

Micropropagation, Molecular and Biochemical Analyses of *Hypoxis* Species

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Cover: Map of Swaziland, insets - *Hypoxis* flower, seed capsule, plantlet and *in vitro* work

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Abstract

Plants are an important source of food, nutrition and medicine. An estimated 80% of the global population relies on herbal medicinal products and supplements. In developing countries plant extracts are the major source of medicine, more especially in rural communities where modern health facilities are not easily accessible. *Hypoxis* species are associated with medicinal properties and have been used for many generations by the indigenous people and traditional healers of southern Africa to treat various ailments. Some contain hypoxoside, a phytochemical that has immune regulatory properties and is used in the composition of medicinal products in the pharmaceutical industry. *Hypoxis* species grow naturally in most warm temperate and tropical regions of the world except Europe, northern and central Asia, north Africa, extra-tropical South America and Canada, and are reported to be mostly common in the southern hemisphere.

The objectives of this work were to develop strategies for the sustainable commercial production and maintenance of the medicinal *Hypoxis* species in the near future, to understand how the different species that grow in Swaziland are related, and to determine the phytochemical compounds of medicinal significance by **i)** developing *in vitro* protocols for their propagation and mass production; **ii)** studying the molecular phylogeny of some *Hypoxis* species that grow in Swaziland and **iii)** analyzing the geophytes for the presence of important biochemical compounds known for their medicinal value.

The investigations were carried out at the Swedish University of Agricultural Sciences (SLU), Department of Plant Breeding in Alnarp, Sweden. All species used in this work originated from Swaziland and were identified at the National Herbarium in Swaziland. The major results include development of *in vitro* regeneration protocols for propagating *Hypoxis* on semi-solid media and in liquid medium using bioreactors for mass production, identification of two taxa with a similar evolutionary relationship, extracts from the corms of all the species analyzed were found to contain phenolic compounds and the norlignan diglucoside, hypoxoside. However, in this study, *H. hemerocallidea* the main species used in the pharmaceutical industry for extracting hypoxoside was found to contain less of it compared to most of the other species analyzed.

Keywords: Bioreactor, chloroplast DNA regions, corm, hypoxoside, medicinal plant, micropropagation, phylogeny, total phenolics.

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Dedication

To my family for their great support....and to myself, for finally making it to the finish line

To God be the glory forever and ever!
Galatians 1:5

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Nsibande Busie, Li Xue-Yuan, Ahlman Annelie and Zhu Li-Hua. (2015). *In vitro* Regeneration of Endangered Medicinal *Hypoxis* Species. *American Journal of Plant Sciences* 6 (16), 2585-95.
- II Nsibande Busie and Zhu Li-Hua (2017). Developing an *in vitro* Propagation Method for Mass Production of Medicinal *Hypoxis* Species Using Bioreactors. *Asian Journal of Science and Research* 6 (3), 52-59.
- III Nsibande Busie, Geleta Mulatu and Zhu Li-Hua. Molecular Phylogeny of Some *Hypoxis* Inferred through Sequencing of Four Chloroplast DNA Regions (manuscript).
- IV Nsibande Busie, Gustavsson Carl-Erik and Zhu Li-Hua. Analysis of Some Phytochemical Compounds in Seven Species of *Hypoxis* (submitted manuscript).

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The contribution of Busie Nsibande to the papers included in this thesis was as follows:

- I Planned the experiment with the main supervisor, performed all the experimental work and data analysis with assistance from co-authors and wrote manuscript with input from the co-authors.
- II Participated in planning the experiment with the main supervisor, performed the experimental work with assistance from co-authors, analysed the data and wrote manuscript with input from the co-author.
- III Planned the experiment with the supervisors, performed the experimental work with assistance from co-authors, analysed the data and wrote manuscript with input from the co-authors.
- IV Participated in planning the work with the main supervisor, performed the experimental work, analysed the data and wrote the manuscript with contributions from the co-authors.

Abbreviations

IAA	Indole-3-acetic acid
BA	Benzyladenine
Ca(ClO) ₂	Calcium chloride
CIM	Callus inducing medium
cpDNA	Chloroplast DNA
DW	Dry weight
HCOOH	Methanoic acid
HgCl ₂	Mercuric chloride
HPLC	High Performance Liquid Chromatography
Kin	Kinetin
LC	Liquid chromatography
MS	Murashige and Skoog
NAA	Naphthaleneacetic acid
PGRs	Plant growth regulators
Sida	Swedish international development agency
SIM	Shoot inducing medium
SLU	Swedish University of Agricultural Sciences
TPC	Total phenolic content

1 Introduction

Plants are an important source of food, nutrition and medicine. An estimated 80% of the global population relies on herbal medicinal products and supplements (Ekor, 2013). In developing countries plant extracts are the major source of medicine, more especially in rural communities where modern health facilities are not easily accessible (Brown *et al.*, 2008). Herbal medicines from plant parts such as leaves, roots or barks are usually extracted by boiling or percolating in water, alcohol or other solvents to release the biologically active constituents (du Plessis-Stoman *et al.*, 2009). In addition to being a source of medicine, nutrients and vitamins, some of the indigenous plant resources are used by the poor in society as a source of income where they are often utilized unsustainably by destructive harvesting of whole plants (Dzerefos & Witkowski, 2001).

