Plant growth and development depend on light for photosynthesis and photomorphogenesis. Plant tissue cultures also require light, to a lesser degree, to regulate morphogenetic processes. The effect of light on photosynthesis of *in vitro* cultures is of less importance than that of *in vivo* plants. In tissue culture, photosynthesis is not actually necessary since a carbohydrate source is provided (MURASHIGE, 1974). According to ECONOMOU and READ (1987) light influences the success of micropropagation through its three parameters of duration (photoperiod), illuminance (light intensity) and spectral quality (wavelength).

These three light factors affect shoot growth and morphogenesis in addition to having a role in photosynthesis. Light intensity regulates the size of leaves and stems, as well as their morphogenic pathway and is involved in pigment formation and hyperhydricity. Light quality has been reported in different plant cultures to play an important role on several morphologic characteristics which include plant elongation (e.g. chrysanthemum and tomato), axillary shoots (e.g. grapevine), leaf anatomy and leaf size (e.g. birch) as well as rhizogenesis (e.g. pear) (MURASHIGE, 1974; DA SILVA & DEBERGH, 1997).

Experiments were conducted by ECONOMOU and READ (1987) which looked at light treatments to improve efficiency of *in vitro* propagation systems. All three parameters: duration, illumination and quality were looked at and it was reported that both root and shoot formation was affected by light duration (ECONOMOU & READ, 1987). The photoperiod extends from 0 h of light (continuous darkness) to 24 h of light (continuous light). Plant species and explants respond differently to photoperiods, however, most plants need a photoperiod of 8 to 18 h of light from cool white light bulbs (ECONOMOU & READ, 1987).

LESHEM *et al.* (1982) who worked on *Lilium longiflorum* and KROMER (1989) who worked on *Muscari racemosum* both found that light affected bulblet growth and the number of roots produced. LESHEM *et al.* (1982) however, also found that light affected the number of bulblets produced per explant. KROMER (1989) reported that in the light and in the dark a proportional

number of explants formed roots, but the explants grown in the light produced a greater number of roots than those in the dark. KROMER (1989) also observed there was an essential difference in the number of emerging leaves per bulblet. In the light the number of leaves was 20 % higher than in the dark. This finding was confirmed by LESHEM *et al.* (1982) who found that bulblets cultured in the light bore many leaves, while those in the dark bore fewer leaves.

LESHEM *et al.* (1982) found that bulblets in the light were noticeably different from the bulblets developing in the dark. Scale sections growing in the light turned green and so did the bulblets developing on them. More bulblets developed in the light but they were smaller than those produced in the dark.

In the study carried out by KROMER (1989) it was noted that light slightly increased the percentage of regenerating bulblets but did not influence the number of bulblets produced per explant. The results obtained by LESHEM *et al.* (1982) are not in agreement with KROMER's findings. LESHEM *et al.* (1982) found no difference between effects of light and dark in the percentage of scales regenerating bulblets although there was a higher number of bulblets produced per explant in the light.

GUDE and DIJKEMA (1992) found that light treatments had no effect on the number of newly formed bulbs in *Hyacinth*. These findings agreed with LESHEM *et al.* (1982). However, the appearance of the bulbs was greatly affected by the light treatment. Dark-treated bulbs were relatively large, white and undifferentiated as only bulb scales were visible.

VARSHNEY *et al.* (2000) developed a protocol for the *in vitro* mass production of Asiatic lily hybrids. Previous research showed that the effect of photoperiod on bulblet formation varied with various *Lilium* species especially *L. longiflorum, L. speciosum, L. auratum* and *L. rubellum*. Bulb formation and growth was stimulated by continuous darkness in some species, while in other species it was observed that light significantly affected bulblet growth. Bulblets grown in the light were small and had many leaves while those grown in the dark were large and had few leaves (LESHEM *et al.,* 1982). NIIMI *et al.* (1999) found that there was an increase in the percentage of regenerating bulb scales and the number of bulblets per scale when the cultures were grown in the light. TAKAYAMA and MISAWA (1983) and MAESATO *et al.* (1994) found that continuous light was optimal for bulblet formation for *L. speciosum, L. auratum* and *L. japonicum*. VARSHNEY *et al.* (2000) reported that continuous light was not essential for

growth and bulblet multiplication of the Asiatic lily hybrids. The number, size and weight of bulblets increased under a 16 h photoperiod for both cultivars compared to 24 h light and complete darkness.

2.5.2 Materials and methods

The preparation of bulb material and culture media is as described in Section 2.5.2, however, the cultures were stored in E7H Convirons at 25 ± 2 °C with varying photoperiods. The photoperiods tested were continuous light, continuous dark, 16 h light/ 8 h dark and 8 h light/ 16 h dark. The Convirons were fitted with Osram L58W/640 cool white fluorescent bulbs and the light intensity range in the growth chambers was 70 to 90 µmol/m^2/s.

2.5.3 Results and discussion

The photoperiod 16 h light/ 8 h dark had one successful treatment (3 % glucose, 0 µM NAA: 4.44 µM BA). Only one bulblet formed and it had a diameter of 7.78 mm and a mass of 331 mg. VARSHNEY *et al*. (2000) and MORÁN *et al*. (2003) found that a greater number of bulblets were formed on twin-scales of *Lilium* species and *Cyrtanthus* species when grown in a 16 h photoperiod.

Bulblet formation is stimulated by darkness in many bulbous species (VAN AARTRIJK & VAN DER LINDE, 1986). In *Nerine*, bulblet formation was greatest when twin-scales were exposed to a period of darkness (PIERIK & IPPEL, 1997). Twin-scale explants are derived from bulbs and as bulbs are soil-bound, they are exposed to little light in their natural environment. Therefore, dark conditions simulate the underground conditions in which bulbs normally grow (ULRICH *et al*., 1999). Looking at Table 2.6, it can be seen that in the dark two treatments were successful in stimulating bulblet formation. The first (9 % sucrose, 0 NAA: 4.44 µM BA) successfully produced one bulblet which had a diameter of 2.16 mm and a mass of 30 mg. The second successful treatment (3 % glucose, 0 NAA: 4.44 µM BA) produced multiple bulblets with a mean diameter of 5 mm and a mean mass of 167 mg.

When culture tubes were placed in the dark, the twin-scales remained white and the bulblets which formed were also white in colour (Figure 2.6). This is in agreement with GUDE and DIJKEMA (1992) who found that the appearance of *Hyacinth* bulbs was greatly affected by the light treatment. Dark-treated bulbs were relatively large, white and undifferentiated as only bulb scales were visible (GUDE & DIJKEMA, 1992). Once the *B. disticha* cultures were placed in the light, the twin-scale explants turned a browny-black colour and the bulblets turned green.

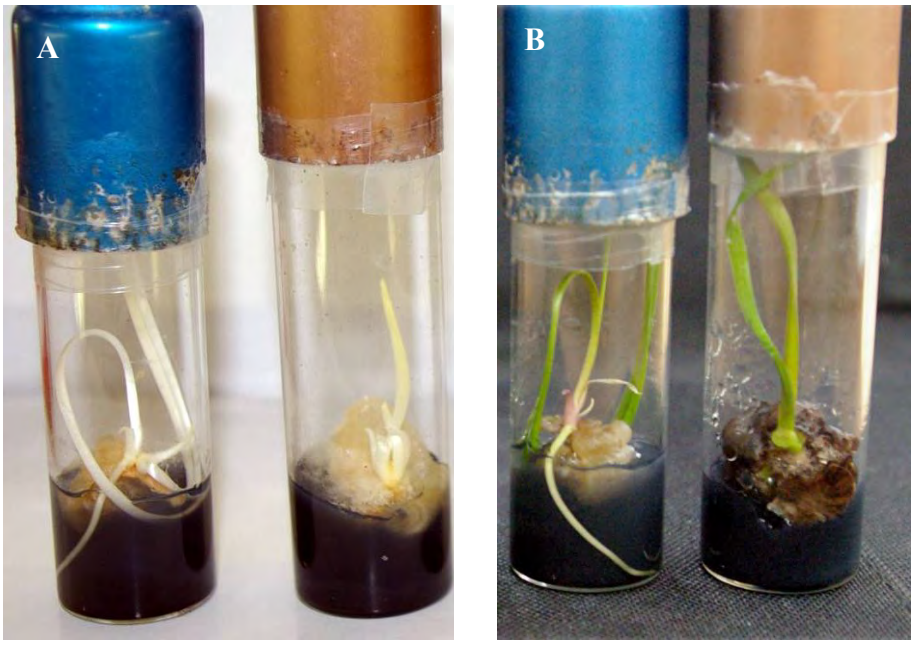


Figure 2.6: Explants and bublets stored in the dark are white in colour (A), but once removed and placed in the light, turn green (B).

For continuous light, only one treatment (3 % glucose and 0 µM NAA: 4.44 µM BA) produced results. *Hippeastrum* is cultured in continuous light (HUANG *et al*., 1990a) and it is believed that the exposure to a greater number of hours of light will enable greater photosynthesis by the explants in this photoperiod. Greater photosynthesis would mean greater production of carbohydrates and thus increased growth and regeneration. This could possibly account for the bulblet produced in this photoperiod having the largest mass of 384 mg.

As results, although very few, were obtained for three out of the four photoperiods tested (the photoperiod 8 h light/ 16 h dark was unsuccessful), it seems that photoperiod does not really have an effect on the induction of bulblets from twin-scales of *B. disticha*. LESHEM *et al.* (1982) found no difference between the effects of light and dark in the percentage of scales

regenerating bulblets although there were a higher number of bulblets produced per explant in the light. Treatments which had successful results for *B. disticha*, all had media supplemented with 3 % glucose, 0 µM NAA: 4.44 µM BA, while the fourth successful treatment was media with 9 % sucrose and 0 µM NAA: 4.44 µM BA. Therefore, it seems that the plant growth regulator combination, or rather just BA as no NAA was present, had more of an effect on the induction of bulblets than the photoperiod did. Overall, however, the results were poor.

Table 2.5: Effect of various photoperiods on percentage bulblet induction, mean number, size and mass of bulblets for *B. disticha* twin-scale cultures.

Photoperiod	Carbohydrate concentration (%)	NAA: BA concentration (µM)	Bulblet induction (%)	No. of bulblets per twin-scale	Bulblet diameter (mm)	Bulblet mass (mg)
16 L/ 8 D	0 Sucrose	0: 0	-	-	-	-
	6 Sucrose	5.37: 4.44	-	-	-	-
	9 Sucrose	0: 4.44	-	-	-	-
	3 Glucose	0: 4.44	5	1	7.78	331.00
	3 Glucose	10.74: 4.44	-	-	-	-
Continuous light	0 Sucrose	0: 0	-	-	-	-
	6 Sucrose	5.37: 4.44	-	-	-	-
	9 Sucrose	0: 4.44	-	-	-	-
	3 Glucose	0: 4.44	5	1	7.40	384.00
	3 Glucose	10.74: 4.44	-	-	-	-
Continuous dark	0 Sucrose	0: 0	-	-	-	-
	6 Sucrose	5.37: 4.44	-	-	-	-
	9 Sucrose	0: 4.44	5	1	2.16	30.00
	3 Glucose	0: 4.44	5	2	5.00 ± 0.71	167.00 ± 5.66
	3 Glucose	10.74: 4.44	-	-	-	-

2.6 MICROPROPAGATION OF *B. DISTICHA*

On the whole, bulblet formation from twin-scale explants of *B. disticha* was poor. A possible reason for this is because *B. disticha* is a monocotyledonous plant and monocotyledons are more difficult to regenerate *in vitro* than dicotyledons (MOREL & WETMORE, 1951; KAMO, 1994).

Dicotyledons have meristematic cells in the vascular cambium and secondary growth is produced by this cambium. Vascular bundles are situated in a ring in dicotyledons. In monocotyledons, vascular cambium is absent and the vascular bundles are scattered. Therefore monocotyledons lack areas of meristematic tissue and thus secondary growth is limited.

Generally callus is difficult to obtain from monocotyledons such as species from the Amaryllidaceae (HUSSEY, 1975). Therefore, HUSSEY (1982a) states that in the Liliaceae, Iridaceae and Amaryllidaceae the induction of direct adventitious shoots from explants *in vitro* is the principle method of propagation. From the current study it was found that propagation was quicker and more successful when using seedling material as the explant. This could possibly be because the meristematic tissue is still very active. However, different areas have different activity. The leaves on the seedling did not respond as perhaps the meristematic activity had already been lost due to growth. Therefore, for successful propagation of *B. disticha*, young bulbs or seedlings should be used.

Lily callus was successfully produced on the relatively simple media of LINSMAIER and SKOOG (1965). This result was surprising considering the general failure of establishing monocotyledons in tissue culture. The successful tissue culture of oats (CARTER *et al*., 1967) and rice (YAMADA *et al*., 1967) were also accomplished on similar media of MURASHIGE and SKOOG (1962) or of LINSMAIER and SKOOG (1965). These media differ from the others in general use in that they are much higher in their mineral salt content, particularly nitrogenous and potassium salts. The micropropagation of *B. disticha* had limited success, therefore, in future studies it might be useful to try to induce bulblet formation from *B. disticha* twin-scales on LINSMAIER and SKOOG (1965) media or even on quarter or half-strength media. However, it has been reported that certain monocotyledonous species may respond differently in culture.

The establishment of an effective micropropagation protocol for *B. disticha* was unsuccessful. In the present study, a number of variations on a tissue culture protocol were tried and tested such

as various photoperiods, temperatures, carbohydrates and plant growth regulators. Although these experiments were not successful, it was noted that activated charcoal, ascorbic acid and BA are important and should be incorporated into the culture media. In future experiments, different culture media as well as various cytokinins (including the newly discovered topolins) should be tested. As successful protocols have been established for other Amaryllidaceae species (Section 1.5.3), all that can be deduced is that different species (owing to their genetics or endogenous plant growth regulators) have different reactions even when placed on the same culture medium in the same environmental conditions. This, therefore, makes it nearly impossible to predict an outcome.

2.7 SUMMARY

- Twin-scales proved to be the most successful explants
- It was necessary for the culture media for *B. disticha* to contain activated charcoal and ascorbic acid
- Bulblet induction was best when the media was supplemented with a cytokinin
- Addition of BA had more of an effect on bulblet induction than carbohydrate, temperature or photoperiod
- Overall, the micropropagation of *B. disticha* showed limited success
-

Chapter Three

ANTIMICROBIAL ACTIVITY

3.1 ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY

3.1.1 Introduction

Global antibacterial and antifungal resistance along with the emergence of new infections is becoming an increasing public health problem; therefore the need for new antimicrobial agents is of utmost importance (BAX *et al.*, 2000; FABRICANT & FARNSWORTH, 2001; SPELLBERG *et al.*, 2004). Infectious diseases have been reported to be the second-leading cause of death worldwide and with the emergence of multidrug-resistant pathogens, a situation has been created where there are few or no treatment options for infections with certain micro-organisms (SPELLBERG *et al.*, 2004). The rise in drug-resistant bacteria is blamed on the overuse and misuse of antibiotics and an increase in resistance has been paralleled by an overall increase in the number of prescriptions for antibiotics (BAX *et al.*, 2000). In spite of the serious need for new antimicrobial agents, the development of these agents is decreasing. The approval of new antibacterial agents, by the United States Food and Drug Administration, has decreased by 56% over the past 20 years and some of the large pharmaceutical companies have shown that they are reducing or completely abandoning anti-infective research (SPELLBERG *et al.*, 2004; THOMSON *et al.*, 2004). Therefore, not only is the development of completely new antimicrobials needed to help restore the balance of resistance versus available antibiotics but so is the use of natural products to treat these infections.

Natural products are becoming more important in society as the move away from synthetic products increases. Synthetic products are believed to be detrimental to the environment as well as to human health; therefore there has been renewed interest in traditional pharmacopoeias (LOUW *et al.*, 2002). There are several approaches to selecting plant species as candidates for biological activity screening. These include: a random selection approach where plants or plant parts are randomly selected and then subjected to chemical or biological screening; a chemotaxonomic approach which is based on correlation between plant taxonomy and the

occurrence of chemical compounds; the use of databases such as NAPRALERT which contains information on 43 879 species of higher plants covering ethnomedical, chemical and pharmacologic uses; and lastly the ethnobotanical approach where plants that are used in traditional medicine are screened for biological activity (FABRICANT & FARNSWORTH, 2001). To date, 122 compounds have been discovered from only 94 species of plants and three quarters of these 122 plant-based drugs in use were discovered by following leads provided by traditional medicine in various parts of the world (SCOTT, 1993; FABRICANT & FARNSWORTH, 2001). Thus the ethnobotanical approach was used in this study to screen *Boophone disticha* for antimicrobial activity.

Various assays can be used to test for biological activity, firstly *in vitro* and later for promising products, *in vivo*. Microbial assays are an important technique for evaluating the antimicrobial activity of medicinal plants and when it comes to testing for antibacterial activity, there are three such methods (HEWITT & VINCENT, 1989). These include: disc-diffusion, dilution and bioautographic assays. The disc-diffusion method allows more than one plant extract to be screened against various bacteria at the same time hence this method is useful for preliminary screenings. This method however is no longer used as the data obtained is not accepted by international journals. The dilution assay on the other hand is more readily used as it determines more precisely the antibacterial activity of extracts by giving the minimum inhibitory concentration (MIC) or the minimum bactericidal concentration (MBC) values for a particular micro-organism. Lastly, the bioautographic assay is used for the rapid detection of bioactive constituents of plant extracts during bioassay-guided fractionation (RASOANAIVO & RATSIMAMANGA-URVERG, 1993).

The plant extract or isolated compound determines which assay is to be used and the results are often influenced by various factors such as extraction method, culture medium and incubation temperature (RIOS *et al.*, 1988). JANSSEN *et al.* (1987) and THOMAS (1989) reported that the age of the plant, the state of the plant material (whether it is fresh or dried) and the strain of bacteria used for testing are also factors which influence the results. Bacteria are some of the most plentiful organisms on earth and are able to adapt to virtually any environment. Bacteria can be divided into two major groups based on differences in cell wall structure: Gram-positive or Gram-negative bacteria. These differences were first seen as differences in the ability of the bacteria to retain a crystal violet-iodine complex known as Gram's stain (SALYERS & WHITT, 1994).

Gram-positive bacteria have a peptidoglycan cell wall that is many layers thick (15-50 nm), is porous and can be easily separated from the plasma membrane. Interwoven in the peptidoglycan matrix are teichoic acids which are polymers of sugar alcohol phosphate. The outside surface of Gram-positive peptidoglycan is usually studded with proteins. Different strains and species of Gram-positive bacteria differ in teichoic acid structure and in surface proteins (RANG & DALE, 1987; SALYERS & WHITT, 1994). The structure of Gram-negative bacteria peptidoglycan is very similar to that of Gram-positive bacteria but is much thinner (2 nm) and attached to a second membrane (outer membrane) by lipoproteins. The outer membrane has an unusual structure. Phospholipids are located mainly on the inner side next to the peptidoglycan layer and the outer side contains a lipopolysaccharide that has its lipid portion embedded in the membrane and its polysaccharide portion sticking out from the bacterial surface. The outer membrane contains a number of proteins; porins, being one of the most important. Porins create small channels across the outer membrane that admit low molecular weight compounds such as nutrients. The porins restrict the access of many antibiotics and it is this that is thought to be the reason why Gram-negative bacteria, in general, are more resistant to antibiotics than Gram-positive bacteria (SALYERS & WHITT, 1994).