Hypoxis species are associated with medicinal properties and have been used for many generations by the indigenous people and traditional healers of southern Africa to treat various ailments (Singh, 1999). The popularity of these species in recent years, most specifically *H. hemerocallidea* commonly known as African potato, in the treatment of HIV and aids sparked great interest from scientists to consumers resulting in pressure being exerted to the populations of these species in the wild due to indiscriminate harvesting (Appleton *et al.*, 2012). This species is counted amongst the top five of African medicinal species (Figure 1). *Hypoxis* species grow naturally in most warm temperate and tropical regions of the world except Europe, northern and central Asia, north Africa, extra-tropical South America and Canada (Singh, 1999), and are reported to be mostly common in the southern hemisphere (Kocyan *et al.*, 2011). Some of the species, such as *H. hemerocallidea*, *H. stellipilis*, *H. sobolifera* var. *sobolifera* (Boukes *et al.*, 2008) and *H. obtusa* (Msonthi *et al.*, 1990; Vinesi *et al.*, 1990), to name a few, have been scientifically proven to

contain hypoxoside, a phytochemical that has immune regulatory properties and whose extracts are now widely used in the pharmaceutical industry. Unfortunately *Hypoxis* species like *H. hemerocallidea* are difficult to propagate mainly because vegetative multiplication is uncommon (Appleton *et al.*, 2012) and the seeds have a deep dormancy (Hammerton & Vanstaden, 1988), thus leaving micropropagation the most feasible alternative that could be used for cultivating the species. This situation clearly puts the survival of these species at risk as most often the rhizomes or corms are harvested for medicinal use without replacement or plans for sustainability and continued survival of the species. This is further exacerbated by the fact that harvesting of the plants from the wild is not only limited to those that are medicinal, but to other species of the genus that bear close resemblance to the medicinal ones whose medicinal properties have not been authenticated. However, *H. hemerocallidea*, is characterized by a unique three-way growth pattern (Bihrmann.com, 2005) that can be used to distinguish this species from others. Other species do have a similar growth pattern, but it is not as distinct as it is in *H. hemerocallidea*.

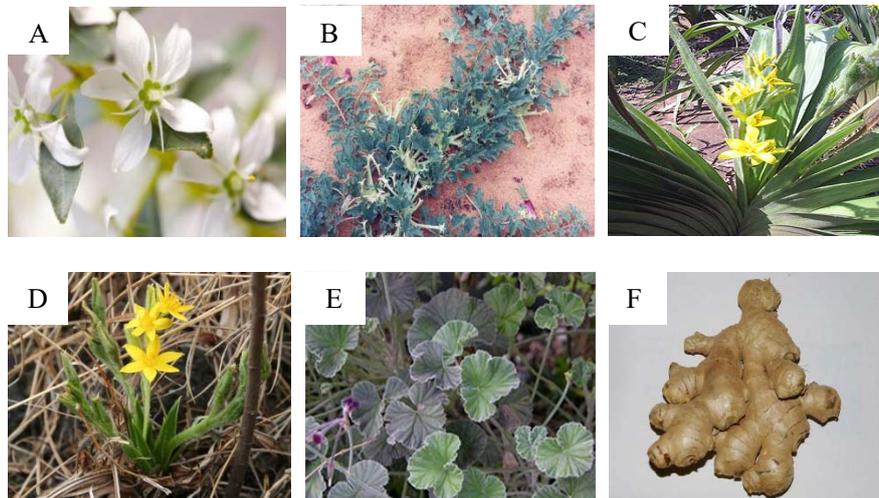


Figure 1. Five top African medicinal plants: A – Buchu; B – Devil's claw; C and D – African potato; E – South African geranium; F – African ginger.

Photos taken from:

<http://www.arabella.co.za/2013/08/27/5-top-performing-african-medicinal-herbs>

2 Background

2.1 Botanical Information on *Hypoxis*

Hypoxis is a genus belonging to the Hypoxidaceae family (Kocyan *et al.*, 2011; Sathekge *et al.*, 2010; Singh, 2007; Ndong *et al.*, 2006; Singh, 2004; Vinesi *et al.*, 1990) and comprises of perennial grasses (Figure 2) that are common in sub-Saharan Africa, America, south-east Asia, and Australia (Singh, 2004; Appleton & van Staden, 1995b; Nordal *et al.*, 1985). This monocotyledonous family also known as Asparagales, has nine genera and is mostly found in the Southern Hemisphere (Kocyan *et al.*, 2011) or the Torrid Zone (Cheng *et al.*, 2009). The genus *Hypoxis* is reported to have its center of variation in South Africa (Wiland-Szymańska, 2009; Singh, 2007; Nordal *et al.*, 1985) where it occurs in open undisturbed grasslands. It is characterized under the Hypoxidaceae family based on the presence of hairy flowers with six stamens, trilocular ovaries without a beak, and free perianth segments (Nordal *et al.*, 1985). The name “Hypoxis” was invented by Linnaeus in 1759 from the Greek words *hypo* meaning below and *xis*, sharp, referring to the fruit which is pointed at the base (Drewes & Khan, 2004; Singh, 1999).

Plants of the Hypoxidaceae family are herbaceous geophytes that often have a tuberous rhizome or corm (Figure 3C) where nutrients are stored. The type that forms rhizomes is mostly found in the tropics and sub-tropics, whereas the one with corms is common in the southern temperate regions of Africa. Growth of the tubers or rhizomes occur when new tissue is added gradually by the apical meristem; this underground trunk referred to as the “vertical stock” is the primitive type that is also found in *Hypoxis*. The inflorescences are presumed to be indeterminate with the scapes being produced continuously

from the axils of the successively produced leaves. Flower development can either be acropetalous or basipetalous, and the narrowing of the rachis from the base towards the apex is usually stepwise with cluster of flowers produced at each node. In *Hypoxis*, the basic type of the inflorescence may be transitional between a rhipidium and a bostryx; and often one to two bracts per flower are present (Nordal, 1998). According to Baker (1878), the Hypoxidaceae family is formed by a group of plants with four genera that has between 60 and 70 species, which differ from Amaryllidaceae by their tuberous rootstocks; persistent leaves of grass-like or coriaceous, never fleshy texture; more persistent or firmer perianth-segments of which three outer are generally green and hairy on the outside; the general tendency of their leaves, scapes, and other parts to become clothed with hairs; and by the thick crustaceous testa of the seeds. However, Nordal (1998) stated that the family has nine genera and about 100 species as the genus *Hypoxis* is to a large extent apomictic and thus delimitation of species was a problem. Hypoxidaceae are herbaceous plants and plant height can range between 20 cm and 1 metre, or more. Flowers are less than 2 cm in diameter with colours ranging from yellow to white or pink, or orange in some species (Kocyan *et al.*, 2011). Baker (1878) demarcated the species by predominantly using leaf characters and inflorescence type (Singh, 2009). Flowers of most *Hypoxis* species are bright yellow (Figure 3A), hence the common name “yellow stars”. The corms are hard, fleshy and mucilaginous with white or yellow-orange flesh that turns black when exposed to the atmosphere as a result of oxidation (Singh, 2004).

Reports by most recent authors are that this family has 55 genera and an estimated 155 species, of which *Hypoxis* is the largest (Kocyan *et al.*, 2011; Singh, 2007). Singh (2009) reported that about 50 *Hypoxis* species occur in sub Saharan Africa, while Singh (2004) previously reported that 41 occur in southern Africa, mostly in the eastern region, and only 30 are reportedly in the Flora of Southern Africa (FSA) region that includes Botswana, Lesotho, Namibia, South Africa and Swaziland. Out of the 38 species described by Baker (1878) to be occurring in Africa, according to a revision made by Singh (2009), 28 are recognized of which 22 are indigenous to this region.

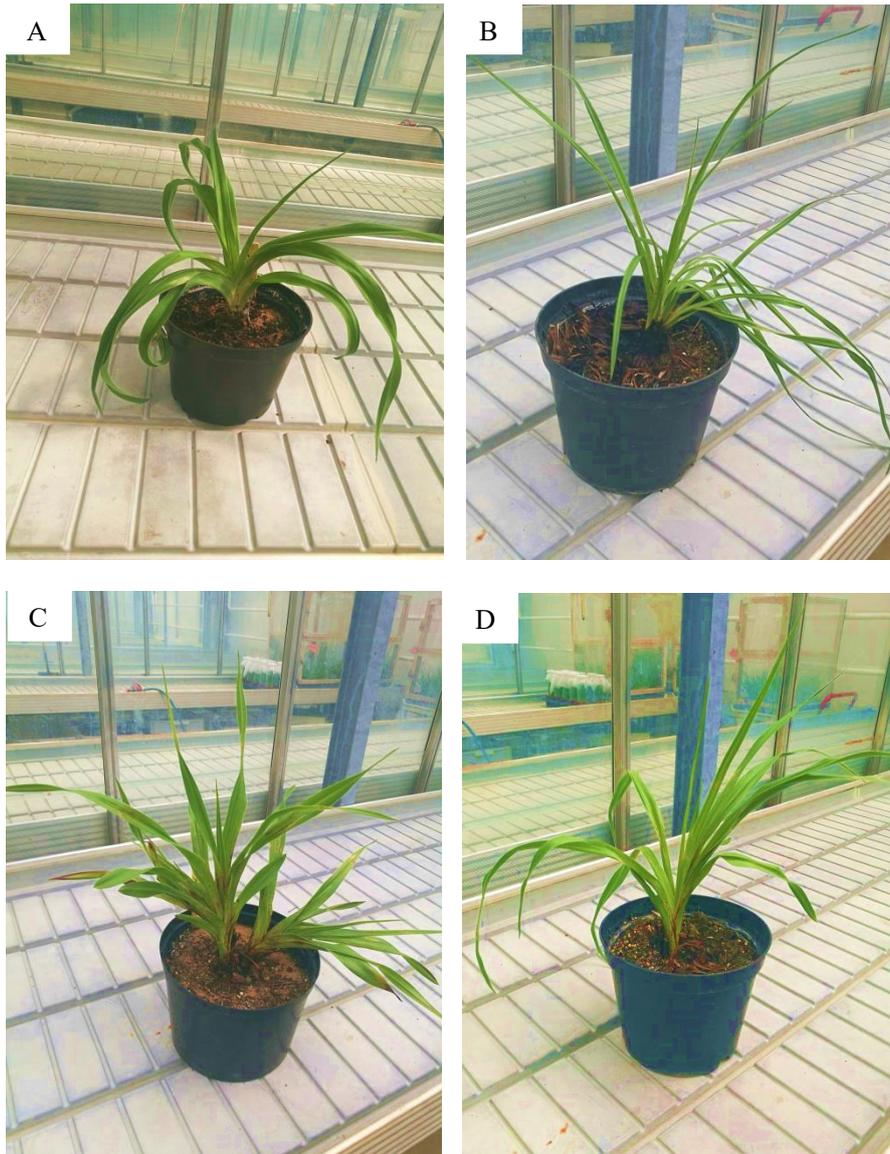


Figure 2: Images of four *Hypoxis* species grown in the greenhouse at the Swedish University of Agricultural Sciences, Alnarp. **A** – *H. hemerocallidea*; **B** – *H. filiformis*; **C** – *H. iridifolia*; **D** – *H. argentea*