Four species of bacteria, two from each class of bacteria, were used to screen for antibacterial activity. The two Gram-positive bacteria were *Bacillus subtilis* and *Staphylococcus aureus* and the two Gram-negative were *Escherichia coli* and *Klebsiella pneumoniae*. *B. subtilis* is rod-shaped, catalase-positive and commonly found in soil (MADIGAN & MARTINKO, 2006). It may contaminate food but it rarely causes food poisoning. *B. subtilis* is only known to cause disease in severely immuno-compromised patients, or can alternatively be used as a probiotic in healthy individuals (RYAN & RAY, 2004). *S. aureus* is a facultative anaerobic, catalase-positive, coccal bacterium (RYAN & RAY, 2004). It is often found as part of the normal skin flora on the skin and nasal passages as well as in the colon and in urine. *S. aureus* can cause a range of illnesses from minor skin infections, such as pimples, boils, carbuncles and abscesses, to life-threatening diseases such as pneumonia, meningitis, toxic shock syndrome and sepsis (HEYMANN, 2004). *S. aureus* ranks second to *E. coli* in causing hospital-acquired infections (EVANS & FELDMAN, 1982).

E. coli is a rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. It is a facultative anaerobic organism which cannot sporulate (MADIGAN & MARTINKO, 2006). Most *E. coli* strains are harmless however some serotypes can cause

serious food poisoning in humans. Virulent strains of *E. coli* are known to cause gastroenteritis, urinary tract infections and neonatal meningitis (SALYERS & WHITT, 1994). *K. pneumoniae* is a non-motile, encapsulated, facultative anaerobic bacterium. It is rod-shaped and found in the normal flora of the mouth, skin, and intestines as well as in water, soil and occasionally food (PODSCHUN & ULLMAN, 1998). In recent years, *K. pneumoniae* has become an important pathogen in nosocomial infections. It is known to cause intra-abdominal infections, urinary tract infections and pneumonia (PODSCHUN & ULLMAN, 1998; RYAN & RAY, 2004). Respiratory infections in the form of pneumonias make up approximately 15% of all nosocomial infections recorded and three quarters of these are reported to be caused by *K. pneumoniae* (EVANS & FELDMAN, 1982).

During the past decade there has been an increase in the number of patients with weakened immune systems as a result of the human immunodeficiency virus (HIV). This has been linked to an increase in the incidence of human systemic mycoses (MASOKO *et al.*, 2007). Worldwide studies of acquired immune deficiency syndrome (AIDS) show that 58-81% of all patients contract a fungal infection at some time during the early stages or after developing AIDS and 10-20% have died as a direct result of fungal infections. In HIV patients the presence of oral candidiasis is one of the earliest opportunistic infections (FAN-HARVARD *et al.,* 1991; MOTSEI *et al.*, 2003).

Candidiasis is caused by *Candida albicans* which is a fungus that grows both as yeast and filamentous cells. It is a constituent of the normal gut flora that lives in the human mouth and gastrointestinal tract. *C. albicans* lives in 80% of the human population without causing harmful effects, but stress, antibiotics, birth control pills and excessive sugar in the diet causes overgrowth of the fungus which results in candidiasis (SALTARELLI, 1989). When *C. albicans* is active, it can affect every part of the body and its toxins can even affect the brain and bloodstream (ODDS, 1987). This disease affects people of all ages and shows either mild symptoms or chronic conditions (ODDS, 1987). Amphotericin B and the azole group of antifungal agents are often used in the treatment of fungal infections, however, the widespread and incorrect use of these drugs has led to the emergence of drug resistance in several common pathogenic fungi (MASOKO *et al.*, 2007). Due to this emergence, it is important to develop new antifungal agents.

Two screening methods may be used to test plant extracts for possible antifungal activity. The first method is a modification of the National Committee for Clinical Laboratory Standards proposed method (M27-P) broth dilution test (ESPINEL-INGROFF & PFALLER, 1995) and the second is the microdilution bioassay (ELOFF, 1998) with slight modifications to suit fungal growth. The microdilution bioassay was used in this study to assess both antibacterial and antifungal activity of various plant parts of *Boophone disticha* collected in different seasons. This assay is widely used and is a well recognized technique in the screening of plant extracts for antimicrobial activity (FENNELL *et al.*, 2004).

3.1.2 Materials and methods

Collection of plant material

Boophone disticha bulbs were collected in Spring, Summer, Autumn and Winter from the outskirts of Mpophomeni, along the Underberg road, 29° 33.296' S & 30° 11.468' E, KwaZulu-Natal, South Africa. A voucher specimen (Cheesman 01 NU) was deposited in the University of KwaZulu-Natal Herbarium, Pietermaritzburg. The bulbs were divided into the various plant parts; inner scales, outer scales, roots and leaves. The plant parts were oven dried at 50°C, ground and stored in airtight containers in the dark.

Preparation of plant extracts

Dried, ground plant parts were extracted sequentially with 20 ml/g of petroleum ether, dichloromethane, 80% ethanol and water with sonication for 1 h. The temperature was kept low by adding ice to the water bath. The plant extracts were then filtered using Whatman No. 1 filter paper. The filtrates were concentrated using a rotary evaporator and then air-dried under a stream of cold air. The aqueous extracts were freeze-dried. The dried extracts were kept in the dark at 10 °C until ready for use.

Antibacterial assays

Minimum inhibitory concentration (MIC) of the extracts for antibacterial activity was determined using the micro-dilution bioassay in 96-well micro-plates (ELOFF, 1998). Overnight cultures (incubated at 37 °C in a water bath with an orbital shaker) of four bacterial strains: 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) were diluted with sterile Mueller-Hinton (MH) broth (1 ml bacterial suspension/50 ml MH). The crude plant extracts were re-dissolved to a concentration of 50 mg/ml in ethanol for organic extracts and water for aqueous extracts. One hundred microlitres of each extract were two-fold serially diluted with sterile distilled water in a 96-well micro-plate for each of the four bacteria in triplicate. A similar two-fold serial dilution of neomycin (Sigma) (0.1mg/ml) was used as a positive control against each bacterium. The ethanol solvent, bacteria-free broth and distilled water were included as negative controls. One hundred microlitres of each bacterial culture were added to each well. The plates were covered with parafilm and incubated overnight at 37 °C. Bacterial growth was indicated by adding 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) to each well and the plates incubated at 37 °C for 1 h. Bacterial growth in the wells was indicated by a reddish-pink colour, whereas clear wells indicated growth inhibition by the tested extracts. MIC values were recorded as the lowest concentrations of extracts showing clear wells. The assay was repeated twice with three replicates each.

Antifungal screening

The antifungal activity of the extracts was evaluated against *Candida albicans* (ATCC 10231) using the micro-dilution assay (ELOFF, 1998) as modified for fungi (MASOKO *et al*., 2007). An overnight fungal culture was prepared in Yeast Malt (YM) broth. Four millilitres of sterile saline was added to 400 µl of a 24-h-old *C. albicans* culture. The absorbance was read at 530 nm and adjusted with sterile saline to match that of a 0.5 M McFarland standard solution. From this prepared stock, a 1:1000 dilution with sterile YM broth was prepared. Plant extracts were re-suspended in dimethyl sulfoxide (DMSO) and distilled water to produce organic solvent and aqueous extracts to a concentration of 100 mg/ml. The assay was repeated twice with three replicates per extract. MIC and minimum fungicidal concentration (MFC) values were recorded as lowest concentrations of extracts showing clear wells.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple range test was used for pair-wise comparison at a 5 % level of significance ($P < 0.005$) (Table 3.5). General ANOVA was conducted for main effects and their interactions (Table 3.6).

GenStat® 14th edition (VSN International, Hemel Hempstead, U.K.) statistical package was used to analyze the data of the study.

3.1.3 Results and discussion

An estimated 27 million people in South Africa are using traditional plant medicine for primary health care purposes (MULHOLLAND & DREWES, 2004). Indigenous bulbous plants that are important to South African traditional healers mainly belong to the Amaryllidaceae and Hyacinthaceae families and some of these plants are traditionally used against infection related ailments (LOUW *et al.*, 2002). When it comes to bulbous plants, the bulbs are regarded as the most valuable material as herbalists believe that the underground plant parts contain the highest concentration of potent healing agents (LIKHITWITAYAWUID *et al.*, 1993; SHALE *et al.*, 1999; ZSCHOCKE *et al.*, 2000). Leaves of bulbous plants are not often used in traditional practice as they cannot be kept for long periods of time nor do they apparently retain their medicinal compounds for later use like the slower growing storage organs do (ZSCHOCKE *et al.*, 2000; KELMANSON *et al.*, 2000). Therefore, this study aimed at testing the various plant parts of *Boophone disticha* for antimicrobial activity, in the hopes that the leaves could be used instead of bulb material. MIC values less than 1 mg/ml were considered as good activity (ALIGIANNIS *et al.*, 2001).

DILIKA *et al.* (1996) examined the antimicrobial properties of plants used by the Xhosa to bandage circumcision wounds after the cultural ceremony which separates men from boys. The wound is bandaged with mashed leaves of *Helichrysum pedunculatum, Helichrysum appendiculatum* or *Helichrysum longifolium*. The wound is then dressed with the dry outer scales of the bulb of *B. disticha.* DILIKA *et al.* (1996) showed that *H. pedunculatum* and *H. longifolium* had positive antimicrobial activity against all four bacterial strains used in the disc-diffusion assay. DILIKA *et al.* (1996) did not test the antimicrobial activity of *B. disticha*. However, in our study all extracts of the outer scales of *B.disticha* showed poor activity against all the bacterial strains tested (Table 3.1 to 3.4). The best MIC value for the outer scales was 1.56 mg/ml. Therefore, it is apparently the positive antimicrobial activity of the *Helichrysum* plants used to treat circumcision wounds, and not the outer scales of *B. disticha,* which could explain the fact that most traditionally circumcised patients, remain free from infection.

KELMANSON *et al.* (2000) screened Zulu medicinal plants for antibacterial activity and found that *Dioscorea sylvatica* tuber bark extracts showed higher antibacterial activity than any other plant part. KELMANSON *et al.* (2000) was of the opinion that the tuber bark possesses the highest antifungal and antibacterial activity as it is the plant part that is in constant contact with soil, therefore, the above result was expected. FROHNE and PFÄNDER (1984) also reported that bulbs contain high concentrations of alkaloids and the epidermis of the outer scale leaves appears to be particularly rich in the alkaloids. This, however, was not found to be the case with *B. disticha* outer scales which exhibited poor antimicrobial activity.

With regards to the petroleum ether (PE) extracts, the season which showed the most activity was summer as three out of the four plant parts tested were active (Table 3.1). The inner scales, leaves and roots were all active against *B. subtilis* with a minimum inhibitory concentration (MIC) value of 0.78 mg/ml. This can be attributed to the fact that Gram-positive bacteria are generally more susceptible to antimicrobial substances than Gram-negative bacteria due to differences in the cell wall structure (RANG & DALE, 1987). In summer, the inner bulb scales were also active against *E. coli* with a MIC value of 0.78 mg/ml. Summer roots were active against both *E. coli* and *K. pneumoniae* with values of 0.78 mg/ml. Among the PE extracts, the roots of all the plant parts, had the lowest MIC values in winter, summer and autumn.

Table 3.1: Minimum inhibitory concentration (MIC) of petroleum ether (PE) extracts from various plant parts of *Boophone disticha* collected in the four seasons. Values in bold are considered to have noteworthy antimicrobial activity.

Season	Plant part	Antibacterial activity MIC (mg/ml)				Antifungal activity *Candida albicans*	
		Bacteria					
		Bs	***Sa***	***Ec***	***Kp***	**MIC (mg/ml)**	**MFC (mg/ml)**
Winter	Inner scales	6.25	8.33	3.13	6.25	6.25	6.25
	Outer scales	6.25	6.25	3.13	5.21	6.25	6.25
	Roots	**0.78**	3.13	1.56	1.56	3.13	3.13
Spring	Inner scales	3.13	6.25	3.13	2.60	1.56	12.5
	Outer scales	2.08	4.17	2.60	2.60	3.13	12.5
	Leaves	3.13	3.13	1.56	1.56	3.13	6.25
	Roots	1.56	3.13	3.13	1.56	1.56	5.21
Summer	Inner scales	**0.78**	3.13	**0.78**	1.56	3.13	4.17
	Outer scales	2.08	6.25	4.17	2.60	>12.5	>12.5
	Leaves	**0.78**	1.56	1.56	1.56	3.13	3.13
	Roots	**0.78**	1.56	**0.78**	**0.78**	3.13	4.17
Autumn	Inner scales	**0.78**	3.13	1.56	3.13	10.42	12.5
	Outer scales	3.13	6.25	2.08	3.13	>12.5	>12.5
	Leaves	1.56	12.5	3.13	6.25	3.13	12.5
	Roots	**0.78**	3.13	1.56	1.56	3.13	4.17
Neomycin (µg/ml)		1.56	1.56	0.39	0.78	-	-
Amphotericin B (µg/ml)		-	-	-	-	0.15	9.80

The dichloromethane (DCM) extracts from all seasons showed inhibitory activity against at least one of the test bacteria (Table 3.2). The DCM summer root extract showed the lowest MIC of 0.33 mg/ml against *K. pneumoniae.* When plants were collected in winter, only the root extract showed activity against *B. subtilis* and *K. pneumoniae* while the roots from autumn-collected plants exhibited antibacterial activity against three of the four bacterial strains. Plants collected in spring only had activity in the leaves and only inhibited *B. subtilis* growth. KOPTUR (1985)

believes the shift in the activity between plant organs and different seasons suggests a possible shift in and/or accumulation of some compounds responsible for the activity. This is possible as most of the phytochemicals found in the plant are produced in response to external stimuli such as temperature, moisture stress and light intensity (KUBO *et al.*, 1976; DERITA *et al*., 2009). Therefore, it is possible that according to the season, the content and presence of the bioactive compounds could vary with the absence or presence of the stimuli and thus result in changing antibacterial properties (NCUBE *et al.*, 2011). NCUBE *et al.* (2011) suggested that along with the morphological, physiological and biochemical differences between leaves and bulbs, the differences in activity between these parts of the same plant could also be explained by dynamics in the production of the active compounds in response to stimulation factors. This was observed in summer plants where various plant parts; inner scales, leaves and roots were active against at least one bacterium with MIC values of 0.78 mg/ml (Table 3.2).

ELGORASHI and VAN STADEN (2004) screened different plant parts of six Amaryllidaceae species: *Cyrtanthus falcatus, Cyrtanthus mackenii, Cyrtanthus suaveolens, Gethyllis ciliaris, Gethyllis multifolia* and *Gethyllis villosa*, for antibacterial activity. Seven out of twelve DCM extracts exhibited activity against at least one bacterial strain. Bulb extracts of *C. falcatus,* bulb/root extracts of *C. suaveolens* and bulb extracts of *G. ciliaris* were the three DCM extracts that showed activity against Gram-negative bacteria. Like *C. suaveolens*, only the root extracts of summer, winter and autumn *B. disticha* plants were active against *K. pneumoniae,* one of the Gram-negative bacterial strains tested. *C. falcatus* leaves and root extracts and *C. suaveolens* leaves and bulb/root extracts displayed activity against Gram-positive bacteria. *B. disticha*, like *C. falcatus* and *C. suaveolens* are similar in that their leaves showed activity against only Gram-positive bacteria, and in *B. disticha*'s case only against *B. subtilis* with a MIC value of 0.78 mg/ml.

Table 3.2: Minimum inhibitory concentration of dichloromethane (DCM) extracts from various plant parts of *B. disticha* as detected over four seasons.

Season	Plant part	Antibacterial activity MIC (mg/ml)				Antifungal activity *Candida albicans*	
		Bacteria					
		Bs	***Sa***	***Ec***	***Kp***	**MIC (mg/ml)**	**MFC (mg/ml)**
Winter	Inner scales	6.25	6.25	6.25	6.25	6.25	8.33
	Outer scales	6.25	12.5	6.25	6.25	6.25	8.33
	Roots	**0.78**	3.13	1.56	**0.78**	1.56	3.13
Spring	Inner scales	1.56	1.56	1.56	1.56	12.5	>12.5
	Outer scales	3.13	6.25	1.56	2.60	12.5	>12.5
	Leaves	**0.78**	3.13	1.56	1.56	3.13	3.13
	Roots	1.56	3.13	1.56	1.56	12.5	>12.5
Summer	Inner scales	**0.78**	3.13	**0.78**	1.56	1.56	3.13
	Outer scales	3.13	6.25	1.56	3.13	3.13	12.5
	Leaves	**0.78**	1.56	1.56	2.08	3.13	3.13
	Roots	**0.78**	3.13	1.56	**0.33**	1.56	3.13
Autumn	Inner scales	2.08	3.13	1.56	1.56	3.13	6.25
	Outer scales	1.56	3.13	1.30	1.56	2.60	12.5
	Leaves	3.13	3.13	1.56	1.56	3.13	3.13
	Roots	**0.78**	3.13	**0.78**	**0.78**	3.13	6.25
Neomycin (µg/ml)		1.56	1.56	0.39	0.78	-	-
Amphotericin B (µg/ml)		-	-	-	-	0.15	9.80

In ELGORASHI and VAN STADEN's (2004) study, other than bacteriostatic effects, none of the 90% methanol extracts exhibited antibacterial activity. RABE and VAN STADEN (1997) showed that methanol extracts of bulb material of *B. disticha* were not active in the antibacterial disc-dilution assay. However, in our study, spring leaves were the only 80% ethanol (EtOH) extract out of all seasons and plant parts tested to show any antibacterial activity (Table 3.3).

Table 3.3: Minimum inhibitory concentration of ethanol (EtOH) extracts from various plant parts of *B. disticha* collected in the four seasons.

Season	Plant part	Antibacterial activity MIC (mg/ml)				Antifungal activity *Candida albicans*	
		Bacteria					
		Bs	***Sa***	***Ec***	***Kp***	**MIC (mg/ml)**	**MFC (mg/ml)**
Winter	Inner scales	6.25	6.25	6.25	6.25	1.56	3.13
	Outer scales	3.13	4.17	5.21	6.25	6.25	6.25
	Roots	3.13	5.21	3.13	2.60	3.13	6.25
Spring	Inner scales	3.13	3.13	1.56	1.56	6.25	6.25
	Outer scales	3.13	3.13	1.56	1.56	6.25	6.25
	Leaves	**0.78**	3.13	1.56	1.56	3.13	6.25
	Roots	3.13	3.13	1.56	1.56	3.13	6.25
Summer	Inner scales	2.08	3.13	1.56	2.08	3.13	4.17
	Outer scales	3.13	5.21	3.13	2.60	3.13	6.25
	Leaves	3.13	4.17	4.17	1.56	3.13	6.25
	Roots	1.30	4.17	1.56	1.56	1.56	3.13
Autumn	Inner scales	2.08	12.5	6.25	4.17	3.13	5.21
	Outer scales	3.13	10.42	3.65	5.21	2.60	3.13
	Leaves	1.56	4.17	1.56	3.13	3.13	6.25
	Roots	1.56	6.25	3.13	3.13	3.13	3.13
Neomycin (µg/ml)		1.56	1.56	0.39	0.78	-	-
Amphotericin B (µg/ml)		-	-	-	-	0.15	9.80

Aqueous infusions or decoctions are common preparations in traditional medicine while access to less polar solvents is limited (FENNELL *et al.,* 2004). In cases where water is traditionally used as the solvent for preparation of decoctions and infusions, the use of organic solvents such as PE and DCM might be considered irrelevant. However, chemical investigations of plant extracts is usually accomplished using organic extracts leading to a full recognition of the chemical compounds present in the plant (ANDREO *et al.,* 2006). In terms of validation of the use of plants in traditional medicine, it is suggested that methanol-water or ethanol-water

extracts be used (ELGORASHI & VAN STADEN, 2004). No inhibitory activity against all the test bacteria was detected with the aqueous extracts. MIC values were greater than 6 mg/ml in all extracts (Table 3.4). This is in agreement with RABE and VAN STADEN (1997) who found no activity against any of the bacteria when water extracts of bulb material were screened in the disc-dilution assay.