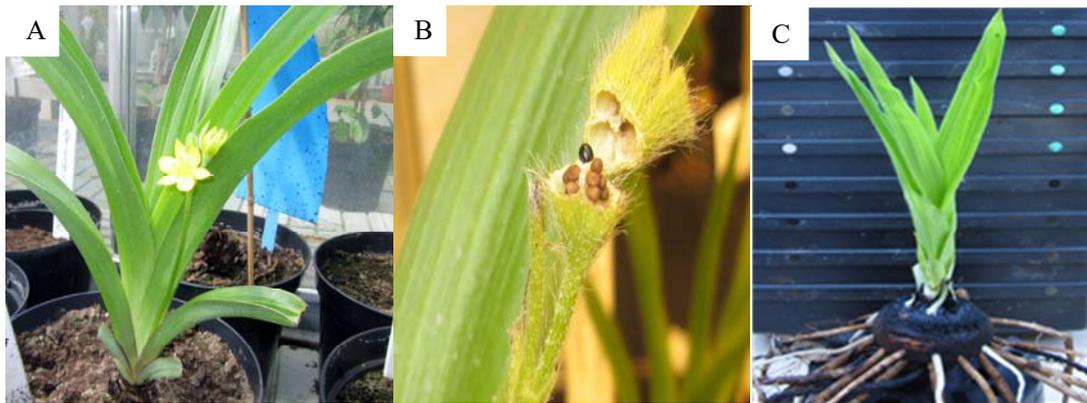


Figure 3: Images of different plant parts of *Hypoxis*. **A** – Potted flowering plant of *H. hemerocallidea* in greenhouse; **B** – Seed capsule with black shiny seed; **C** – Rhizome with shoot and roots.

2.2 Agronomic Information

The genus *Hypoxis*, which mainly contains phenolic glycosides (Cheng *et al.*, 2009) seemingly thrive on poor soils with minimum nutrition. A study carried out by Mcalister and Vanstaden (1995) on the effect of artificially induced stress conditions on the growth of *H. hemerocallidea* showed that the control plants that had no nutrients added grew better than the plants that had nutrients added, and moreover, yielded the highest hypoxoside content, a natural compound reported by Bayley and Vanstaden (1990a) as having potential medicinal value. The study also showed that plants grown in clay/shale soil survived better than when grown on sandy or grey prismic columnar soils, and yielded the highest hypoxoside content. However, in the initial growth stages, high levels of nitrogen, phosphorus, and potassium are required for production of a good biomass (Mcalister & Vanstaden, 1995). Birmann (2005) reported that in southern Africa *H. hemerocallidea* is found growing in sandy or any other well-drained soil.

2.3 Medicinal uses

The importance of the genus is not only based on its use as traditional medicine in some African countries, but it has also become important in pharmaceutical preparations (Betto *et al.*, 1992). In southern Africa, eleven species are

reportedly used for medicinal purposes (Appleton *et al.*, 2012), whilst *H. hemerocallidea* and *H. colchifolia* are reported to be the most popular (Appleton *et al.*, 2012; Singh, 2004). Species of *Hypoxis* are famous for their use in traditional medicine by the native people of South Africa as anti-inflammatory and anti-tumor drugs (Nicoletti *et al.*, 1992), however, *H. hemerocallidea*, whose synonym is *H. rooperii* T. Moore (Drewes & Khan, 2004), is the only species that has been extensively studied, whilst very little has been done on the medicinal value of other species (Boukes & van de Venter, 2011). *H. hemerocallidea*, or African potato as it is popularly known in southern Africa, is one of the most widely used plants in traditional medicine as an immune booster and source of nutrition (Nair & Kanfer, 2006), and in the treatment of various ailments such as urinary diseases, prostate hypertrophy, and cancer (Nair *et al.*, 2007), urinary infections, heart weakness, internal tumours, and nervous breakdown (Singh, 2004). Studies on the medicinal properties of *H. hemerocallidea* date back to 1982 when unknowingly the corms were simultaneously studied for the first time in two continents by scientists in Italy and South Africa (Drewes & Khan, 2004). Extracts of *H. hemerocallidea*, the phytosterols including their main constituents, hypoxoside and its active derivative rooperol, are now being used in fields of anti-oxidants, anti-inflammatories, anti-diabetes, anti-convulsants, inhibitors of drug marker substances, and anti-cancerous and premalignant cancer cells (Drewes *et al.*, 2008). Despite the use of *H. hemerocallidea* extracts by traditional healers to treat HIV/AIDS, controversy exists on its effect, but its future use cannot be overlooked. Furthermore, the pharmacological properties of rooperol in studies conducted by several scientists have demonstrated its potency towards cancer, inflammation, and HIV (Drewes & Khan, 2004). Drewes (2008), described the booming trade of the sale of *Hypoxis* corm extracts, together with other medicinal plants, by the pharmaceutical industry in South Africa as the “enormous expansion of over-the-counter trade”. Market brands such as Harzol and Moducare containing *Hypoxis* phytosterols are freely sold, with the former gaining popularity in Germany around the late eighties and early nineties as a treatment for benign prostate hypertrophy (Drewes *et al.*, 2008; Laporta *et al.*, 2007).

2.4 Phytochemical Compounds

Drewes and Khan (2004) reported that corms of *H. hemerocallidea* that were collected and extracted from the wet tissues showed that among other compounds separated, 7.3% were phenolic. Hypoxoside, or (E)-1,5-bis(3'-

hydroxy-4'-O- β -D-glucopyranosylphenyl)pent-1-en-4-yne (Kruger *et al.*, 1994), is a norlignan diglucoside that is found in the corms of *Hypoxis hemerocallidea* (Nair & Kanfer, 2006). Drewes *et al.* (1984) reported the presence of this compound in *H. rooperi*, *H. acuminata*, *H. nitida*, *H. obtusa*, *H. rigidula*, and *H. latifolia*. The glucoside hypoxoside was first isolated and characterized from *H. obtusa* by Marini-Betolo *et al.* in 1982 four years before the findings by Drewes *et al.* (1984), and Vinesi *et al.* (1990). Hypoxoside and the sterol, beta-sitosterol, are believed to be the most important phytochemicals derived from *H. hemerocallidea* that are in the market (Nair & Kanfer, 2008). The corm appears to be the main source of the compound hypoxoside; Bayley and Vanstaden (1990b) found that extracts of leaves, corms, and roots of nine-month old *H. hemerocallidea* showed that the corm contained the highest concentration of the compound, followed by the roots whilst the leaves had insignificant levels. Page and Vanstaden (1987) found that only the root type cultures contained hypoxoside when they compared undifferentiated callus, malformed roots from flower buds, and malformed root tissue from corm explants of *H. rooperi*. Katerere and Eloff (2008) discovered that there was a distinct difference in the chemical composition of leaves and corms, with leaf extracts showing a more complex composition compared to corm extracts. Corm extracts were found to contain phytosterols that were not present in leaf extracts, leading to the conclusion that leaves should, ideally, not be substituted for corms.

Species of *Hypoxis* may not contain the same chemical compounds, a study conducted by Sathekge (2008) on *H. argentea*, *H. hemerocallidea*, *H. interjecta*, *H. iridifolia*, and *H. rigidula* showed that there were differences in the chemical composition of the five species when analyzed using high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). Other compounds other than hypoxoside are also found; Nicoletti *et al.* (1992) reported isolating a rare form of glycosides from the rhizomes of some African species that were characterized by the Ph-C5-Ph skeleton, which were also found to occur in other Hypoxidaceae species. A geraniol glycoside, acuminoside was isolated from *H. acuminata* (Bredenkamp *et al.*, 1989), whereas in *H. filiformis*, ellagic acid, a natural phenol antioxidant compound with a rigid structure and poor solubility was reported to be present (Yang *et al.*, 1998). This latter claim was refuted as being erroneous by Hillis (2014) who purported that no member of the monocotyledons contains ellagic acid.

The phenolic glucoside, hypoxoside, found in *Hypoxis* species cannot be easily produced synthetically making these plant species an important source of this phytochemical (Page & Vanstaden, 1987). Efforts were made by

Drewes and Khan (2004) to prepare analogues of rooperol because they believed that isolation of the hypoxoside on a large scale from natural sources would most likely not be viable. The intention was to find a compound that was much simpler but with the same or better activity as rooperol, but this was unsuccessful. However, they were able to isolate the major constituents from the corms of *H. hemerocallidea*, hypoxoside (Figure 4 (1)), a pentenyne derivative, and rooperol (Figure 4(2)), the active compound that is derived through hydrolysis of hypoxoside by the enzyme β -glucosidase (Drewes *et al.*, 2008; Drewes & Khan, 2004).

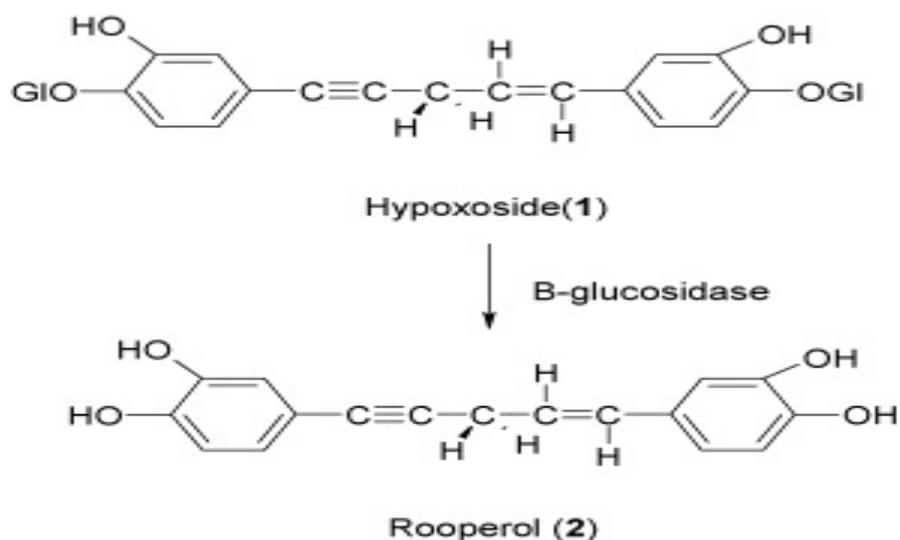


Figure 4: Chemical structure of hypoxoside (1) and rooperol (2)

2.5 *In vitro* Studies

Hypoxis species are some of the most important wild medicinal plants of southern Africa. Harvesting them from the wild is proving to be unsustainable and exposing them to the risk of extinction, alternative means of ensuring their availability have to be developed. In the case of *Hypoxis*, a genus that is not easily propagated by seeds, *in vitro* methods offer the much needed solution. Numerous studies on this subject have been done, more specifically on *H.*

hemerocallidea, and have shown the potential that in vitro regeneration techniques have on the cultivation of this genus for commercial use, as well as for conservation.