Table 3.4: Minimum inhibitory concentration (MIC) of water extracts from various plant parts of *B. disticha* which were collected over the four seasons.

Season	Plant part	Antibacterial activity MIC (mg/ml)				Antifungal activity *Candida albicans*	
		Bacteria					
		Bs	***Sa***	***Ec***	***Kp***	**MIC (mg/ml)**	**MFC (mg/ml)**
Winter	Inner scales	>12.5	12.5	12.5	12.5	3.13	6.25
	Outer scales	>12.5	8.33	8.33	10.42	6.25	6.25
	Roots	>12.5	>12.5	>12.5	>12.5	6.25	12.5
Spring	Inner scales	>12.5	12.5	>12.5	6.25	12.5	>12.5
	Outer scales	12.5	12.5	12.5	10.42	6.25	6.25
	Leaves	>12.5	12.5	12.5	6.25	6.25	6.25
	Roots	>12.5	10.42	>12.5	6.25	12.5	>12.5
Summer	Inner scales	>12.5	>12.5	>12.5	6.25	>12.5	>12.5
	Outer scales	12.5	12.5	12.5	12.5	6.25	12.5
	Leaves	12.5	>12.5	>12.5	8.33	12.5	>12.5
	Roots	12.5	>12.5	>12.5	8.33	12.5	>12.5
Autumn	Inner scales	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5
	Outer scales	12.5	>12.5	10.42	12.5	12.5	12.5
	Leaves	12.5	>12.5	10.42	12.5	6.25	>12.5
	Roots	>12.5	>12.5	>12.5	>12.5	10.42	>12.5
Neomycin (µg/ml)		1.56	1.56	0.39	0.78	-	-
Amphotericin B (µg/ml)		-	-	-	-	0.15	9.80

Several plants are used by traditional healers to treat oral candidiasis which is rife in HIV-patients. Therefore *B. disticha* extracts were tested for antifungal activity against *Candida albicans* using the microdilution assay. Inhibition of *C. albicans* was observed after 24 h however inhibition decreased after 48 h with the lowest MIC value being 1.56 mg/ml. This was observed for the plant parts, inner scales and roots in the solvents PE, DCM and EtOH. After 48 h once the MIC values had been recorded, more broth was added to determine whether the activity was fungistatic (a state where fungal growth is inhibited but not dead) or fungicidal (the fungus is destroyed/killed). After a further 24 h of incubation fungal growth resumed in most of the wells. Extracts which initially had an MIC value of 1.56 mg/ml after additional broth and further incubation had MFC values greater than this value all showed fungistatic activity. Extracts which had an MIC value of 3.13 mg/ml and a MFC value of 3.13 mg/ml indicated fungicidal activity against *C. albicans*. Examples of fungicidal activity were PE winter roots, PE summer leaves, DCM spring, summer and autumn leaves and EtOH autumn roots. The relatively low potency values (MIC > 1 mg/ml) in the extracts evaluated in the antifungal assay could be due to the impure form and/or low concentration of the active compounds in the crude extracts (RABE & VAN STADEN, 1997). Overall, the results against *C. albicans* were poor. There were no real differences in activity between the different plant parts. The best PE extract was with spring material while summer and autumn were the best seasons for DCM and EtOH extracts (Table 3.5).

Medicinal plants are threatened by large scale collection for local use and recently for the export trade. Some species are able to recover quickly and adapt to continuous harvesting, while other species, in particular species that are harvested for their underground bulbs or roots, are more sensitive and are unable to recover from uncontrolled harvesting (CUNNINGHAM, 1991; SIEBERT, 2004). The leaves and roots of *Pelargonium sidoides* were screened for antibacterial activity in the hopes that shoots could substitute roots in medicinal formulations (LEWU *et al.*, 2006). From the study, LEWU *et al*. (2006) showed that both the shoots and roots had antimicrobial properties and that there was no significant difference between the MIC of the extracts. Therefore, shoots/leaves of *P. sidoides* may substitute for its roots in the treatment of bacterial infections.

Leaves of *B. disticha* that were collected in spring and summer showed the same level of antibacterial activity (MIC of 0.78 mg/ml) as the roots and inner scales, however, the leaves were not active against as many bacteria as the roots and inner scales were. This however was

not the case with the upper and lower leaves of *Crinum bulbispermum*¸ which showed no antibacterial activity (KELMANSON *et al.,* 2000). Therefore, the leaves of *B. disticha* could possibly be used in spring and summer to treat certain infections.

The least affected bacterium by all the PE, DCM and EtOH *B. disticha* extracts was *S. aureus*. This result was significantly different from the other 3 bacteria tested. Looking at the water extract, only *B. subtilis* was significantly different from *K. pneumoniae* (Table 3.5). As *S. aureus* and *B. subtilis* are Gram-positive bacteria, these results contradict the belief that Gram-positive bacteria are more susceptible to antimicrobial substances than Gram-negative bacteria.

Looking at the overall results for the different seasons and solvents, PE extracts with summer plant material were better than the other seasons. For DCM extracts, there was no significant difference between spring, summer and autumn. Spring and summer were the best seasons for EtOH extracts and they were significantly different from both autumn and winter. Lastly, there was no significant difference in the seasons when it came to water extracts (Table 3.5). This is expected as all the water extracts had poor results for the antimicrobial assays.

The results obtained from this screening confirm the therapeutic potency of *B. disticha* and therefore lends support to the use of it in traditional medicine. The leaves only had antibacterial activity in spring and summer and this could be due to the fact that the leaves are actively growing in these seasons. All plants are vulnerable to predation at this particular time because of new growth, however, *B. disticha* has the alkaloid, lycorine present and this has been shown to have antifeedant activity (EVIDENTE *et al*., 1986). Therefore it can be presumed that the likes of this alkaloid is sent to the leaves during spring and summer as a defence mechanism and this could account for the antibacterial activity.

Overall, the roots had the best antimicrobial activity as activity was found against both Gram-positive and Gram-negative bacteria. The activity of the roots in solvents PE, DCM and EtOH were significantly different from all the other plant parts in the same solvents (Table 3.5). The roots of *B. disticha* can be harvested and then the bulb can be placed back into the ground. The bulb will be able to carry on growing and produce new roots as long as the basal plate is intact and undamaged. This will allow *B. disticha* to be used in traditional medicine but in a more sustainable manner as harvesting the bulb is destructive.

From this current study, the seasonal and different plant part experiments were a success (Table 3.6) as the F- probability is <0.001, which is a highly significant result. The interactions were also significant (see Table 3.6). In conclusion, the factors: season, plant part, solvent and bacteria had an effect and caused *B. disticha* to respond differently to the antimicrobial assays.

Table 3.5: Statistical data showing the overall results for the different parameters tested. Results are mean MIC values for the antibacterial assay and mean MIC and MFC values for the antifungal assay.

		Solvents											
		Antibacterial activity				Antifungal activity *Candida albicans*							
Treatment		**PE**	**DCM**	**EtOH**	**Water**	**PE**	**DCM**	**EtOH**	**Water**	**PE**	**DCM**	**EtOH**	**Water**
		MIC (mg/ml)				**MIC (mg/ml)**				**MFC (mg/ml)**			
Season	Winter	5.3 ± 0.5 c	5.8 ± 0.6 b	5.6 ± 0.5 c	9.7 ± 0.6 a	5.6 ± 1.0 b	5.3 ± 1.1 b	4.8 ± 1.1 b	5.6 ± 1.0 a	7.5 ± 0.8 a	8.4 ± 0.9 ab	7.5 ± 0.9 b	9.4 ± 0.7 a
	Spring	2.5 ± 0.2 ab	1.9 ± 0.2 a	1.9 ± 0.1 a	9.0 ± 0.6 a	1.9 ± 0.3 a	8.1 ± 1.4 c	3.8 ± 0.6 ab	7.5 ± 1.2 a	9.2 ±0.8 ab	10.0 ± 0.9 b	6.9 ± 0.4 ab	9.4 ± 0.7 a
	Summer	1.7 ± 0.2 a	1.8 ± 0.2 a	2.4 ± 0.2 a	9.4 ± 0.6 a	4.4 ± 1.1 ab	1.9 ± 0.3 a	2.2 ± 0.3 a	8.7 ± 1.3 a	6.7 ± 1.0 a	6.3 ± 1.0 a	5.9 ± 0.6 ab	11.9 ± 0.3 b
	Autumn	2.9 ± 0.3 b	1.7 ± 0.1 a	3.8 ± 0.4 b	10.0 ± 0.6 a	5.8 ± 1.3 b	2.4 ± 0.3 a	2.2 ± 0.3 a	8.3 ± 1.3 a	10.3 ± 0.9 b	7.5 ± 0.8 ab	5.5 ± 0.6 a	11.9 ± 0.3 b
Plant parts	Inner scale	3.4 ± 0.3 b	2.9 ± 0.3 b	4.2 ± 0.4 b	11.7 ± 0.3 a	5.4 ± 1.1 a	5.9 ± 1.2 a	3.5 ± 0.5 ab	10.1 ± 1.2 b	8.8 ± 1.1 b	7.5 ± 1.1 ab	4.7 ± 0.5 a	10.9 ± 0.8 a
	Outer scales	3.9 ± 0.2 b	4.1 ± 0.4 c	4.0 ± 0.3 b	11.5 ± 0.3 a	8.6 ± 1.2 b	6.1 ± 1.2 a	4.2 ± 0.6 ab	7.8 ± 0.8 b	10.9 ± 0.8 b	11.4 ± 0.7 c	5.5 ± 0.4 a	9.3 ± 0.9 a
	Leaves	5.5 ± 0.7 c	4.5 ± 0.7 c	5.0 ± 0.6 b	11.7 ± 0.3 a	5.5 ± 1.2 a	5.5 ± 1.2 a	5.5 ± 1.2 b	9.3 ± 0.9 b	8.5 ± 1.2 b	5.4 ± 1.2 a	7.8 ± 0.8 b	10.9 ± 0.8 a
	Roots	1.7 ± 0.1 a	1.5 ± 0.1 a	2.9 ± 0.2 a	11.7 ± 0.3 a	2.7 ± 0.2 a	4.7 ± 1.3 a	2.7 ± 0.2 a	10.4 ± 0.9 b	4.1 ± 0.4 a	6.2 ± 1.1 a	4.7 ± 0.4 a	12.5 ± 0.0 b
Bacteria	*Bs*	2.6 ± 0.4 a	2.6 ± 0.4 a	2.9 ± 0.3 a	10.3 ± 0.5 b	-	-	-	-	-	-	-	-
	Sa	4.5 ± 0.4 b	4.0 ± 0.4 b	4.8 ± 0.4 b	10.0 ± 0.5 ab	-	-	-	-	-	-	-	-
	Ec	2.4 ± 0.3 a	2.2 ± 0.4 a	2.9 ± 0.4 a	9.7 ± 0.6 ab	-	-	-	-	-	-	-	-
	Kp	2.9 ± 0.4 a	2.4 ± 0.4 a	3.0 ± 0.4 a	8.3 ± 0.6 a	-	-	-	-	-	-	-	-
Control (µg/ml)	Neomycin	1.072	1.072	1.072	1.072	-	-	-	-	-	-	-	-
	Amphotericin B	-	-	-	-	0.150	0.150	0.150	0.150	9.8	9.8	9.8	9.8

Mean values (± SE) in a column of each treatment with different letters are significantly different according to Duncan's multiple range test ($P < 0.005$).

Table 3.6: Analysis of variance showing the seasonal/plant part experiments for *B. disticha* were successful.

Source of variation	Degrees of freedom	Sum of Squares	Mean Squares	Variance	F-probability
Variate- Antibacterial activity (MIC)					
Season (S)	3	1204.8154	401.6051	633.40	<.001
Part (P)	4	3724.8680	931.2170	1468.68	<.001
Solvent (SV)	3	7486.0469	2495.3490	3935.57	<.001
Bacteria (B)	3	426.4144	142.1381	224.18	<.001
S X P	12	1486.5116	123.8760	195.37	<.001
S X SV	9	446.6246	49.6250	78.27	<.001
P X SV	12	2105.8392	175.4866	276.77	<.001
S X B	9	139.0762	15.4529	24.37	<.001
P X B	12	92.7416	7.7285	12.19	<.001
SV X B	9	162.7136	18.0793	28.51	<.001
S X P X SV	36	719.1820	19.9773	31.51	<.001
S X P X B	36	87.6863	2.4357	3.84	<.001
S X SV X B	27	210.6753	7.8028	12.31	<.001
P X SV X B	36	156.8778	4.3577	6.87	<.001
S X P X SV X B	108	357.9283	3.3142	5.23	<.001
Residual	640	405.7919	0.6340		
Total	959	19213.7929			
Variate- Antifungal activity (MIC)					
S	3	44.9593	14.9864	44.64	<.001
P	4	1439.9299	359.9825	1072.25	<.001
SV	3	621.7270	207.2423	617.30	<.001
S X P	12	789.5522	65.7960	195.98	<.001
S X SV	9	635.4967	70.6107	210.32	<.001
P X SV	12	407.0520	33.9210	101.04	<.001
S X P X SV	36	680.5983	18.9055	56.31	<.001
Residual	160	53.7162	0.3357		
Total	239	4673.0317			
Variate- Antifungal activity (MFC)					
S	3	55.5013	18.5004	32.48	<.001
P	4	250.4406	62.6101	109.91	<.001
SV	3	543.7826	181.2609	318.19	<.001
S X P	12	718.8314	59.9026	105.15	<.001
S X SV	9	305.5013	33.9446	59.59	<.001
P X SV	12	679.7689	56.6474	99.44	<.001
S X P X SV	36	378.4993	10.5139	18.46	<.001
Residual	160	91.1458	0.5697		
Total	239	3023.4712			

'X' denotes interaction of parameters

3.2 ANTIMICROBIAL ACTIVITY OF *IN VITRO* BULBLETS

3.2.1 Introduction

The Amaryllidaceae are important sources of pharmaceuticals, however, the accumulation of compounds is highly susceptible to geographical and environmental conditions. *In vitro* cultures can be used to produce secondary metabolites in large quantities and these can provide a stable supply for clinical and chemical studies (FENNELL, 2002). BAJAJ *et al*. (1988) believes that plant tissue culture can be used to discover new biochemicals, as products may accumulate in the plant cells as well as in the culture media.

One of the advantages of tissue culture is that the culture conditions can be controlled, therefore, the optimal conditions for compound production could be determined. MISAWA and NAKANISHI (1988) suggested that cultures which showed increased production could be selected and improved by inducing mutation or differentiation either chemically or by genetic engineering. According to BAJAJ *et al*. (1988) tissue culture provides a unique system for studying secondary metabolite production as well as the relationship between this production and organogenesis.

The Amaryllidaceae are amongst the first plants to be studied *in vitro* to determine whether the alkaloids produced in the plant could also be produced in culture. *Hippeastrum* has been cultured *in vitro* for the production of Amaryllidaceae alkaloids while *Narcissus pseudonarcissus* has been cultured for haemanthine (BERGOÑÓN *et al*., 1992). However, KRIKORIAN and KANN (1986) reported that the production and maintenance of callus containing secondary metabolites from monocotyledons is difficult.

The aim of this experiment was to determine whether *in vitro* grown *B. disticha* bulblets were active in the antimicrobial assays, thereby indicating that the cultured plants maintained their medicinal properties.

3.2.2 Materials and methods

Preparation of in vitro bulblet extract

In vitro grown bulblets from the micropropagation experiments were collect and dried. The dried material (5.533g) was ground and extracted with 60 ml of 100% ethanol. The extract was sonicated for 1 h and the temperature was kept low by adding ice to the water bath.

Antibacterial and antifungal assay

The antimicrobial assays were carried out as described in Section 3.1.2 but with slight modifications. Test samples were re-dissolved in methanol at a concentration of 1 mg/ml and 100 µl of each solution was two-fold serially diluted with sterile distilled water in a 96-well micro-plate, in triplicate for each bacterial strain and *Candida albicans*. MIC values were recorded as the lowest concentration of sample to affect clear wells. Data collected was subjected to one-way analysis of variance (ANOVA). Where there was significant difference (p=0.05), the means were separated using Duncan Multiple Range Test (DMRT). Data analysis was done using GenStat (version 14, Rothamsted Research, Harpenden, UK).

3.2.3 Results and discussion

Table 3.7: Antimicrobial minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of *in vitro* grown bulblets of *B. disticha*.

Sample	Antibacterial activity MIC (mg/ml)				Antifungal activity *Candida albicans*	
	Bacteria				MIC (mg/ml)	MFC (mg/ml)
	Bs	***Sa***	***Ec***	***Kp***		
Crude ethanol extract	0.250b	0.125a	0.125a	0.125a	0.125a	>0.250b
In vitro bulblet extract	>0.250b	0.167a	0.125a	0.125a	0.125a	0.250b
Neomycin (µg/ml)	$1.6x10^{-3}$	$0.8x10^{-3}$	$0.8x10^{-3}$	$1.6x10^{-3}$	-	-
Amphotericin B (µg/ml)	-	-	-	-	$9.77x10^{-3}$	$7.81x10^{-2}$

In vitro grown bulblets showed good antimicrobial activity. The best activity was against the Gram-positive bacteria, *E. coli* and *K. pneumoniae,* which had MIC values of 0.125 mg/ml. The activity against *S. aureus* was also good and had an MIC value of 0.167 mg/ml. Activity against all three of these bacteria were similar and only significantly different from *B. subtilis* which had an MIC > 0.25 mg/ml. The *in vitro* bulblet extract showed fungicidal activity against *C. albicans*, with an MFC value of 0.250 mg/ml.

Both the crude ethanol extract and *in vitro* bulblet extract may be so active compared to the seasonal/plant part results, because both of these extracts contained all the plant parts (leaves, roots and scales). There could be a synergistic effect occurring between the various plant parts which as a whole, make the crude and *in vitro* extract more active than the separate plant parts.

From these positive antimicrobial results, if a successful micropropagation protocol can be established for *B. disticha*, the tissue culture technique can be used to possibly provide phytochemists and the indigenous people with high quality material and help reduce large-scale collection from the wild.

3.3 SUMMARY

- *Boophone disticha* showed broad-spectrum antibacterial activity against all four tested bacteria
- The petroleum ether and dichloromethane extracts showed the best inhibition against the bacteria
- Strong inhibition of the Gram-positive bacterium, *Bacillus subtilis,* was detected
- Weak inhibitory activity against *Candida albicans* was observed
- Activity of plant parts varied according to the season of collection
- In spring and summer, the leaves can be used to treat microbial infections as opposed to bulb material
- *In vitro* grown bulblets have maintained their Amaryllidaceae alkaloids and have good antimicrobial activity

Chapter Four

MUTAGENIC EFFECTS

4.1 TOXICITY TESTING

4.1.1 Introduction

The investigation of traditionally used medicinal plants is important for two reasons: firstly, as a source of potential chemotherapeutic drugs and secondly, as a measure of safety for the continued use of medicinal plants (VERSCHAEVE *et al.*, 2004). Plants which are frequently used in traditional medicine are assumed to be safe, due to their long-term use and are considered to be harmless and have no side effects because they are 'natural' (POPAT *et al.*, 2001; ELGORASHI *et al.*, 2002). This belief is largely circumstantial and it is important to determine the toxicology of plant extracts, especially those that are used for long periods of time (REID *et al.*, 2006).

The use of some traditional medicines has resulted in several cases of acute poisoning, leading to increased morbidity and mortality. Due to insufficient data, estimates of the mortality rate vary widely from 10 000 to 20 000 per annum (POPAT *et al.*, 2001). POPAT *et al.* (2001) referred to three case studies in South Africa and reported that poisoning with traditional medicines resulted in the highest mortality, accounting for 51.7% of all deaths that were due to acute poisoning. However, many cases of poisoning remain unreported and mortality from traditional medicines may thus be higher than currently known (FENNELL *et al*., 2004). Therefore, the determination of toxicity of South African medicinal plants is particularly necessary, given their widespread use.

The identification of substances which are capable of inducing mutations has become an important procedure in safety assessment. Mutagenicity can be defined as the property of being able to induce genetic mutations. A mutation is defined as any permanent change in deoxyribonucleic acid (DNA): that is, a change in the nucleotide sequence or re-arrangement of DNA in the genome (VOGEL & MOTULSKY, 1986). In broad terms, mutations can be

classified into three categories; genome mutations, chromosome mutations and gene mutations. All three types of mutations occur at considerable frequencies and underlie not only all genetic or heritable diseases but also many instances of cancer. Mutations can occur in any cell, germline cells and somatic cells. Only germline mutations can be carried from one generation to the next and are responsible for inherited diseases. Somatic mutations in a number of genes can give rise to a significant proportion of cancers as a result of any of the three mutations. Therefore, cancer is fundamentally a 'genetic' disease and mutations are central to its etiology or progression (VOGEL & MOTULSKY, 1986).

Mutagens are physical, biological or chemical agents that change the DNA of an organism and thus increase the frequency of mutations. As many mutations cause cancer, mutagens are also carcinogens. Thus, mutagenic chemicals which are capable of causing cancer have driven most of the mutagenicity testing programs (MORTELMANS & ZEIGER, 2000). Many plants are known to contain mutagenic compounds such as flavonoids, quercetin, furoquinoline alkaloids and tannins (ELGORASHI *et al.*, 2003b; VERSCHAEVE *et al.*, 2004). Therefore, the search for inhibitors of mutagenesis may be useful as a tool to discover anticarcinogenic agents (VERSCHAEVE & VAN STADEN, 2008).

Of Africa's many bulbous plants, *Boophone disticha* is possibly the most widely known for its poisonous and medicinal properties (MANNING *et al.*, 2002), refer to Chapter 1. Although *B. disticha* is one of the most important medicinal bulbs, its extremely high toxicity has led to several human deaths. Poisoning with *B. disticha* usually occurs due to the underestimation of the toxicity of the bulb or the administration of too large a dose of the decoction. Further more, decoctions of the bulb have been used to commit suicide and murder (NEUWINGER, 1994). NEUWINGER (1994) reported that in the rural districts of South Africa at least 30 deaths in one year were a result of *B. disticha* poisoning; however the true fatality rate for *B. disticha* poisoning is likely to be much higher. Such poisonings are based mainly on its medicinal use as a remedy against gastrointestinal problems, abdominal pains or general weakness.

Mutagenicity testing

Mutagenicity assays are important tests for determining mutagens in substances, as well as extracts from medicinal plants. There are a number of tests that can be used for this purpose and the selection of a test depends on the specific characteristics of the substances to be examined. There are four methods for assessing mutagenicity in plants and these include; tests for gene mutations in bacteria, tests for chromosomal aberrations in mammalian cells *in vitro*, tests for gene mutations in eukaryotic systems and *in vivo* tests for genetic damage (DIRECTIVE 75/318/EEC, 1987).

Testing for gene mutations in bacteria is the most common and widely used method for evaluating mutagenic properties. The test uses well established bacterial strains which have been designed to detect various types of genetic change such as frame-shift and base change mutations and the test is carried out with or without extrinsic metabolic activation (MORTELMANS & ZEIGER, 2000). It has been found that chemicals which are able to mutate bacteria may also be carcinogenic to animals (DÉCIGA-CAMPOS *et al.*, 2007). The test for chromosomal aberrations in mammalian cells uses human lymphocytes along with several mammalian cell lines and the damage is scored by microscopic examination of chromosomes at metaphase during mitosis (www.genpharmtox.com). The test for gene mutations in eukaryotic systems is helpful in that positive results found in bacteria can be additionally studied in a system which has the complex eukaryotic chromosomal structure. This complex system also detects mutations which arise through mechanisms that cannot occur in the relatively simple bacterial genome (KNAAP *et al.*, 1988). Lastly, the main role of the *in vivo* test is to determine if a mutagenic compound has been missed by the *in vitro* tests due to inappropriate metabolic activation systems being used (JACOBSON-KRAM, 2008).

Ames test

The Ames *Salmonella/*microsome mutagenicity assay, also known as the Ames test or *Salmonella* test, is a short-term bacterial reverse mutation assay which is designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations (MORTELMANS & ZEIGER, 2000). The test uses several *Salmonella* strains which have pre-existing mutations that leave the bacteria unable to synthesize the required

amino acid, histidine, and are, therefore, unable to grow and form colonies in the absence of histidine. New mutations at the site of, or nearby, the pre-existing mutations in the genes can restore gene function and allow the cells to synthesize histidine (MORTELMANS & ZEIGER, 2000). These newly mutated cells can grow in the absence of histidine and form colonies. The *Salmonella* strains have different mutations in various genes in the histidine operon, and each of these mutations is responsive to mutagens that act via different mechanisms (MORTELMANS & ZEIGER, 2000).

The *Salmonella* test was specifically designed to detect chemically induced mutagenesis (AMES *et al.,* 1975). The test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs as there is a high predictive value for rodent carcinogenicity (MORTELMANS & ZEIGER, 2000). In some instances, chemicals are not mutagenic by themselves, but become mutagens as they are metabolized in the liver. The *Salmonella* test includes an evaluation in the presence of a mammalian mixture of liver enzymes, also known as the S9 microsomal fraction, to mimic the *in vivo* activation process (AMES *et al.*, 1973). Different types of the *Salmonella* strains are used to identify different types of mutations. The strains TA 100 and TA 98 are most often used as they detect the great majority of mutagens. Strain TA 98 gives an indication of frame-shift mutations, while strain TA 100 indicates base-pair substitution, and strain TA 102 indicates transitions/transversions (VERSCHAEVE *et al.*, 2004). These mutants, therefore, could be used to identify and characterize mutagenic chemicals by their ability to revert to wild-type (histidine-independence) in the presence of mutagens (MORTELMANS & ZEIGER, 2000). A positive response in any single bacterial strain, either with or without metabolic activation is enough to designate a substance as a mutagen (ZEIGER, 2001). Therefore, the Ames test was used in this study to evaluate the toxicity of extracts of *B. disticha* as it is reliable, quick and easy to perform.

4.1.2 Materials and methods

Plant material

Only plants collected in summer were used in this experiment as they had previously shown the best antimicrobial activity (Chapter 3). The plants were divided into the various plant parts; inner scales, outer scales, roots and leaves and were oven dried at 50 °C, ground and

stored in airtight containers in the dark. The preparation of plant extracts is as described in Section 3.1.2. A voucher specimen (Cheesman 01 NU) is retained at the UKZN Herbarium.

Ames test

Mutagenicity was tested using the *Salmonella*/microsome assay with and without metabolic activation, based on the plate-incorporation method (MARON & AMES, 1983). Aqueous extracts were redissolved in water and organic extracts were dissolved in 10% DMSO. For each sample, three different concentrations were used to test for mutagencity: 50 µg/ml, 500 µg/ml and 5000 µg/ml. One hundred microlitres of *Salmonella typhimurium* bacterial stock cultures (TA98 or TA100) were inoculated in 20 ml of Oxoid Nutrient Broth No. 2 and incubated for 16 h at 37 °C in a water bath on an orbital shaker. The bacterial cultures (100 µl) were added to 100 µl of plant extract and 500 µl of phosphate buffer (without metabolic activation) or 500 µl metabolic activation mixture containing S9 rat liver enzyme. The top agar (2 ml) containing histidine-biotin (0.5 mM) was added to this mixture, which was poured over the surface of a minimal agar plate and incubated (inverted in the dark) at 37 °C for 48 h. Positive controls were 2-aminoanthracene (2AA) with S9 and 4-Nitroquinoline-N-oxide (4NQO) without S9. Water and 10% DMSO were the negative controls.

After incubation, his^+ revertant colonies were counted and compared to the number of revertant colonies formed in the negative control. An extract was considered mutagenic when the mean number of colonies was equal to or more than twice that of the negative control, and when the number of colonies showed dose dependent increases. Each concentration per sample was tested three times in two different experiments.

The mutagenic index (MI) was calculated for each concentration per sample. The average number of revertants per plate for each sample was divided by the average number of revertants per plate in the negative control. A sample was considered positive when $MI \geq 2$ for at least one of the tested doses and where there was a reproducible dose-response curve (VARELLA *et al*., 2004).

4.1.3 Results and discussion

Information about the safety and effective use of medicinal plants is difficult to find due to the lack of thorough clinical studies and inadequate toxicological data (ELGORASHI & VAN STADEN, 2004). Therefore, the assessment of bacterial mutagenicity is very important as an initial test for complex mixtures because there is a possibility that one or more components can be positive (REID *et al.*, 2006). The Ames test is a recommended assay for initial screening of medicinal plants since studies have shown that the proportion of carcinogens identified as mutagens ranges from 50 to 90% (ZEIGER, 2001). This study will hopefully add some important mutagenicity information about *B. disticha* to the scientific literature.

Table 4.1 and Table 4.2 give the mean number of revertants per plate in *Salmonella typhimurium* strain TA 98 and TA 100, with and without metabolic activation. None of the extracts tested were found to induce mutations, since the extracts did not give double the number of revertant colonies in relation to the negative control. The extracts also did not modify the effect of the mutagenic compounds (2AA with S9 and 4NQO without S9). The number of revertant colonies shown by the positive and negative controls was within the normal range found in the laboratory and in accordance with the literature (MORTELMANS & ZEIGER, 2000).

All extracts of *B. disticha* were neither toxic nor mutagenic in the range of concentrations tested (Table 4.1 and Table 4.2). The absence of toxicity was examined by observing the background bacterial growth, which was present. Increase in the concentration of extracts did not influence the viability and mutation frequencies of the indicator bacterium. There was no significant difference in the values obtained between different extracts, solvents or concentrations and the negative control where p was < 0.05 (Table 4.1 and Table 4.2).
The mutagenic index (MI) shown in Table 4.1 and Table 4.2 indicates that there was no significant increase in revertants due to treatment with the various plant part extracts. The MI values were all below 2 and the dose response increase was not significant, showing an absence of any mutagenic effect by the *B. disticha* extracts.

Only summer plants were tested in this study as the summer plants previously showed the most activity in the antifungal and antibacterial assays (Section 3.1.3). POPAT *et al.* (2001)

suggested that consideration of factors that affect the toxicity of the plant itself be given. The level of pharmacologically active constituents found in plants is influenced by environmental factors such as climate and soil and it has been shown that the toxicity of some plants vary with season (HUTCHINGS & TERBLANCHE, 1989). Therefore, one should possibly test the plant extracts across the four seasons in order to be sure about the mutagenicity and toxic properties of the plant.

In another study on Amaryllidaceae species, ELGORASHI and VAN STADEN (2004) showed that dichloromethane extracts of leaves and roots of *Cyrtanthus falcatus* and bulbs/roots and leaves of *Cyrtanthus suaveolens* induced mutation in strain TA 98. When determining the genotoxic effects of various medicinal plants used in South African traditional medicine, ELGORASHI *et al.* (2003b) discovered that the dichloromethane extracts from the bulbs of *Crinum macowanii* was mutagenic for strain TA 98, with and without metabolic activation, indicating that *C. macowanii* causes frame-shift mutations. The extract however gave a negative response to strain TA 100, with and without metabolic activation. *Brunsvigia grandiflora* tested negative in the Ames test and so was devoid of any mutagenic properties (REID *et al.*, 2006). The bulb extracts from *B. disticha* were found to have negative responses to both *S. typhimurium* strains (ELGORASHI *et al.,* 2003b), which is in keeping with the results obtained in the current investigation. The detection of mutagenic effects for these extracts demonstrated that plants used in traditional medicine should be administered with caution and only after careful toxicological testing.

The absence of a mutagenic response by extracts from *B. disticha* against *S. typhimurium* strains TA 98 and TA 100 is a positive step towards establishing the safe use of this plant for medicinal purposes. However, if a substance/extract screened does not indicate a mutagenic response, this does not necessarily confirm that it is neither mutagenic nor carcinogenic. It just confirms that it is not mutagenic to that particular bacterial strain used and for the genetic endpoint tested (REID *et al.*, 2006), and further testing may be necessary.

The fact that plant extracts are complex mixtures of organic compounds makes it difficult to speculate on the compounds responsible for the mutagenic response detected previously with these Amaryllidaceae extracts (ELGORASHI *et al.*, 2003b). Therefore, the isolation of alkaloids from *B. disticha* and their mutagenic effects will be dealt with in Chapter 5.

4.2 SUMMARY

- *B. disticha* collected in summer did not test positive for toxicity or mutagenicity when using the *in vitro Salmonella*/microsome assay, with and without metabolic activation
- A more comprehensive toxicological profile needs to be established for extracts of *B. disticha* before it can be considered completely safe for use in traditional medicine

Table 4.1: Mean number (± S.D.) of histidine+ revertants/plate and mutagenic index (MI) in *Salmonella typhimurium* strain TA 98 with extracts from different plant parts of *B. disticha,* without or with metabolic activation (S9).

Plant part analysed	Extract	Number of TA 98 revertants											
		5000 µg/ml				500 µg/ml				50 µg/ml			
		Without S9	MI	With S9	MI	Without S9	MI	With S9	MI	Without S9	MI	With S9	MI
Roots	PE	7.33 ± 1.53	0.50	18.50 ± 2.12	0.75	11.67 ± 1.53	0.80	14.00 ± 2.83	0.57	13.00 ± 3.00	0.89	17.00 ± 1.41	0.69
	DCM	10.00 ± 1.00	0.69	26.00 ± 1.41	1.05	10.67 ± 1.53	0.73	18.50 ± 0.71	0.75	12.00 ± 2.65	0.82	22.00 ± 1.41	0.89
	EtOH	13.00 ± 2.65	0.89	17.50 ± 2.12	0.71	13.67 ± 5.86	0.94	23.50 ± 2.12	0.95	14.33 ± 2.08	0.98	17.50 ± 2.12	0.71
	Water	16.67 ± 2.52	0.94	16.50 ± 2.12	0.77	11.33 ± 3.06	0.64	17.50 ± 3.54	0.81	13.33 ± 1.53	0.75	16.50 ± 3.54	0.77
Leaves	PE	10.67 ± 3.51	0.73	19.50 ± 2.12	0.79	15.00 ± 2.65	1.03	13.00 ± 2.83	0.53	12.33 ± 1.15	0.85	15.50 ± 3.54	0.63
	DCM	12.00 ± 1.73	0.82	25.50 ± 0.71	1.03	11.67 ± 4.16	0.80	18.50 ± 0.71	0.75	11.00 ± 1.00	0.76	20.00 ± 0.00	0.81
	EtOH	15.33 ± 2.52	1.05	14.50 ± 2.12	0.59	13.33 ± 4.73	0.92	18.00 ± 1.41	0.73	13.67 ± 2.31	0.94	17.00 ± 1.41	0.69
	Water	11.67 ± 1.15	0.66	20.50 ± 2.12	0.95	27.33 ± 19.09	1.55	13.50 ± 0.71	0.63	13.00 ± 1.73	0.74	17.00 ± 2.83	0.79
Inner Scales	PE	11.33 ± 0.58	0.78	12.50 ± 0.71	0.51	13.00 ± 2.00	0.89	14.50 ± 0.71	0.59	12.00 ± 2.00	0.82	16.00 ± 4.24	0.65
	DCM	16.67 ± 1.15	1.14	21.50 ± 3.54	0.87	11.67 ± 1.53	0.80	21.50 ± 2.12	0.87	13.67 ± 1.53	0.94	18.00 ± 2.83	0.73
	EtOH	12.00 ± 2.00	0.82	30.00 ± 7.07	1.21	11.67 ± 2.31	0.80	17.50 ± 0.71	0.71	10.33 ± 1.53	0.71	24.00 ± 8.49	0.97

Plant part analysed	Extract	Number of TA 98 revertants											
		5000 µg/ml				500 µg/ml				50 µg/ml			
		Without S9	MI	With S9	MI	Without S9	MI	With S9	MI	Without S9	MI	With S9	MI
	Water	17.00 ± 3.61	0.96	17.50 ± 0.71	0.81	17.67 ± 1.53	1.00	11.00 ± 2.83	0.51	20.00 ± 1.73	1.13	18.00 ± 0.00	0.84
Outer Scales	PE	13.33 ± 4.16	0.92	17.00 ± 2.83	0.69	15.33 ± 1.53	1.05	15.00 ± 1.41	0.61	15.00 ± 3.46	1.03	21.50 ± 0.71	0.87
	DCM	11.67 ± 3.06	0.80	23.50 ± 0.71	0.95	13.33 ± 3.21	0.92	21.00 ± 1.41	0.85	14.33 ± 1.15	0.98	23.50 ± 0.71	0.95
	EtOH	14.33 ± 1.53	0.98	23.00 ± 0.00	0.93	14.33 ± 1.53	0.98	22.50 ± 6.36	0.91	13.67 ± 1.53	0.94	16.50 ± 0.71	0.67
	Water	11.33 ± 1.15	0.64	17.50 ± 0.71	0.81	14.67 ± 2.52	0.83	17.50 ± 0.71	0.81	16.67 ± 7.09	0.94	18.00 ± 1.41	0.84
4NQO		223.83 ± 13.76	15.37	-	-								
2AA		-	-	229.25 ± 4.92	9.26								
Water		17.67 ± 4.93	1.00	21.50 ± 3.40	1.00								
10% DMSO		14.56 ± 3.50	1.00	24.75 ± 1.71	1.00								

Number of histidine+ revertants/plate: mean values of three triplicates, repeated twice.

Table 4.2: Mean number (± S.D.) of histidine+ revertants/plate and mutagenic index in *Salmonella typhimurium* strain TA 100 with extracts from different plant parts of *B. disticha,* without or with metabolic activation (S9).