Most *in vitro* studies of plants involve the use of artificial medium such as Murashige and Skoog (MS) (Murashige & Skoog, 1962) and plant growth regulators (PGRs) on an agar base. Vinesi *et al.* (1990) tested three species of *Hypoxis in vitro*, *H. angustifolia*, *H. nyasica*, and *H. obtusa*. In their study only corm explants of *H. obtusa* gave rise to shoots when cultured on the MS medium without PGRs and the MS supplemented with 1 mg BAP L⁻¹, however, in another by study Appleton and van Staden (1995a) shoot regeneration was obtained from the corm explants of *H. angustifolia* cultured on the MS PGR-free basal medium, and on the MS containing either 0.5 mg BA L⁻¹ or 2 mg BA L⁻¹. Appleton and van Staden (1995b) obtained the greatest growth response from corm explants of *H. hemerocallidea* on the initiation MS medium supplemented with 1 mg NAA L⁻¹ and 1 mg BAP L⁻¹. Generally, the corms are the most in vitro responsive part of the genus, however, Appleton *et al.* (2012) managed to obtain regeneration and multiple shoots from explants of unopened flower buds of *H. colchicifolia* cultured on various MS-based initiation medium. Ndong *et al.* (2006) obtained a high regeneration frequency vial callus on corm explants of *H. hemerocallidea* when the explants were first cultured on callus induction medium comprising 3 mg L⁻¹ kinetin with 1 mg L⁻¹ NAA for several weeks before being transferred onto shoot inducing medium with 1.5 mg L⁻¹ BA and 0.5 mg L⁻¹ NAA. They were able to obtain 100% regeneration frequency with 30 to 35 shoots per explant. Most recently Nsibandé *et al.* (2015) reported achieving 100% regeneration in corm explants of *H. filiformis* cultured with a portion of shoot on MS supplemented with 3 mg L⁻¹ kinetin; and 29% germination of seeds of *H. argentea* when cultured with the seed coat crushed on the half-strength MS.

2.6 Molecular Studies

Despite the importance of *Hypoxis* species as medicinal plants in southern Africa, and their wide distribution in the Southern Hemisphere, very few studies on genetic diversity and phylogeny of the Hypoxidaceae family have been conducted. This family belongs to the order Asparagales together with twelve others: Asteliaceae, Blandfordiaceae, Boryaceae, Doryanthaceae, Iridaceae, Ixioliriaceae, Lanariaceae, Orchidaceae, Tecophilaeaceae, Xanthorrhoeaceae s.l., and Xeronemataceae, which form the lower asparagoid grade of this order. A phylogenetic analysis conducted in the plastid DNA

region identified three major clades: the *Curculigo* s.l. clade that includes *Curculigo*, *Hypoxidia*, and *Molineria* species; the *Hypoxis* clade, which includes *Hypoxis* s.s and *Rhodohypoxis*; and the *Pauridia-Empodium* clade that comprises *Empodium*, *Hypoxis* p.p., *Pauridia*, *Saniella* and *Spiloxene*. The *Hypoxis* clade is reported to be the most morphologically uniform group of Hypoxidaceae with the exception of the southern African *Rhodohypoxis* which differs in that it has sharply incurved claws at the base of the inner tepals that close the perianth throat and conceal the stamens and style (Kocyan *et al.*, 2011).

The spread of the Hypoxidaceae members from continent to continent can only be speculated, for instance it is believed that there was a long distance dispersal of *Hypoxis glabella* and *Hypoxis occidentalis* between southern Africa and southern Australia; and that *Hypoxis* sect. *Ianthe* may have entered Australia through the vicariance process, which occurred about 100 million years ago (Kocyan *et al.*, 2011).

In phylogenetic studies chloroplast markers seem to be the most common molecular tool used since they target the coding region of the chloroplast genome (Hamilton, 1999). Chloroplast DNA sequence variations are widely used to investigate interspecific relationships between angiosperms and other plants (Taberlet *et al.*, 1991). They are the main source of data for inferring plant phylogenies and may only be superseded by nuclear ribosomal ITS sequences. Two regions, the *trnL-trnF* and *trnK/matK* have been able to provide sufficient information in resolving issues related to species relationships in some taxa but fall short in resolving issues at low taxonomic levels (Shaw *et al.*, 2005). The low evolutionary rate of the chloroplast DNA molecule has been cited as the main limitation at the intraspecific level. Taberlet *et al* (1991) designed six universal primers for the amplification of non-coding regions of chloroplast DNA using the polymerase chain reaction (PCR) that were able to amplify a wide range of species including algae, bryophytes, pteridophytes, gymnosperms and angiosperms.

3 Aim and Objectives

The major aim of this work was to develop strategies for the sustainable production and maintenance of the medicinal *Hypoxis* species; and meanwhile understand how different species collected in Swaziland are related with each other at molecular phylogenetic level, and to evaluate if these species have any differences in amount of bioactive compounds of medicinal value.

The specific objectives of the thesis were to:

- Develop protocols for *in vitro* establishment and regeneration of *Hypoxis* using semi-solid medium.
- Develop *in vitro* based protocols for mass production of *Hypoxis* that could be used for commercial production.
- Evaluate the relationship of some of the *Hypoxis* species found in Swaziland by studying the similarities and dissimilarities within four chloroplast DNA regions.
- Determine the levels of some important biochemical compounds with medicinal implications.

4 Materials and Methods

Species used in this thesis originated from Swaziland and were identified at the National Herbarium in Swaziland. Whole plants were collected from the four different geographical regions of Swaziland, which are Hhohho, Lubombo, Manzini and Shiselweni (Figure 5), and identified before the corms were planted in the greenhouse at the Swedish University of Agricultural Sciences (SLU). All the thesis work was carried out at SLU, Department of Plant Breeding in Alnarp, Sweden.

4.1 Micropropagation

Development of *in vitro* propagation protocols of *Hypoxis* in this thesis was carried out in two parts. The first part involved micropropagation of four species of *Hypoxis* on semi-solid medium to determine the suitable types of explants for regenerating *Hypoxis*. The second part dealt with developing *in vitro* propagating methods that could be used for mass production of *Hypoxis* using bioreactors.

4.1.1 Micropropagation on semi-solid medium

The four *Hypoxis* species used for this investigation were: *H. acuminata* Baker, *H. argentea* Harv. ex Baker, *H. filiformis* Baker and *H. hemerocallidea* Fisch., C.A. Mey. & Ave`-Lall. Plant materials were taken from plants grown in the greenhouse. Several plant parts including leaves, flower buds, roots, corms and seeds, were tested as explants on agar-based Murashige and Skoog (MS) (Murashige & Skoog, 1962) medium either with or without plant growth regulators (PGRs) of different types and concentrations. Corms were cultured with or without a portion of the shoot attached. Seeds were obtained from three of the four species: *H. argentea*, *H. filiformis* and *H. hemerocallidea*; and were cultured on half-strength MS without PGRs either intact or with the seed coats

crushed. All the cultures were maintained in a growth chamber with the following conditions: temperature of 25/18°C (day/night), 16 h photoperiod and light intensity of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent tubes.

For initial *in vitro* establishment explants were surface sterilized with 6% calcium hypochlorite ($\text{Ca}(\text{ClO}_2)$) solution before culturing on the media. The basal MS medium consisted of 30.0 g/l sucrose, 8.0 g/l Bacto agar, and 1.0% activated charcoal. This basal medium was then supplemented with the different types and concentrations of auxins or cytokinins, separately or in combination, and the pH was adjusted to 5.8. The explants were either maintained on the same type of medium for the whole duration of the culture period or transferred to medium with a different PGR combination.

4.1.2 Micropropagation using bioreactors

In this series of experiments cormlets of *H. argentea* and *H. filiformis* and shoots of *H. filiformis* that had been regenerated *in vitro* from the semi-solid medium were used as explants: The explants were cultured in the liquid basal MS medium consisting of 30.0 g/l sucrose and 1.0 g/l calcium hydrolysate with pH 5.8. The basal medium was supplemented with different types and concentrations of PGRs and the explants were kept in the same type of medium for the whole culture duration.

The PlantForm® bioreactors using the temporary immersion principle (Persson, 2011), were used as the vessel for culturing the explants. Explants were immersed in the media twice a day for the duration of 6 min per immersion, and 8 h between each immersion. Aeration was provided every hour during the day for the duration of 4 min for 8 h. All cultures were grown under the same climate conditions as described for the semi-solid medium above.

Regenerated shoots were acclimatized well *in vitro* in the climate chamber before they were planted in soil in the greenhouse.

4.2 Phylogenetic Analysis

Eight known *Hypoxis* species: *H. acuminata* Baker, *H. argentea* Harv. ex Baker, *H. filiformis* Baker, *H. gerrardii* Baker, *H. hemerocallidea* Fisch., C.A. Mey. & Ave'-Lall, *H. iridifolia* Baker (*H. nitida*), *H. parvifolia* Baker (*H. parviflora*), *H. rigidula* Baker and 17 unclassified species were used in this study. Analyses were carried out using molecular markers on four chloroplast DNA regions (cpDNA): the *rbcL* gene, the *matK* gene, the *trnL-trnF* intergenic spacer and the *trnS-trnG* intergenic spacer. Genomic DNA was extracted from

fresh leaf tissue for the known species, which were grown in the greenhouse at SLU. For the unclassified species, the DNA was extracted from dried leaf samples taken from plants collected directly from the wild and dried with silica gel. The target regions were amplified by PCR using different sets of primers. Primers 1F, 724R and 1460R were used to amplify part of the *rbcL* gene, primers MatK 6 and MatK 6'R to amplify a portion of the *matK* gene, primers c-B49327, d-A49855, and f-A50272 to amplify a fragment of the *trnL-trnF* intergenic spacer and primers S and G to amplify a section of the *trnS-trnG* intergenic spacer. The same primers were used to sequence the amplified DNA, which was done at Eurofins Genomics in Germany. Prior to sequencing, the PCR products were purified using the QIAquick® PCR Purification kit.

Sequences were aligned using ClustalX 2.0.11, edited manually in BioEdit Sequence Alignment Editor and analyzed for evolutionary distance and maximum parsimony using MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar *et al.*, 2016).

4.3 Analyses of Biochemical Compounds

The biochemical compounds ellagic acid, total phenolic content and hypoxoside were analyzed from corm extracts of seven *Hypoxis* species: *H. acuminata* Baker, *H. argentea* Harv. ex Baker, *H. filiformis* Baker, *H. gerrardii* Baker, *H. hemerocallidea* Fisch., C.A. Mey. & Ave'-Lall, *H. iridifolia* Baker (*H. nitida*), *H. parvifolia* Baker (*H. parviflora*). The corms were harvested, immediately freeze-dried and pulverized into a fine powder. All assays used the same raw extract derived from mixing 1 g sample powder with 25 ml 70% acetone and extracted overnight with gentle shaking.