Plant part analysed	Extract	Number of TA 100 revertants											
		5000 µg/ml				500 µg/ml				50 µg/ml			
		Without S9	MI	With S9	MI	Without S9	MI	With S9	MI	Without S9	MI	With S9	MI
Roots	PE	169.00 ± 1.00	0.94	163.00 ± 8.49	1.11	172.67 ± 0.58	0.96	141.50 ± 3.54	0.96	172.67 ± 1.15	0.96	156.00 ± 0.00	1.06
	DCM	172.33 ± 1.53	0.95	138.00 ± 0.00	0.94	171.67 ± 1.53	0.95	152.00 ± 1.41	1.04	154.67 ± 1.53	0.86	146.50 ± 0.71	1.00
	EtOH	149.67 ± 6.35	0.83	138.00 ± 7.07	0.94	161.67 ± 1.53	0.89	125.50 ± 6.36	0.86	164.33 ± 1.53	0.91	138.00 ± 12.73	0.94
	Water	125.00 ± 2.65	0.87	109.00 ± 1.41	0.90	120.33 ± 1.15	0.83	104.00 ± 1.41	0.86	150.00 ± 12.77	1.04	118.50 ± 9.19	0.98
Leaves	PE	175.67 ± 2.08	0.97	137.50 ± 9.19	0.94	179.33 ± 2.08	0.99	139.50 ± 4.95	0.95	137.33 ± 1.53	0.76	139.00 ± 0.00	0.95
	DCM	156.67 ± 0.58	0.87	145.00 ± 5.66	0.99	175.00 ± 1.73	0.97	149.00 ± 1.41	1.02	150.67 ± 0.58	0.83	124.00 ± 1.41	0.85
	EtOH	163.00 ± 2.65	0.90	104.50 ± 0.71	0.71	151.67 ± 0.58	0.84	129.50 ± 10.61	0.88	162.33 ± 2.08	0.90	140.00 ± 1.41	0.95
	Water	129.67 ± 3.79	0.90	114.00 ± 2.83	0.94	123.67 ± 1.53	0.86	110.00 ± 2.83	0.91	145.33 ± 1.15	1.01	104.00 ± 2.83	0.86
Inner Scales	PE	167.67 ± 19.09	0.93	154.00 ± 8.49	1.05	175.33 ± 1.15	0.97	132.50 ± 0.71	0.90	172.00 ± 9.17	0.95	119.00 ± 7.07	0.81
	DCM	166.67 ± 1.53	0.92	128.00 ± 1.41	0.87	178.67 ± 1.53	0.99	135.50 ± 2.12	0.92	169.00 ± 1.00	0.94	140.00 ± 7.07	0.95
	EtOH	170.33 ± 3.51	0.94	133.00 ± 5.66	0.91	143.33 ± 1.15	0.79	127.00 ± 1.41	0.87	165.67 ± 2.89	0.92	132.50 ± 0.71	0.90

Plant part analysed	Extract	Number of TA 100 revertants											
		5000 µg/ml				500 µg/ml				50 µg/ml			
		Without S9	MI	With S9	MI	Without S9	MI	With S9	MI	Without S9	MI	With S9	MI
	Water	142.67 ± 3.06	0.98	111.00 ± 1.41	0.92	128.00 ± 1.00	0.89	121.00 ± 4.24	1.00	140.00 ± 1.73	0.97	121.00 ± 5.66	1.00
Outer Scales	PE	173.33 ± 4.04	0.96	143.00 ± 9.90	0.97	137.00 ± 3.00	0.76	138.50 ± 7.78	0.94	165.67 ± 1.53	0.92	133.00 ± 8.49	0.91
	DCM	148.67 ± 1.15	0.82	143.50 ± 14.85	0.98	188.33 ± 1.53	1.04	138.00 ± 12.73	0.94	158.00 ± 5.00	0.87	137.50 ± 3.54	0.94
	EtOH	158.67 ± 3.06	0.88	160.00 ± 1.41	1.09	165.67 ± 4.51	0.92	138.50 ± 0.71	0.94	160.67 ± 1.53	0.89	136.50 ± 2.12	0.93
	Water	136.67 ± 4.16	0.95	117.00 ± 4.24	0.97	145.67 ± 1.15	1.01	112.00 ± 5.66	0.93	123.33 ± 1.53	0.85	122.50 ± 6.36	1.01
4NQO		1219.08 ± 97.07	6.75	-	-								
2AA		-	-	1194.67 ± 32.33	8.15								
Water		144.33 ± 4.89	1.00	121.00 ± 3.61	1.00								
10% DMSO		180.67 ± 9.77	1.00	146.67 ± 8.33	1.00								

Number of histidine+ revertants/plate: mean values of three triplicates, repeated twice.

Chapter Five

AMARYLLIDACEAE ALKALOIDS FROM *BOOPHONE DISTICHA*

5.1 ISOLATION AND IDENTIFICATION OF ALKALOIDS

5.1.1 Introduction

The Amaryllidaceae is a uniform group of monocotyledonous species (KOOPOWITZ, 1986). Plants of this family are richly represented in the southern African region which houses at least 250 of the approximately 850 species known worldwide (MEEROW & SNIJMAN, 1998). The family includes many desirable ornamentals and many species are heavily exploited in traditional medicine, most likely due to their alkaloidal constituents (GHOSAL *et al.*, 1985). The alkaloids are classically grouped as the Amaryllidaceae alkaloids because of their limited taxonomic distribution (Refer to Section 1.4). Many secondary metabolites such as the Amaryllidaceae alkaloids have biological activities that can be assayed in the laboratory, such as antifungal, antibacterial and anticancer activities, providing a basis for the use of a particular plant. From some plants, active compounds are isolated and then commercialized in the development of a drug. It is estimated that about a quarter of modern drugs are derived from higher plants (KINGHORN & BALANDRIN, 1993). Thus, the presence of the Amaryllidaceae alkaloids in the family has been the focus of many pharmacological investigations (GHOSAL *et al.*, 1985).

The study of Amaryllidaceae alkaloids began with the isolation of lycorine from *Narcissus pseudonarcissus* in 1877. Since then, 150 species belonging to 36 genera of this family have been studied for alkaloids. Over 100 alkaloids with a wide range of structural variation, consisting of 12 distinct ring types have been isolated from different plant parts of Amaryllidaceae species (GHOSAL *et al.*, 1985). Investigations of the alkaloids of *Boophone disticha* were among the first to be reported in the family (WILDMAN, 1960).

The use of *Boophone disticha* for medicinal purposes amongst the indigenous peoples of southern Africa has been known for several centuries (WATT & BREYER-BRANDWIJK, 1962). As discussed in Section 1.3.1, its most striking usage is for narcotic purposes; for example, inducing stupor in newly circumcised initiates, for sedation of psychotic patients and to produce hallucinatory effects during divination rituals (DE SMET, 1996). The chemicals likely responsible for many of these effects are alkaloidal constituents, structural variations of which occur exclusively within the family Amaryllidaceae (VILADOMAT *et al*., 1997). Previous phytochemical investigations of *Boophone disticha* have led to the identification of a total of eleven alkaloids (Table 5.1) (HAUTH & STAUFFACHER, 1961; SANDAGER *et al*., 2005; NEERGAARD *et al*., 2009), mainly of the crinane group of compounds which are known for a diverse array of biological activities (VILADOMAT *et al*., 1997). These alkaloids have also been previously mentioned in Section 1.4.2 and their chemical structures can be seen in Figure 1.3.

Table 5.1: Alkaloids in the bulb of *Boophone disticha* (DE SMET, 1996).

Alkaloid	Relative contribution to total alkaloids (%)
Buphanidrine	19.4
Undulatine	18.6
Buphanisine	16.9
Buphanamine	14.1
Nerbowdine	11.1
Crinine	7.2
Distichamine	5.4
Crinamidine	1.2
Acetylnerbowdine	0.6
Lycorine	0.4
Buphacetine	0.3

Of these compounds, buphanidrine, buphanamine and distichamine were seen to have a profound effect on the serotonin transporter (SANDAGER *et al*., 2005; NEERGAARD *et al*.,

2009), highlighting their possible involvement in the use of the plant for central nervous system disorders such as Alzheimer's disease and for the treatment of depression. Although diverse extracts from the Amaryllidaceae have exhibited antibacterial effects (VILADOMAT *et al*., 1997), little is known about the activities of single compound isolates of these plants. Therefore, the aim of this study was to investigate the antimicrobial activity of the isolated alkaloids from bulbs of *B. disticha*, buphanidrine and distichamine (as it is currently known to only occur in this species), and to test these for possible mutagenicity in the Ames test (MARON & AMES, 1983).

5.1.2 Materials and methods

General

Melting points (uncorrected) were measured on a Gallenkamp melting point apparatus. Optical rotations were determined on a Perkin-Elmer 241 polarimeter installed with a λ_{589} sodium lamp. IR spectra were measured on a Bio-Rad FTS-40 series spectrometer in dry film. EIMS were run on a Micromass Quattro Ultima spectrometer fitted with a direct injection probe (DIP) with ionization energy set at 70 eV and HRMS (EI) were performed with a Micromass Q-Tof Ultima spectrometer. ^{1}H, ^{13}C NMR, DEPT, COSY, NOESY, HSQC and HMBC spectra were recorded on a Bruker AV400 spectrometer in CD_3OD, chemical shifts are reported in units of δ (ppm) and coupling constants (J) are expressed in Hz. Silica gel Merck KGaA (70-230 mesh) was used for CC and TLC silica gel 60 F_{254} for analytical and preparative TLC (both Merck KGaA). Spots on chromatograms were detected under UV light (254 and 365 nm) and by Dragendorff's reagent stain.

Plant material

Bulbs were collected in March 2011 in the Mpophomeni area (29° 33.296' S & 30° 11.468' E) of KwaZulu-Natal (South Africa) and a voucher specimen (Cheesman 01 NU) retained at the UKZN Herbarium.

Extraction and isolation of alkaloids

Powdered dried bulbs (312 g) were extracted by stirring with EtOH for 48 h after which the solvent was evaporated under reduced pressure. The residue (24 g) was subjected to gravity

column chromatography with silica gel by gradient elution with EtOAc and EtOAc/MeOH mixtures. In this manner, distichamine **2** was obtained from ten 20 ml fractions eluted with 10% MeOH/EtOAc and buphanidrine **1** collected from five similar fractions eluted with 50% MeOH/ EtOAc. Further purification was achieved by preparative TLC using EtOAc/NH_3 as the developing solvent, and by recrystallization with analytically-pure acetone, distichamine (7.4 mg) and buphanidrine (25.0 mg) were each obtained as fine white powders.

*Physical and spectroscopic data for buphanidrine **1***

M.pt. 91-93 ^{o}C. $[\alpha]_D^{25}$ -10.1 (*c* 0.3 in MeOH). IR ν_{MAX}/cm^{-1} (dry film): 3015, 2930, 2398, 1617, 1483, 1316, 1281, 1082, 1046, 938. HRMS (EI): calcd. 315.1471 for $C_{18}H_{21}NO_4$, found 315.1462. LRMS (EI) 70 eV, *m/z* (rel. int.): 315 $[M]^+$ (100), 300 (20), 285 (10), 284 (25), 260 (22), 257 (16), 245 (30), 231 (15), 228 (14), 215 (12), 202 (8), 187 (7), 115 (9). ^{1}H NMR (400 MHz, CD_3OD): δ 1.64 (1H, ddd, *J*= 13.6, 13.5, 4.1 Hz, H-4β), 1.94 (1H, ddd, *J*= 12.6, 10.9, 6.04 Hz, H-11*exo*), 2.03 (1H, brd, *J*= 13.6 Hz, H-4α), 2.13 (1H, ddd, *J*= 12.6, 9.16, 4.0 Hz, H-11*endo*), 2.87 (1H, ddd, *J*= 13.1, 9.1, 6.04 Hz, H-12*endo*), 3.22 (1H, dd, *J*= 13.5, 4.0 Hz, H-4a), 3.30 (1H, m, H-12*exo*), 3.34 (3H, s, 3-OCH_3), 3.79 (1H, d, *J*= 17.2 Hz, H-6β), 3.84 (1H, m, H-3), 3.96 (1H, s, 7-OCH_3), 4.15 (1H, d, *J*= 17.2 Hz, H-6α), 5.84 (2H, s, -OCH_2O-), 5.96 (1H, dd, *J*= 10.0, 5.16 Hz, H-2), 6.61 (1H, s, H-10), 6.62 (1H, d, *J*= 10.0 Hz, H-1). ^{13}C NMR (100 MHz, CD_3OD): δ 30.3 (t, C-4), 45.6 (t, C-11), 46.5 (s, C-10b), 55.0 (t, C-12), 57.6 (q, 3-O*C*H_3), 60.1 (t, C-6), 60.5 (q, 7-O*C*H_3), 64.8 (d, C-4a), 74.7 (d, C-3), 98.8 (d, C-10), 102.9 (t, O*C*H_2O), 118.1 (s, C-6a), 127.1 (d, C-2), 134.6 (d, C-1), 135.7 (s, C-8), 141.3 (s, C-10a), 143.1 (s, C-7), 150.8 (s, C-9).

*Physical and spectroscopic data for distichamine **2***

M.pt. 83-85 ^{o}C. $[\alpha]_D^{25}$ -49.4 (*c* 0.3 in MeOH). IR ν_{MAX}/cm^{-1} (dry film): 3010, 2934, 2400, 1685, 1618, 1483, 1311, 1286, 1080, 1045, 934. HRMS (EI): calcd. 329.1263 for $C_{18}H_{19}NO_5$, found 329.1269. LRMS (EI) 70 eV, *m/z* (rel. int.): 329 $[M]^+$ (100), 314 (22), 300 (10), 286 (18), 270 (10), 231 (27), 215 (11), 204 (22), 181 (10), 149 (8), 129 (12). ^{1}H NMR (400 MHz, CD_3OD): δ 2.25 (1H, ddd, *J*= 12.5, 10.8, 6.60 Hz, H-11*exo*), 2.27 (1H, ddd, *J*= 12.5, 9.10, 4.0 Hz, H-11*endo*), 2.47 (1H, dd, *J*= 17.2, 7.0 Hz, H-4α), 2.52 (1H, ddd, *J*= 17.2, 10.9, 1.24 Hz, H-4β), 2.91 (1H, ddd, *J*= 13.0, 9.1, 6.04 Hz, H-12*endo*), 3.41 (1H, m, H-12*exo*), 3.64 (1H, dd, *J*= 10.8, 7.04 Hz, H-4a), 3.77 (3H, s, 3-OCH_3), 3.78 (1H, d, *J*= 17.3 Hz, H-6β), 3.96 (1H, s, 7-OCH_3),

4.15 (1H, d, *J*= 17.3 Hz, H-6α), 5.42 (1H, d, *J*= 1.28 Hz, H-2), 5.85 (2H, s, -OC*H*$_2$O-), 7.61 (1H, s, H-10). ^{13}C NMR (100 MHz, CD_3OD): δ 32.3 (t, C-4), 43.6 (t, C-11), 52.7 (s, C-10b), 54.4 (t, C-12), 57.6 (q, 3-O*C*H$_3$), 59.6 (t, C-6), 60.5 (q, 7-O*C*H$_3$), 68.4 (d, C-4a), 102.0 (d, C-10), 102.9 (t, O*C*H$_2$O), 103.6 (d, C-2), 118.6 (s, C-6a), 136.1 (s, C-10a), 137.8 (s, C-8), 142.5 (s, C-7), 150.3 (s, C-9), 177.0 (s, C-3), 201.9 (s, C-1).

Antibacterial and antifungal activity

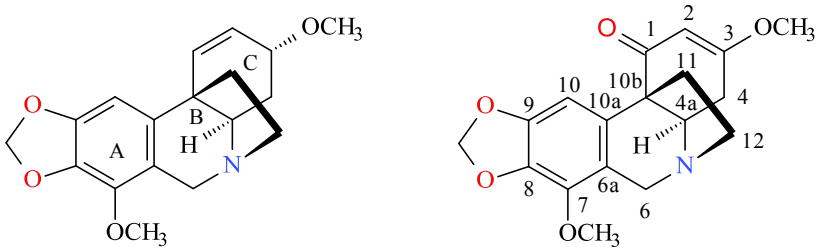
Antibacterial and antifungal activities of samples were determined according to the micro-dilution assay of ELOFF (1998) with modifications for fungi (MASOKO *et al*., 2007). The method is as in Section 3.1.2 but with slight modifications. Test samples were re-dissolved in methanol at a concentration of 1 mg/ml and 100 µl of each solution was two-fold serially diluted with sterile distilled water in a 96-well micro-plate, in duplicate for each bacterial strain and *Candida albicans*. MIC values were recorded as the lowest concentration of sample to affect clear wells. GenStat (Version 14, Rothamsted Research, Harpenden, UK) was used for statistical analysis.

Ames assay

Mutagenicity was tested using the *Salmonella*/microsome assay (Ames test), with and without metabolic activation, based on the plate-incorporation method (MARON & AMES, 1983). The method is as described in Section 4.1.2. For each sample, three different concentrations were used to test for mutagencity: 10 µg/ml, 100 µg/ml and 1000 µg/ml.

5.1.3 Results and discussion

Identification of the respective structures of **1** and **2** as buphanidrine and distichamine (Figure 5.1) was based on both physical and spectroscopic data (Figure 5.2 to 5.5) which were in close comparison to published values (HAUTH & STAUFFACHER, 1961; SANDAGER *et al*., 2005; NEERGAARD *et al*., 2009). However, physical and ^{13}C NMR data for distichamine (Figure 5.3) were absent from the literature and are thus presented. The EIMS spectrum had the molecular ion $[M]^+$ peak as the base peak at *m/z* 329, correct for the molecular mass of distichamine. This was further substantiated by HRMS analysis which gave a mass of 329.1269 g/mol for the compound, which is correct for $C_{18}H_{19}NO_5$ with a calculated mass of 329.1263 g/mol.



1. Buphanidrine **2.** Distichamine

Figure 5.1: β-Crinane alkaloids isolated from *Boophone disticha.*

The low field region of the ^{1}H NMR spectrum was populated by three signals which were assigned to H-10 (δ 7.61, 1H, s) and the methylenedioxy group (δ 5.85, 2H, s) connected to ring A, as well as H-2 (δ 5.42, 1H, d, *J*= 1.28 Hz) of ring C (Figure 5.2). Three-bond HMBC connected H-10 to C-6a, C-8 and C-10b, allowing for the pentasubstituted pattern of ring A. In turn, the B ring heterocycle was characterized by resonance signals for the diastereotropic protons H-6α (δ4.15, *J*= 17.3 Hz) and H-6β (δ3.78, *J*= 17.3 Hz), which had three-bond HMBC connectivity to C-4a, C-7, C-10a and C-12, with H-6α shifted to lower field due to its *syn* proximity to the nitrogen lone pair. Furthermore, the α-axial H-4a proton resonating at δ 3.64 (dd, *J*= 10.8, 7.04 Hz) was connected to C-1, C-3, C-6, C-11 and C-12 via HMBC. The α,β-unsaturated nature of the C ring keto group was indicated by carbon singlet resonances at δ 201.9 (C-1) and 177.0 (C-3), a doublet at δ 103.6 (C-2) as well as a proton doublet at δ 5.42 for H-2 which was seen to be *W*-coupled to H-4β by the small value of the coupling constant (*J*= 1.24 Hz). Spatial correlation via 2D NOESY linked 3-OMe to both H-2 and 2H-4, which together with its proton chemical shift (δ 3.77, 3H, s) established its vinylic positioning. Distichamine **2** is unique amongst the crinane series of compounds in possessing both vinylic methoxyl as well as α,β-unsaturated ketone functionalities. Interestingly, of the Amaryllidaceae species examined, distichamine was previously only isolated from *Boophone disticha*. However, NAIR *et al*. (2012) isolated distichamine from *B. haemanthoides,* therefore, making distichamine a distinctive chemotaxonomic marker for the genus *Boophone*.

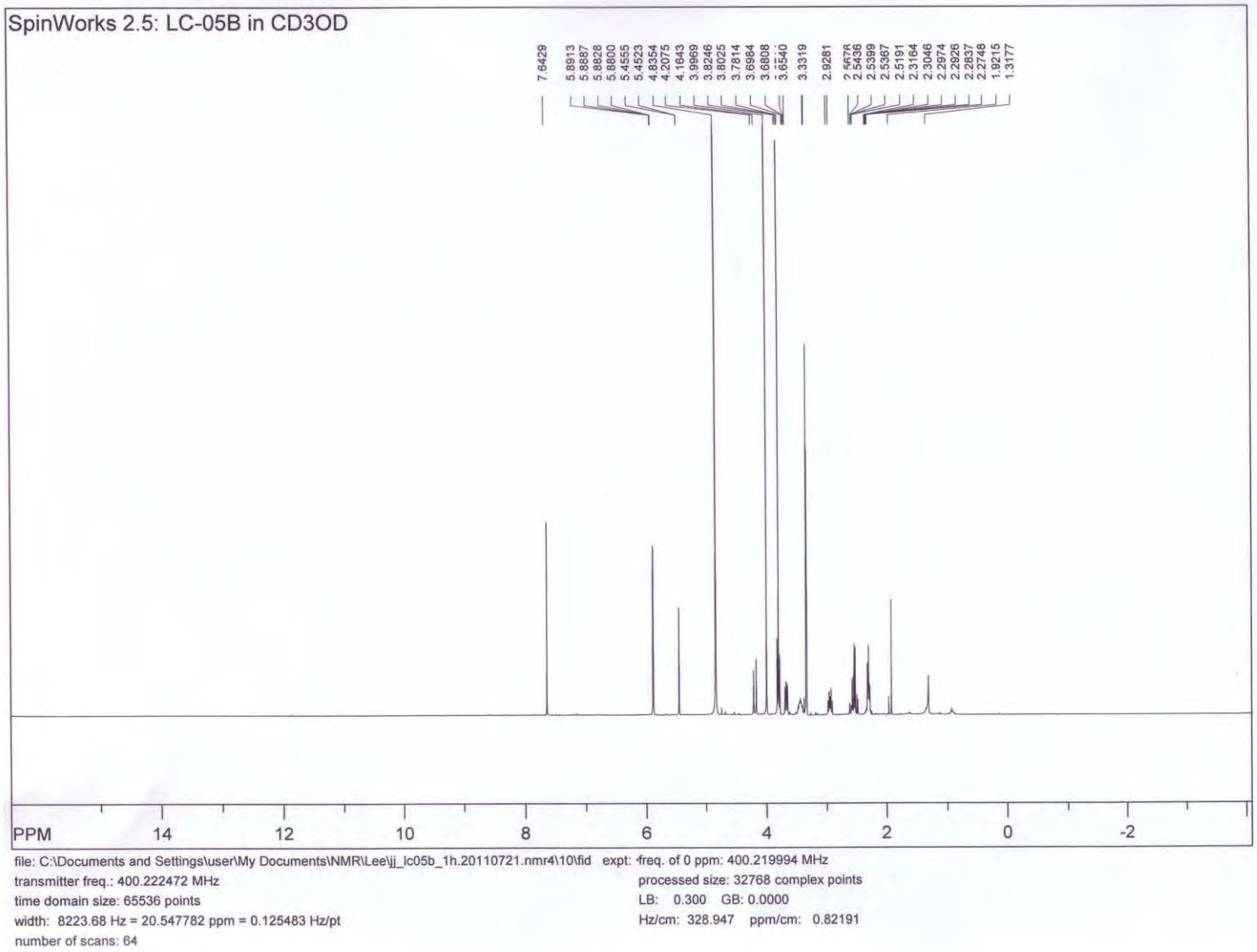


Figure 5.2: ^{1}H NMR spectrum at 400.2199 MHz of distichamine isolated from *B. disticha* bulbs.

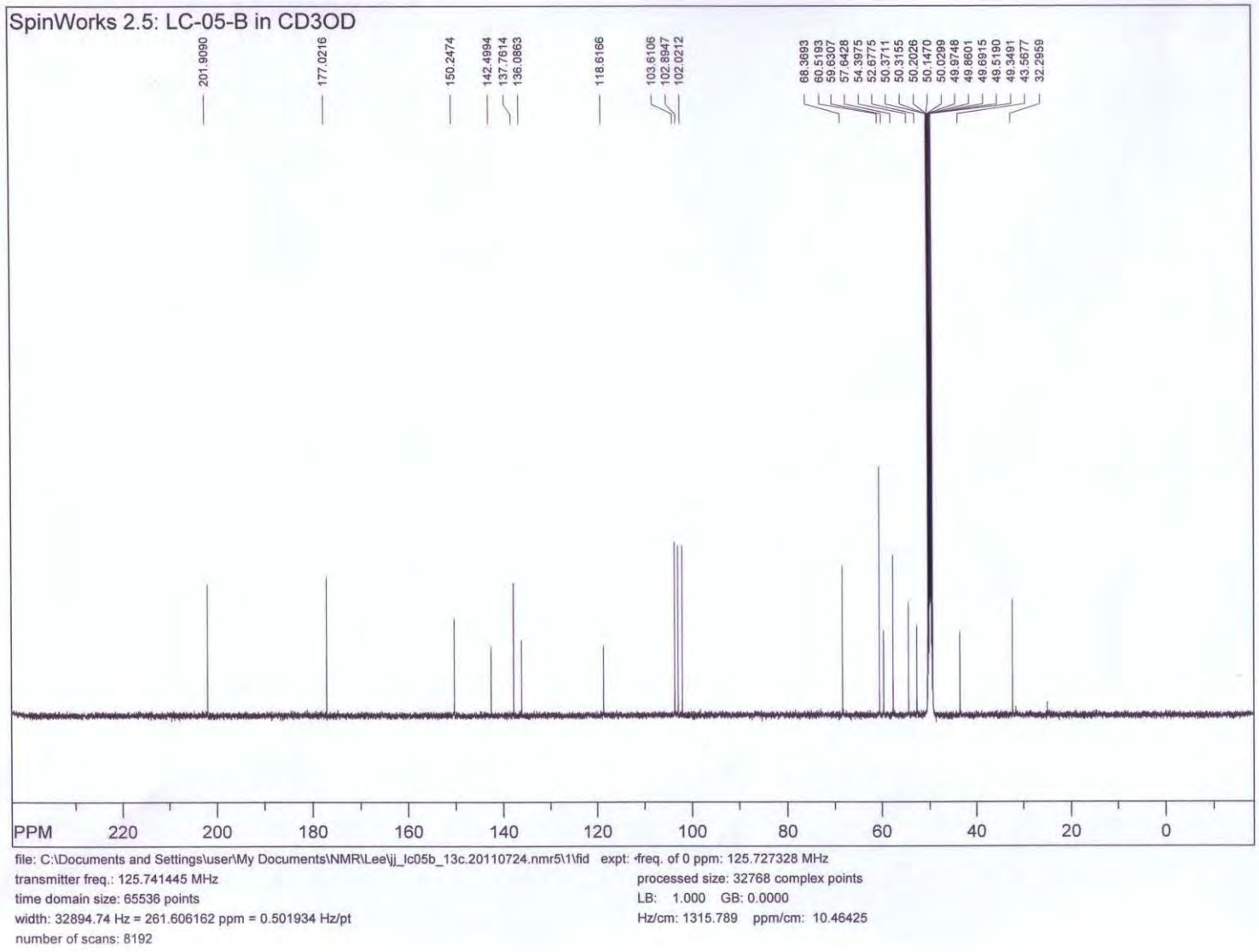


Figure 5.3: ^{13}C NMR spectrum at 125.7273 MHz of distichamine isolated from *B. disticha* bulbs.

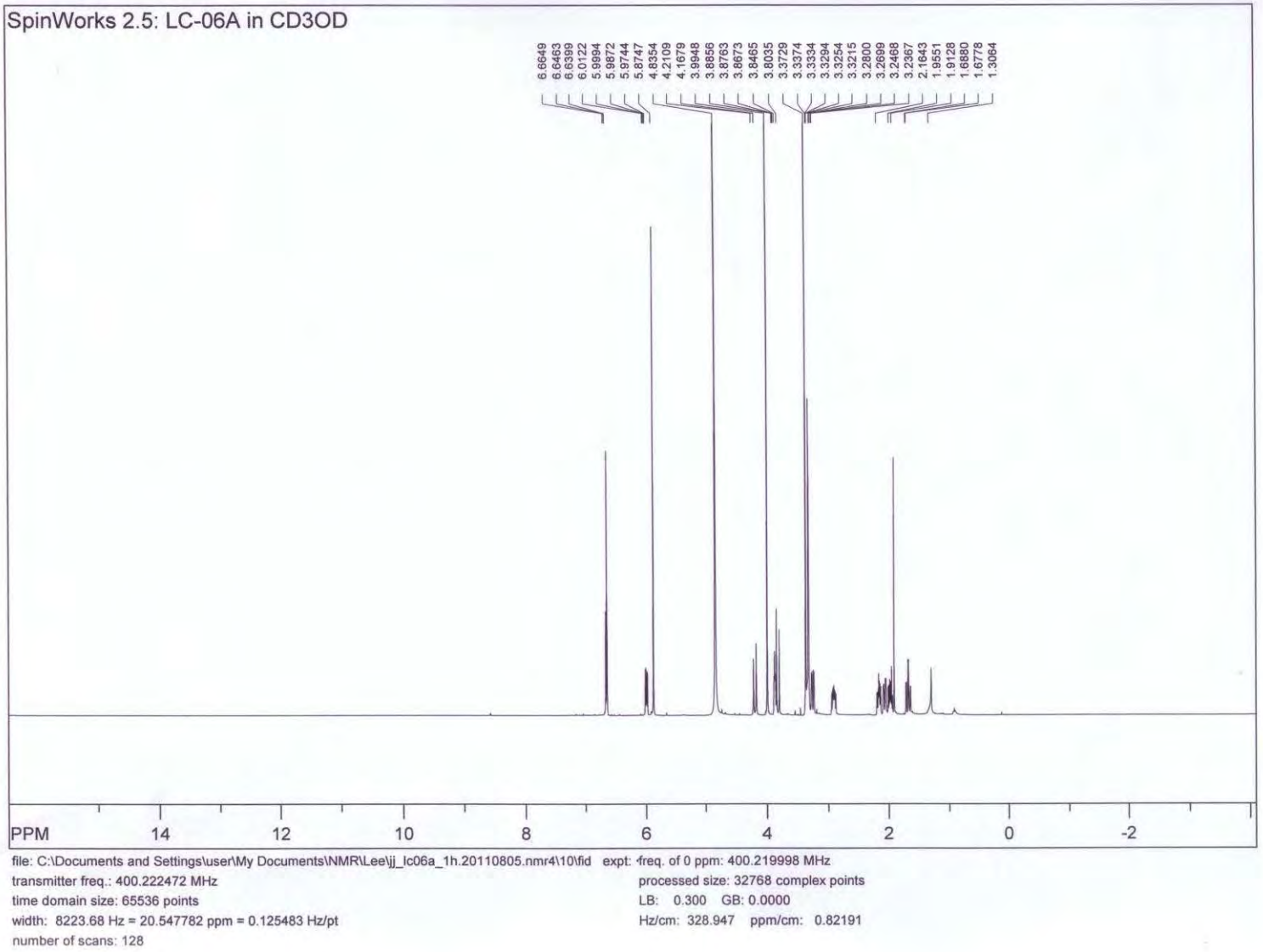


Figure 5.4: ^{1}H NMR spectrum at 400.2199 MHz of buphanidrine isolated from *B. disticha* bulbs.

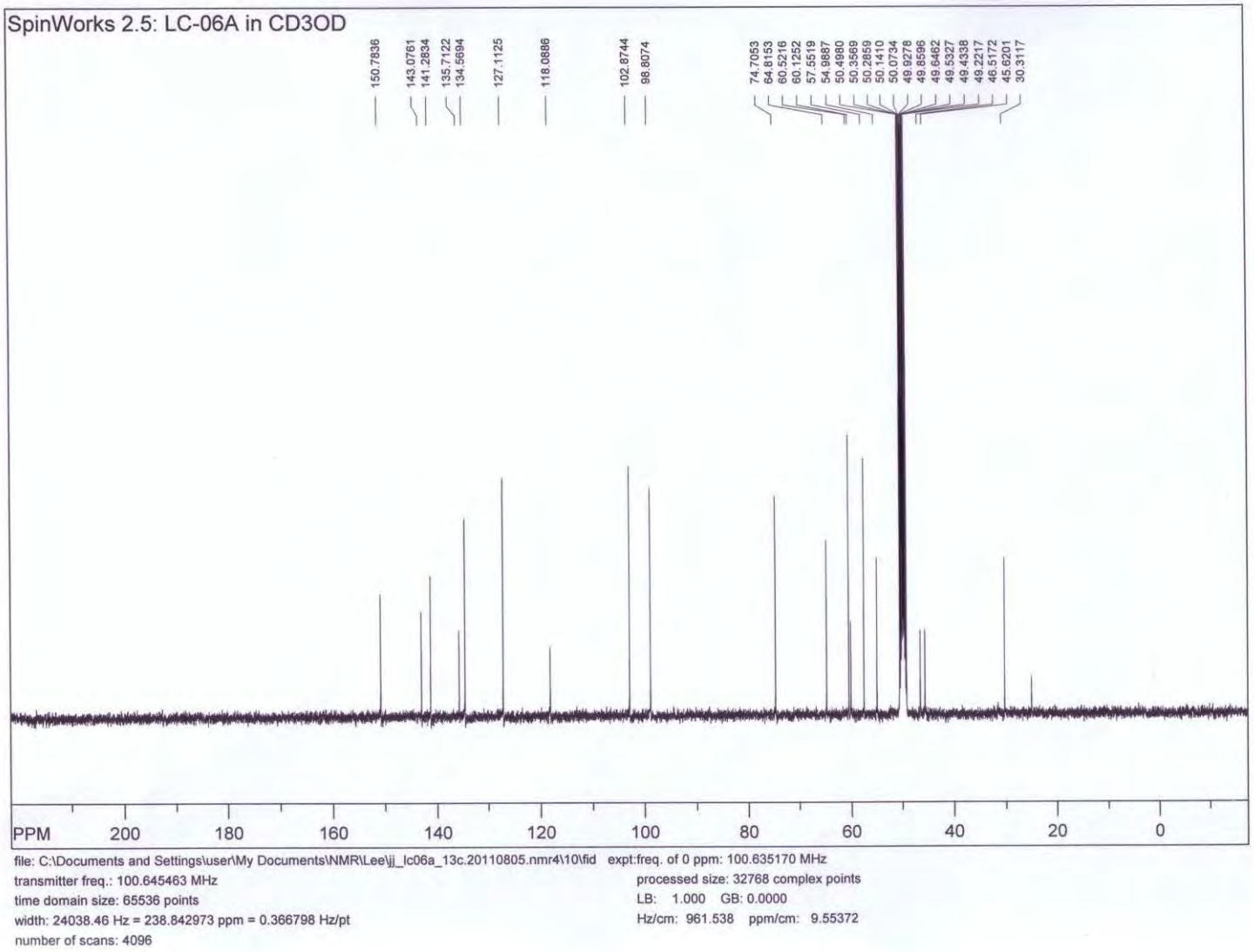


Figure 5.5: ^{13}C NMR spectrum at 100.6351 MHz of buphanidrine isolated from *B. disticha* bulbs.

Given the widespread traditional usage of the plant to treat wounds and infections (HUTCHINGS *et al*., 1996), it is surprising that these responses have not been attributed to specific chemical entities. With this as the basis for investigation, two of the constituents of *Boophone disticha* were screened for antibacterial activity against two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains, and for antifungal activity against *Candida albicans*. These findings (summarized in Table 5.2) showed the crude ethanol extract to be active against all four bacterial strains, but particularly against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* with an MIC at 0.13 mg/ml [$f(3,12) = 2341$, $p < 0.001$]. Furthermore, buphanidrine **1** and distichamine **2** were seen to be twice as active as the crude extract in all four bacterial assays [$f(3,12) = 60244$, $p < 0.001$], with the best MIC value established at 0.063 mg/ml. It is noteworthy that the MIC values for *B. subtilis* were twice that of those observed for the other bacteria in all assays [$f(2,9) = 2363$, $p < 0.001$], suggesting that the extract and pure compounds were selective in their interaction with the bacterial pathogens. The crude extract was active against *C. albicans* with an MIC value of 0.125 mg/ml. Buphanidrine **1** and distichamine **2** were, however, twice as active against *C. albicans* [$f(2,6) = 11532$, $p < 0.001$], compared to the crude extract, with an MIC value of 0.063 mg/ml (Table 5.2). Minimal fungicidal concentration (MFC) of 0.25 mg/ml did not show any significant difference [$f(2,6) = 0.00$, $p > 1.000$].

Table 5.2: Antimicrobial minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of alkaloidal constituents of *B. disticha*.

Sample	Antibacterial activity MIC (mg/ml)				Antifungal activity *Candida albicans*	
	Bacteria				MIC (mg/ml)	MFC (mg/ml)
	B.s	***S.a***	***E.c***	***K.p***		
Crude Ethanol Extract	0.25	0.13	0.13	0.13	0.125	>0.25
Distichamine	0.13	0.063	0.063	0.063	0.063	0.25
Buphanidrine	0.13	0.063	0.063	0.063	0.063	0.25
Neomycin (µg/ml)	$1.6x10^{-3}$	$0.8x10^{-3}$	$0.8x10^{-3}$	$1.6x10^{-3}$	-	-
Amphotericin B (µg/ml)	-	-	-	-	$9.77x10^{-3}$	$7.81x10^{-2}$

B.s = Bacillus subtilis, *S.a = Staphylococcus aureus*, *E.c = Escherichia coli* and *K.p = Klebsiella pneumoniae*

The similar antibacterial and antifungal activity profiles of **1** and **2** may relate directly to their close structural proximity, the apparent difference being the shift of the double bond to C-2,C-3 and oxidation at C-1 in distichamine **2**. Previous findings have shown that the α-crinane compound crinamine, which bears a close structural resemblance to buphanidrine and distichamine, as well as lycorine exhibited antibacterial properties (ADESANYA *et al*., 1992; TAN *et al*., 2011).

In contrast to the present findings, it was earlier reported that both organic and aqueous extracts of *Boophone disticha* were inactive against the above four pathogens (RABE & VAN STADEN, 1997). It has since been established that there is a profound seasonal variation in alkaloid metabolite production in *Boophone disticha* (see Chapter 3), and this could explain the lack of activity found in this prior study.

FENNELL *et al.* (2004) stated that the activity of an extract can be compared between different assays, but not against pure standards, since crude extracts contain a myriad of compounds that may be acting synergistically. In the current study, both the crude extract and isolated compounds were tested for antimicrobial activity and it was found that the isolated compounds were twice as active as the crude extract. In another study by EVIDENTE *et al.* (2004), six lycorine-type alkaloids were isolated from *Amaryllis belladonna* and tested for antimicrobial activity. The results from the antibacterial and antifungal assays showed that (-)-amarbellisine, (-)-pancracine, (+)-vittatine and (+)-11-hydroxyvittatine have antibacterial activity against *S. aureus*. The newly discovered alkaloid, (-)-amarbellisine, and (+)-vittatine exhibited activity against *E. coli* whereas (-)-pancracine showed activity against *Pseudomonas aeruginosea*. All the isolated alkaloids showed antifungal activity against *C. albicans* (EVIDENTE *et al.,* 2004). The results from the current study and EVIDENTE *et al.* (2004)'s study are in contrast to the findings from FENNELL *et al.* (2004) who screened selected medicinal plants for pharmacological activity and toxicology. In their study, the values achieved using the pure compounds were lower than the results obtained with the crude plant extracts. They believed this was due to many other constituents, in addition to the active components, being present in the crude extracts (FENNELL *et al.,* 2004).

During the screening of medicinal plants used in South African traditional medicine for genotoxic effects, ELGORASHI *et al.* (2003b) reported that no compounds from plant extracts were investigated for mutagenicity. ELGORASHI *et al.* (2003b) believed that due to the fact that

as plant extracts are complex mixtures of organic compounds, it is difficult to speculate on the compounds responsible for the mutagenic response detected in these extracts (ELGORASHI *et al.*, 2003b). Therefore, both crude extracts and isolated compounds (distichamine and buphanidrine) from *B. disticha* were tested for mutagenic activity using the Ames test.

The results in the Ames test are based on the number of induced revertant colonies detected. Table 5.3 and Table 5.4 give the mean number of revertants per plate with *Salmonella typhimurium* strain TA 98 and TA 100, with and without metabolic activation. For an extract to be considered mutagenic, the number of revertant colonies on the plate containing the test compounds must be more than twice the number of colonies on the negative control plate. Neither the crude extract nor the isolated compounds (buphanidrine and distichamine) at the concentrations tested were found to induce mutations as the samples did not give double the number of revertant colonies like the negative control. All samples were not toxic in the range of concentrations tested as the absence of toxicity was examined by observing the background bacterial growth, which was present.

In conclusion, the phytochemical investigation of *B. disticha* has led to the identification of two known crinanes, buphanidrine and distichamine. Based on the reputed traditional use of the plant for wounds and infections, both compounds were screened for antibacterial and antifungal activity which revealed them to be novel, broad spectrum antimicrobial agents with the best MIC value set at 0.063 mg/ml. Their close structural similarity may have bearing on their similar activity profiles. Efforts are presently underway to further investigate this interesting antimicrobial pharmacophore.

Table 5.3: Number of histidine+ revertants/plate and mutagenic index (MI) in *Salmonella typhimurium* strain TA 98 produced by a crude extract and isolated compounds of *Boophone disticha* without or with metabolic activation (S9).

Sample	Number of colonies											
	1000 µg/ml				100 µg/ml				10 µg/ml			
	Without S9	MI	With S9	MI	Without S9	MI	With S9	MI	Without S9	MI	With S9	MI
Buphanidrine	163.00 ± 8.49	1.06	137.50 ± 9.19	0.95	141.50 ± 3.54	0.92	139.50 ± 4.95	0.96	156.00 ± 0.00	1.02	139.00 ± 0.00	0.96
Distichamine	138.00 ± 0.00	0.90	145.00 ± 5.66	1.00	152.00 ± 1.41	0.99	149.00 ± 1.41	1.03	146.50 ± 0.71	0.95	124.00 ± 1.41	0.85
Crude Ethanol Extract	138.00 ± 7.07	0.90	104.50 ± 0.71	0.72	125.50 ± 6.36	0.82	129.50 ± 10.61	0.89	138.00 ± 12.73	0.90	140.00 ± 1.41	0.96
4NQO (positive control)	1126.17 ± 13.25	7.34	-	-								
2AA (positive control)	-	-	1148.31 ± 9.84	7.91								
10% DMSO (negative control)	153.52 ± 4.81	1.00	145.25 ± 3.24	1.00								

Table 5.4: Number of histidine+ revertants/plate and mutagenic index in *Salmonella typhimurium* strain TA 100 produced by a crude extract and isolated compounds of *Boophone disticha* without or with metabolic activation (S9).

Sample	Number of colonies											
	1000 µg/ml				100 µg/ml				10 µg/ml			
	Without S9	MI	With S9	MI	Without S9	MI	With S9	MI	Without S9	MI	With S9	MI
Buphanidrine	144.50 ± 2.12	0.96	130.50 ± 6.36	0.95	141.50 ± 3.54	0.94	160.50 ± 9.19	1.17	165.00 ± 26.87	1.10	123.00 ± 0.00	0.90
Distichamine	147.50 ± 7.78	0.98	138.50 ± 7.78	1.01	134.50 ± 12.02	0.89	137.50 ± 13.44	1.01	135.00 ± 2.83	0.99	124.00 ± 21.21	0.91
Crude Ethanol Extract	146.00 ± 1.41	0.97	131.50 ± 9.19	0.96	152.00 ± 2.83	1.01	129.50 ± 4.95	0.95	175.00 ± 25.46	1.16	135.50 ± 2.12	0.99
4NQO (positive control)	1138.29 ± 12.43	7.56	-	-								
2AA (positive control)	-	-	1164.57 ± 17.65	8.52								
10% DMSO (negative control)	150.50 ± 2.77	1.00	136.67 ± 3.21	1.00								

5.2 SUMMARY

- Two alkaloids, buphanidrine and distichamine were isolated from bulbs of *Boophone disticha* collected in summer
- The isolated compounds were twice as active as the crude extract in the antibacterial and antifungal assays
- Neither the crude extract nor the isolated compounds were found to be mutagenic or toxic in the Ames test

Chapter Six

GENETIC STUDY

6.1 GENETIC VARIATION OF SIX WILD *BOOPHONE DISTICHA* POPULATIONS

6.1.1 Introduction

Genetic diversity is a key parameter in evaluating and planning the sustainable management of plant ecosystems. An understanding of the degree of diversity within and between the populations of a species is required to provide information for the conservation of the species' genetic resources, since geographically separated populations are expected to have different genetic compositions. Genetic diversity is of prime importance for species persistence (WANG & SZMIDT, 2001), since the evolutionary adaptive potential of populations depends on genetic variation patterns (SIREGAR, 2000; FINKELDEY & HATTEMER, 2007). Therefore, genetic markers are needed to distinguish between different populations.

Genetic markers were originally used in gene mapping to determine the order of genes along chromosomes. Today, however, genetic markers are used in plant research and plant breeding for characterizing plant germplasm, gene isolation, cultivar identification and to determine genetic diversity (ANDERSEN & LÜBBERSTEDT, 2003). Molecular markers are fragments of DNA that are associated with a certain location within the genome. Molecular markers are phenotypically neutral and are developmentally and environmentally stable. They are identified by different techniques such as Southern Hybridization or the Polymerase Chain Reaction (PCR). Markers identified by Southern Hybridization include Restriction Fragment Length Polymorphisms (RFLPs) and Variable Number of Tandem Repeats (VNTRs). Markers identified by PCR-based methods include: randomly amplified polymorphic DNA (RAPD), amplification fragment length polymorphism (AFLP), cleaved amplified polymorphic site (CAPS), simple sequence repeats (SSR) and single nucleotide polymorphisms (SNP). The best molecular markers are markers which distinguish multiple alleles per locus (i.e. are highly polymorphic) and are co-dominant (each allele can be observed).

Restriction fragment length polymorphism (RFLP) represents a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single restriction enzyme combination. Most RFLP markers are co-dominant (both alleles in heterozygous sample will be detected) and highly locus-specific.

Randomly amplified polymorphic DNAs (RAPDs) include PCR reactions that amplify segments of DNA which are essentially unknown or random. The simplicity of the technique and the number of markers that can be generated has encouraged the use of RAPDs in the investigation of many biodiversity-associated problems. RAPD markers are dominant, which allows for amplification at many loci in one sample of DNA with one PCR reaction. Amplified fragment length polymorphisms (AFLPs), use restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. AFLP-PCR has the ability to detect various polymorphisms in different genomic regions simultaneously. It is highly sensitive and reproducible. AFLP has become widely used for the identification of genetic variation in strains of fungi and bacteria or closely related species of plants and animals. Simple sequence repeats (SSRs), also called microsatellites, are becoming very important molecular markers in both animals and plants. SSRs are stretches of 2 to 6 nucleotide units repeated in tandem and randomly spread in eukaryotic genomes. SSRs are very polymorphic due to the high mutation rate affecting the number of repeat units. The major limitations of these markers are low reproducibility of RAPDs, high cost of AFLPs and the requirement for sequence information from flanking regions to design species specific primers for SSR polymorphism (LIU & WENDEL, 2001; REDDY *et al*., 2002). However, a recently developed modification of SSR-based marker systems, inter simple sequence repeats (ISSRs) overcomes most of these problems.

PCR amplification of inter simple sequence repeats (ISSRs) has been widely used for genetic analysis of plants. The method uses a single oligonucleotide primer composed of 4 to 10 tri- or di-nucleotide repeats ending with a 3'- or 5'- anchor sequence. ISSRs target the highly variable microsatellite regions of the nuclear genome, therefore, providing a large number of polymorphic fragments (GUPTA *et al*., 1994). It provides more reproducibility than similar methods such as RAPDs, AFLPs and SSRs (AWASTHI *et al*., 2004). This technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms.

The aim of this research was to determine whether there was genetic variation between populations of *B. disticha* collected from different locations. From this, it was hoped to establish whether the plants had adapted in any way to their different environments. The bulbs collected from these different populations were also tested to see whether they had similar antimicrobial activity.

6.1.2 Materials and methods

Boophone disticha bulbs were collected from six wild populations (Figure 6.1 and 6.2) and voucher specimens retained at the UKZN Herbarium (Table 6.1).

Table 6.1: GPS co-ordinates for the wild populations of *B. disticha* and voucher specimen numbers.

Location	GPS co-ordinate	Voucher specimen number
Amatikulu	29° 30′ 31″ S, 33° 34′ 35″ E	LC 06
Lincoln Meade	29° 37′ 29″ S, 30° 26′ 88″ E	LC 03
Lions River	29° 29′ 06″ S, 30° 05′ 34″ E	LC 04
Midmar	29° 31′ 56″ S, 30° 12′ 13″ E	LC 02
Mpomphomeni	29° 33′ 23″ S, 30° 11′ 29″ E	LC 01
Umgeni Valley	29° 29′ 26″ S, 30° 15′ 10″ E	LC 05

Figure 6.1: Map of KwaZulu-Natal showing the collection points of the six different populations of *B. disticha*.

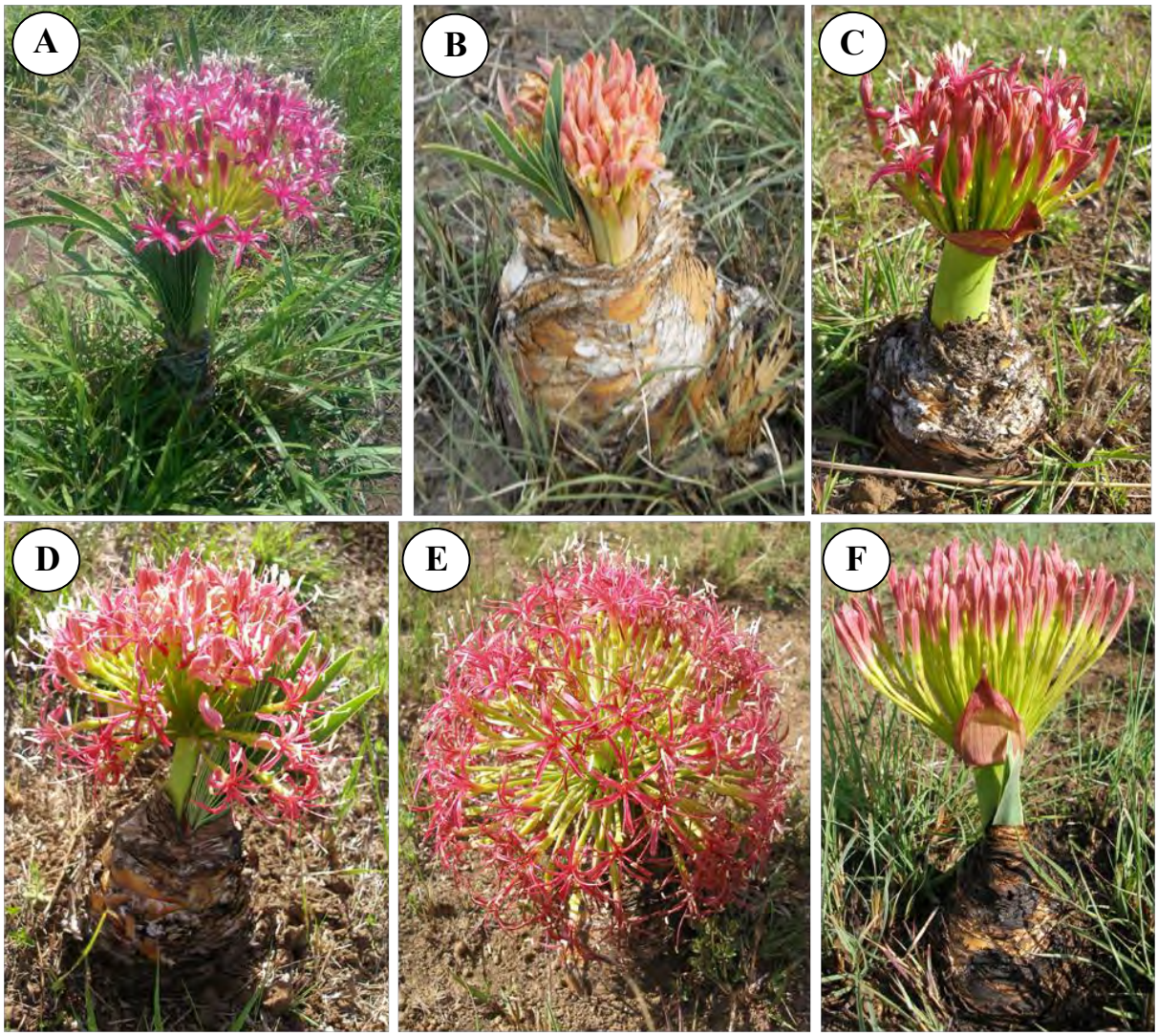


Figure 6.2: **Photographs showing flowering *B. disticha* bulbs from the six different populations. A- Amatikulu, B- Lincoln Meade, C- Lions River, D- Midmar, E- Mpomphomeni and F- Umgeni Valley.**

Isolation of DNA

Bulb scale tissue (100 mg) was ground to a fine powder in liquid nitrogen using a porcelain mortar and pestle. Genomic DNA was isolated using a Genomic DNA Purification Kit from Fermentas (according to manufacturers instructions) and the samples of DNA were quantified using The Thermo Scientific NanoDrop 2000, USA. For each sample, 200 µl of stock was prepared.

DNA amplification

PCR amplification was performed in a 50 µl reaction volume in an Applied Biosystems Veriti™ Thermal Cycler (California, USA). The mixture contained 25 µl Fermentas Life Sciences PCR Master Mix (2x), 0.5 µl primer, 25 ng template DNA (volume dependent on DNA concentration) and nuclease-free water to make up the volume to 50 µl. Ten ISSR primers were used for the analysis (Table 6.2). Amplifications were programmed for an initial denaturation at 95 °C for 2 min, 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 45 °C and 1 min extension at 72 °C, followed by final extension for 15 min at 72 °C. The amplification products were separated on 2 % agarose gels buffered with 0.5 x TAE using electrophoresis and detected by GR Green Nucleic Acid Gel Stain (Lab Supply Mall) staining. Gene Ruler 10 000 bp DNA ladder was used to determine the size of the ISSR fragments (Addendum 1).

Table 6.2: List of primers used in ISSR analysis of the six *Boophone disticha* populations.

Primer	Sequences
1	AGAGAGAGAGAGAGC
2	CACACACACACACAA
3	ACACACACACACACG
4	AGAGAGAGAGAGAGYC[a]
5	GAGAGAGAGAGAGAYC[a]
6	CTCTCTCTCTCTCTRC[b]
7	TGTGTGTGTGTGTGRC[b]
8	TGTGTGTGTGTGTGRA[b]
9	GAAGAAGAAGAAGAAGAA
10	GGAGAGGAGAGGAGA

[a] Y= C or T and [b] R= A or G

Data analysis

Percentage band polymorphism was calculated, which is the number of polymorphic bands x 100/ total number of bands obtained for each primer. Amplified DNA banding patterns

generated by ISSR primers in the lanes were scored as (1) for the presence or (0) for the absence (Addendum 1). All amplifications were repeated twice and only reproducible bands were scored.

POPGENE 32 software was used to obtain: percentage of polymorphic loci, percent band polymorphism (PBP), observed number of alleles (na*), effective number of allele (ne*) and Nei's unbiased gene diversity (h*) which is equivalent to expected heterozyogosity (HE*) of a population. Another genetic diversity parameter Shannon's Information Index (I*) and Gene flow (*Nm) between six populations and genetic distances were also obtained for all these populations.

6.1.3 Results and discussion

The inter-population diversity study primers which were used for the analysis (Table 6.2) generated a total of 173 loci (bands) ranging from molecular weight 419 to 1898 base pairs. Overall, the level of inter-population polymorphism, Percent Band Polymorphism (PBP), detected by these ten ISSR primers ranged between 23 to 39 (Table 6.3). Maximum band polymorphism for Mpomphomeni bulb sample was 39 % followed by the sample from Lincoln Meade at 35 %. The other population samples had lower averages of PBP, with the Lions River sample having the lowest at 23 %. These results show that the populations had low genetic polymorphism which would be expected as the populations are all from the same province, KwaZulu-Natal and are in fairly close proximity to each other.

Table 6.3: Percentage Band Polymorphism (PBP) for six populations of *Boophone disticha* used during inter-population diversity analysis.

Primer	Amatikulu	Lincoln Meade	Lions River	Midmar	Mpomphomeni	Umgeni Valley
1	33	33	0	100	33	33
2	22	44	33	22	44	0
3	17	33	33	0	50	33
4	33	33	33	33	66	33
5	33	11	22	33	22	33
6	29	29	0	57	43	29
7	25	38	13	25	25	13
8	50	50	25	0	25	0
9	33	33	33	11	44	22
10	50	50	33	33	33	33
Average PBP	**33**	**35**	**23**	**31**	**39**	**30**

Inter-population analysis revealed that the observed numbers of alleles (na*) and expected number of alleles (ne*) were 1.8095 ± 0.3958 and 1.5932 ± 0.3622 respectively. NEI's (1972) Gene Diversity (*h) which is equivalent to the Average Heterozygosity (H_E*) was 0.3325 ± 0.1814 (Table 6.4), while another genetic diversity parameter which was obtained was Shannon's Information Index (*I) (LEWONTIN, 1972) with a value of (0.4836 ± 0.2525). Heterozygosity is an important parameter in genetics and it can reveal a great deal about the structure and even the history of a population. High heterozygosity means a lot of genetic variability while low heterozygosity means little genetic variability. Several measures of heterozygosity exist and the value of these measures range from zero (no heterozygosity) to nearly 1.0 (for a system with a large number of equally frequent alleles) (NEI, 1972). Therefore, the results, H_E* = 0.3325 ± 0.1814 and *I = 0.4836 ± 0.2525, indicate that the populations contain moderate inter-population diversity amongst themselves, which will enhance their chances to survive under variable climatic conditions.

Table 6.4: Nei's genetic identity above diagonal and Nei's genetic distance below diagonal.

Location	Amatikulu	Lincoln Meade	Lions River	Midmar	Mpomphomeni	Umgeni Valley
Amatikulu	****	0.5714	0.6508	0.4921	0.5397	0.6032
Lincoln Meade	0.5596	****	0.6032	0.6032	0.6825	0.5873
Lions River	0.4296	0.5055	****	0.5873	0.6032	0.5714
Midmar	0.7091	0.5055	0.5322	****	0.6349	0.6984
Mpomphomeni	0.6168	0.3819	0.5055	0.4543	****	0.5873
Umgeni Valley	0.5055	0.5322	0.5596	0.3589	0.5322	****

Nei's genetic distance values (Table 6.4) were used to construct the dendrogram below (Figure 6.3) which shows the genetic distance between the six *B. disticha* populations. The highest Nei's genetic distance between the populations was 0.7091 which was between Amatikulu and Midmar, whereas the lowest was between Midmar and Umgeni Valley (0.3589). These results indicate that the Midmar population is genetically similar to the Umgeni Valley population. Both populations are in conservation areas through which the Umgeni River flows, therefore, seeds from one of the populations could have been carried by the river to the geographical location of the other, germinated and hence established a new population. From the dendrogram, it can be seen that the Midmar and Umgeni Valley populations are closely related, and these populations are closely related to two sister populations, Mpomphomeni and Lincoln Meade. The Amatikulu and Lions River populations are more similar to each other than to the other four populations.

If the localities are looked at, Clades 1 and 2 are to be expected as the four populations (Midmar, Umgeni Valley, Mpomphomeni and Lincoln Meade) are closely situated. Naturally, it would be presumed that the population at Lions River would be clustered together with Midmar, Umgeni Valley and Mpomphomeni populations, while the Lincoln Meade population might be clustered with the Amatikulu population. However, this is not the case. Amatikulu and Lions River might cluster together because they are the most different from the other four populations. Amatikulu is the most isolated population and Lions River is the furthest north and thus possibly exposed to many small populations in the Midlands which are located further north. Midmar Dam might act as a pollinator or seed disperser barrier for Umgeni Valley, Mpomphomeni and Lincoln Meade. The results for Lions River and Amatikulu show that either the sampling is too small and there is

not a lot of genetic variation between populations, or there is transfer of genes between Lions River and Amatikulu (for example, farmers from the Lions River area bring back plants for the garden after their fishing trip on the North Coast).

Figure 6.3: Dendrogram for genetic distance between all the six studied populations after inter-population genetic diversity analysis (based on NEI's 1972).

These moderate genetic differences amongst the populations could also have come about by the populations having to evolve to adapt to the different climatic conditions. The climate in Amatikulu is more of a maritime environment with high temperatures peaking in summer, whereas the climate where the other populations are found is a more temperate environment, where the night temperatures are much colder and the day temperatures are warmer.

6.2 ANTIMICROBIAL ACTIVITY OF *BOOPHONE DISTICHA* BULBS COLLECTED FROM DIFFERENT WILD POPULATIONS

6.2.1 Introduction

Of Africa's many bulbous plants, *B. disticha* is possibly the most widely known for its poisonous and medicinal properties (MANNING *et al.*, 2002). *Boophone disticha* is used to treat a wide range of ailments such as painful joints, swelling, bruises, sores, rashes, burns, abscesses and boils (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS *et al*., 1996; VAN WYK & GERICKE, 2000; DIEDERICHS, 2006). This study looked at the antimicrobial activity of *B. disticha* bulbs that were collected from populations growing in different parts of KwaZulu-Natal in order to establish if genetic variation has an effect on the medicinal properties of *B. disticha*.

6.2.2 Materials and methods

Preparation of wild B. disticha extracts

Bulbs collected from six populations were dried and ground. The powdered bulbs (5.5 g) were extracted by stirring with 60 ml of 100 % EtOH for 48 h after which the solvent was evaporated under reduced pressure.

Antibacterial and antifungal assay

The antimicrobial assays were carried out as described in Section 3.1.2 but with slight modifications. Test samples were re-dissolved in methanol at a concentration of 1 mg/ml and 100 µl of each solution was two-fold serially diluted with sterile distilled water in a 96-well microtitre plate, in triplicate for each bacterial strain and *Candida albicans*. MIC values were recorded as the lowest concentration of sample to affect clear wells. Data collected was subjected to one-way analysis of variance (ANOVA). Where there was significant differences ($p<0.05$), the means were separated using Duncan's Multiple Range Test (DMRT). Data analysis was done using GenStat (version 14, Rothamsted Research, Harpenden, UK).

6.2.3 Results and discussion

The six wild *B. disticha* populations had low genetic polymorphism and moderate inter-population diversity amongst themselves. This was expected as the populations are all found in KwaZulu-Natal, however, there were slight genetic differences. When the samples were evaluated in the antibacterial and antifungal assays in order to test for variation of activity, differences were observed. All the bulb samples (from the different populations) tested, showed good antimicrobial activity as all values recorded were less than 1 mg/ml.

Table 6.5: Antimicrobial minimal inhibitory concentration (MIC) of six *Boophone disticha* bulbs from six locations.

Sample	Antibacterial activity MIC (mg/ml)				Antifungal activity *Candida albicans*	
	Bacteria				MIC (mg/ml)	MFC (mg/ml)
	Bs	***Sa***	***Ec***	***Kp***		
Amatikulu	0.167bc	0.083a	0.125ab	0.125ab	0.250b	>0.250b
Lincoln Meade	>0.250d	0.208cd	0.167bc	0.167bc	0.125a	>0.250b
Lions River	0.250d	0.125ab	0.125ab	0.125ab	0.250b	>0.250b
Midmar	>0.250d	0.167bc	0.208cd	0.167bc	0.125a	>0.250b
Mpomphomeni	0.250d	0.125ab	0.125ab	0.125ab	0.125a	>0.250b
Umgeni Valley	0.125ab	0.125ab	0.125ab	0.063a	0.250b	>0.250b
Neomycin (µg/ml)	$1.6x10^{-3}$	$0.8x10^{-3}$	$0.8x10^{-3}$	$1.6x10^{-3}$	-	-
Amphotericin B (µg/ml)	-	-	-	-	$9.77x10^{-3}$	$7.81x10^{-2}$

Letters within each column indicate significant differences at $p<0.05$.

Bs = Bacillus subtilis, *Sa = Staphylococcus aureus*, *Ec = Escherichia coli* and *Kp = Klebsiella pneumonia*

The bulbs collected from all the populations showed good antibacterial activity against the Gram-negative bacteria. This is a very useful and positive result since Gram-negative bacteria, in general, are more resistant to antibiotics than Gram-positive bacteria possibly due to differences in the cell wall structure (RANG & DALE, 1987; SALYERS & WHITT, 1994). The Umgeni Valley plants are the most active against *K. pneumonia* with an MIC value of 0.063 mg/ml

which is different from the other populations. The Midmar population was the least active against *E. coli* with an MIC value of 0.208 mg/ml while there was no significant difference in activity between the other populations against *E. coli*.

For the Gram-positive bacteria, bulbs collected from Amatikulu and Umgeni Valley were the most active against *B. subtilis* with MIC values of 0.167 and 0.125 mg/ml, respectively. Lincoln Meade, Lions River, Midmar and Mpomphomeni collections had MIC values of 0.250 mg/ml or greater against *B. subtilis*. For *S. aureus*, bulbs collected from Amatikulu were the most active and was significantly different from all the other populations with a MIC value of 0.083 mg/ml.

Boophone disticha extracts were tested for antifungal activity against *C. albicans* using the microdilution assay. Inhibition of *C. albicans* was observed after 48 h, with populations Lincoln Meade, Midmar and Mpomphomeni having the best activity with a MIC value of 0.125 mg/ml. After 48 h, additional MH broth was added to determine whether the activity was fungistatic or fungicidal. After 72 h of incubation, fungal growth resumed showing fungistatic activity and all populations had MFC values of greater than 0.250 mg/ml.

These small differences in activity could be the result of the slight genetic variation found between the populations. These genetic differences may have arisen as the plants occurred in different locations having to evolve to the different climates. For example, the population in Amatikulu will be subjected to hotter temperatures and, therefore, have evolved to cope with heat stress. This particular climatic condition might have either caused a gene(s) to change/mutate, therefore making the Amatikulu population genetically different from the other populations or the environmental influences could cause a specific gene which is found in all the populations to be under or over-expressed. Possibly one of these genes affects the production of the compounds responsible for the medicinal properties of *B. disticha*. This variation in activity could also be the reason why different tribes and cultures in South Africa and Africa use *B. disticha* to treat different ailments.

This work is, however, part of an ongoing study. Plants need to be collected from other locations in South Africa and other factors such as time of collection, environmental conditions and differences in soil type need to be investigated to determine if they have an effect on genetic variation.

6.3 SUMMARY

- The six different *B. disticha* populations had low genetic polymorphism and moderate inter-population diversity amongst themselves
- The Midmar and Umgeni Valley populations are closely related and these populations are closely related to two sister populations, Mpomphomeni and Lincoln Meade
- The Amatikulu and Lions River populations are similar to each other
- Minor differences in antimicrobial activity were observed for the different *B. disticha* populations. These differences in antimicrobial activity could be due to the genetic variations found between the populations

CONCLUSIONS

Micropropagation is a biotechnological tool that is frequently used for the conservation of threatened plant species and to provide stocks for the horticultural industry. By controlling the *in vitro* conditions, optimal conditions for plantlet regeneration and growth can be established, as well as the production of secondary metabolites. Therefore, micropropagation may be used for rapid, year-round, mass multiplication of ornamental geophytes.

Although the micropropagtion of *Boophone disticha* had limited success in this study, three important points were noted. The first two points are that the culture media must be supplemented with both activated charcoal and ascorbic acid in order to prevent browning and to help adsorb phenolics released by the twin-scale explants. Lastly, a cytokinin should be incorporated into the media in order to promote bublet induction. This plant growth regulator seemed to have a greater effect on the induction of bulblets than any of the other physiological and environmental factors tested. Bulblets grown *in vitro* retained their medicinal properties and were shown to be active against different microorganisms. Since *B. disticha* is a medicinally important species that is widely used and threatened, further studies need to be undertaken to successfully develop a micropropagation protocol to aid in the conservation of this plant.

Boophone disticha is used in traditional medicine to treat a wide range of ailments. This study also aimed to validate the medicinal properties of the plant and to determine whether the use of *B. disticha* is safe by investigating its toxicity. Plants collected in different seasons and separated into different plant parts were investigated for antimicrobial activity. The results validated the use of *B. disticha* in traditional medicine but did not confirm that *B. disticha* is neither mutagenic nor carcinogenic. It was also established that time of collection in the year does, to some extent, affect the activity. Results for the different plant parts demonstrated that the roots (all year round) and the leaves (in spring and summer) were likely to be more effective in treating various ailments. This is a very promising result for the conservation of *B. disticha*, as it means that the bulb does not need to be damaged during harvesting, but rather the leaves or roots collected, and the bulb replanted and allowed to continue to grow.

Having established that extracts from *B. disticha* possess antimicrobial activity, it was questioned whether this activity could be attributed to any specific Amaryllidaceae alkaloids. This led to the isolation of two alkaloids, namely, buphanidrine and distichamine. Both the crude plant extract and isolated compounds were tested for antimicrobial activity and it was found that the isolated compounds exhibited broad spectrum activity, which was twice as active as the crude extract. It is, therefore, quite likely that these compounds are responsible for the antimicrobial effect associated with the medicinal uses of the plant.

Lastly, this study examined whether there was any genetic variation between different populations of *B. disticha* from different locations in KwaZulu-Natal. Moderate inter-population diversity amongst the populations was found, which could have come about by the populations having to adapt to slightly different climatic conditions. The bulbs collected from these different populations were also tested to see whether they had similar antimicrobial activities. Results from the antimicrobial tests showed all the bulbs had good activity, with slight differences in activity observed. These minor differences in activity could be as a result of a number of factors such as genetic variation, time of collection, environmental conditions and differences in soil type.

This thesis has answered a handful of questions which were asked about *B. disticha*. Future studies could include quantitative analysis of the alkaloids from different populations, different seasons as well as from *in vitro* grown bulblets. In addition, alkaloids isolated from *B. disticha* could be tested to see whether there are any synergistic effects. Since the seeds of *B. disticha* are recalcitrant, which does not allow for them to be stored, germplasm conservation or other conservations strategies should be further investigated as this is an important process for maintaining genetic diversity. With regards to the genetic study, future research could involve testing for diversity in bulbs collected from populations across South Africa, as well as seeing how genetically similar *B. disticha* is to *B. haemanthoides*. For the cultivation of *B. disticha*, different methods of cultivation should be investigated, as well as the way in which different cultivation methods may affect the medicinal properties. Lastly, the micropropagation of callus and indirect organogenesis should be further investigated. It would also be interesting to test the callus and culture media for secondary metabolites. As is the case most often in science - from each question asked in this study, an ever increasing number arose. As a final comment, therefore, in the words of the late ALBERT EINSTEIN: “Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning”.

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ADDENDUM 1

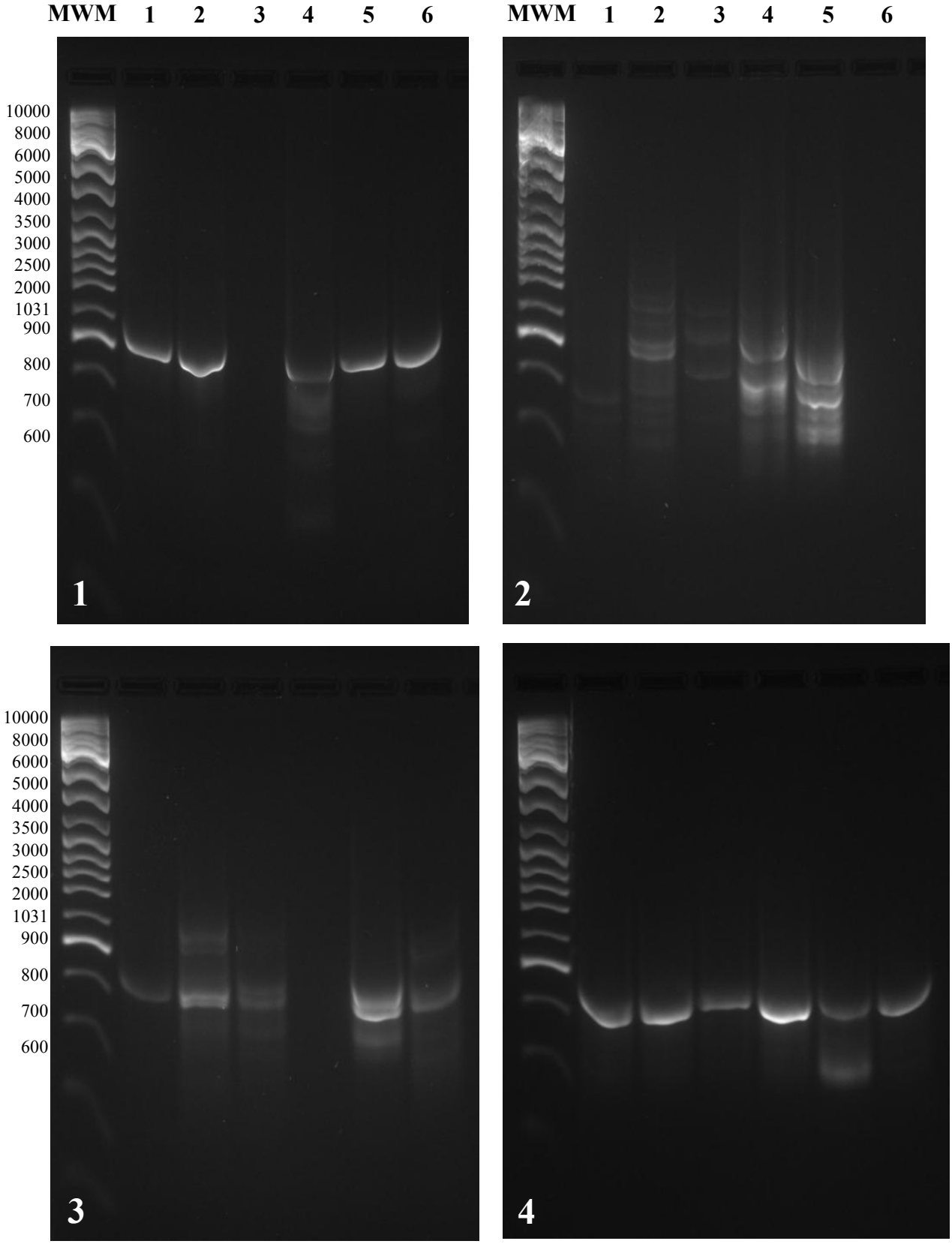

Figure 1: **Agarose gels showing the different ISSR primers used to evaluate the genetic variation of the six populations of *B. disticha.* MWM- molecular weight marker, 1- Amatikulu, 2- Lincoln Meade, 3- Lions River, 4- Midmar, 5- Mpomphomeni and 6- Umgeni Valley. The numbers in the bottom left hand corners represent the ISSR primer number (see Table 6.2).**

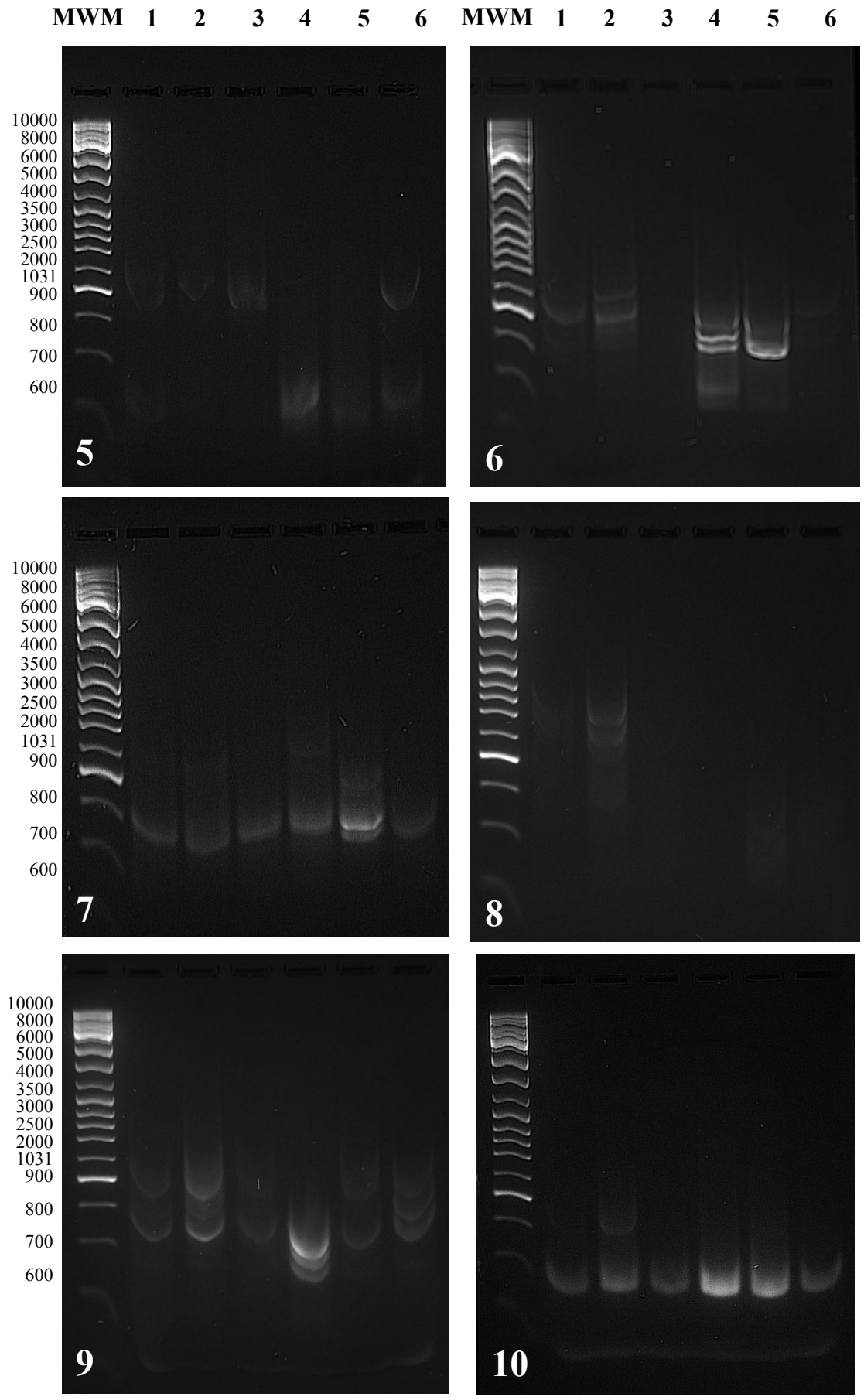

Figure 2: **Agarose gels showing the different ISSR primers used to evaluate the genetic variation of the six populations of *B. disticha.* MWM- molecular weight marker, 1- Amatikulu, 2- Lincoln Meade, 3- Lions River, 4- Midmar, 5- Mpomphomeni and 6- Umgeni Valley. The numbers in the bottom left hand corners represent the ISSR primer number (see Table 6.2).**