4.3.1 Ellagic acid

To extract ellagic acid, the raw extracts from the samples were first centrifuged at 12 500 rpm for 12 min, mixed with 4 M HCl and heated at 95⁰ C for 4 h. The acetone content in samples was then adjusted to 8% using 0.1% HCl. Extraction was done by loading samples onto SPE-Pak columns and eluting with methanol before analyzing eluates by HPLC.

4.3.2 Total Phenolic Content

For determining the total phenolic content in the samples, a modified version of the Folin-Ciocalteu's colometric method (Dewanto *et al.*, 2002) was used. The raw sample extracts were centrifuged at 12 500 rpm for 12 min and the supernatant removed and diluted 50x with 70% acetone before being analyzed. Chlorogenic acid was used as the external standard for calculating the phenolic

content. Total phenolic content (TPC) was expressed as chlorogenic acid equivalents in $\mu\text{g/g}$ of sample dry weight ($\mu\text{g/g DW}$).

4.3.3 Hypoxoside

The raw extracts were first centrifuged at 10 000 rpm for 10 min before running samples in HPLC. The hypoxoside content was expressed as the relative percentage of the compound obtained from the dry matter extracts of corms within the different species.

Epicatechin and hypoxoside extracted from the corms of *H. filiformis* were used as the standards, separately. Hypoxoside was extracted using the HPLC method described in Paper IV with an injection of 100 μl and a flow rate of 5 $\mu\text{l}/\text{min}$. The eluate was collected from the highest point of the peak and dried by vacuum and lyophilization and resulting pellet dissolved in 5 ml methanol followed by centrifugation at 12000 rpm for 10 min. The supernatant was used directly in the HPLC as the standard.

The hypoxoside content was expressed as the relative amount (%) of the compound obtained from extracts of the different species based on the area of the hypoxoside molecules occupied by each in the chromatograph.

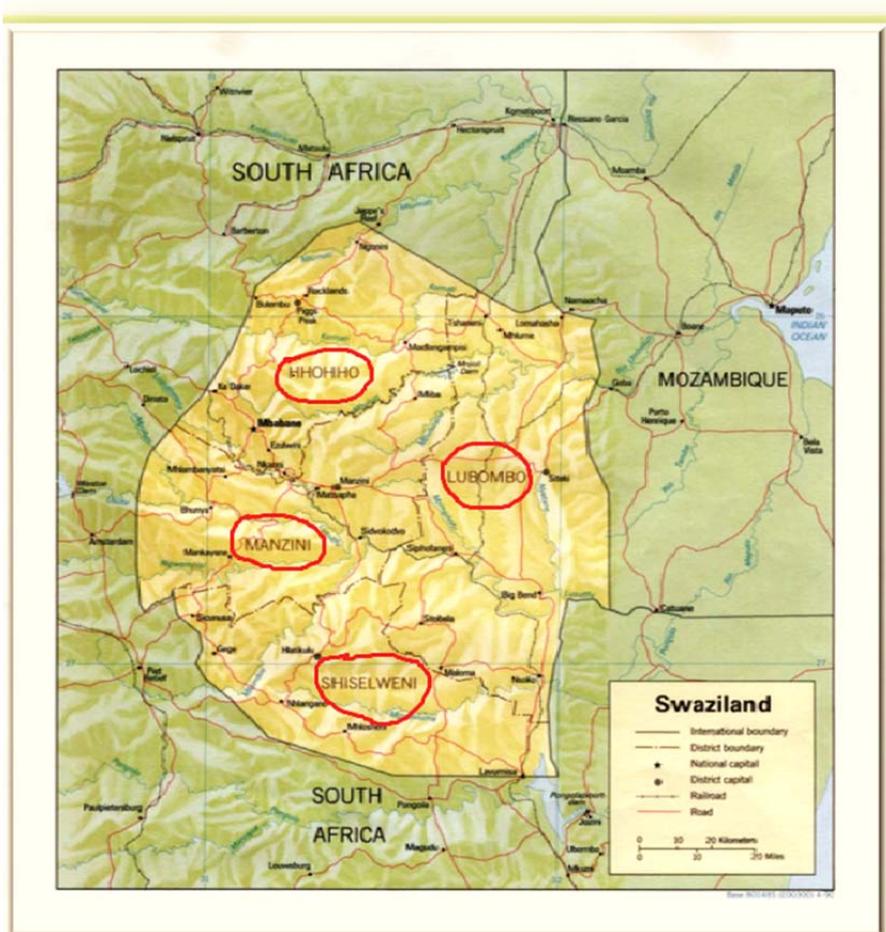


Figure 5. Map of the Kingdom of Swaziland showing the four main regions marked in red where *Hypoxis* species were collected.

5 Summary of Results and Discussion

5.1 Micropropagation of *Hypoxis*

Protocols were established for *in vitro* regeneration and mass production on both semi-solid and liquid media using bioreactors, respectively. In both methods regenerated shoots were successfully acclimatized and planted in soil in the greenhouse. Some of the plants planted in the greenhouse were later transferred to the field genebank at the Malkerns Research Station, Swaziland, where establishment depended on the size of the corms at planting. Plants that had larger corms and had been in the greenhouse for nine months, after *in vitro* survived better than those with smaller-sized and had only been in the greenhouse for three and a half months.

Insufficient plant material was a major limitation for extensive studies, especially when it came to corms as it meant destroying the plants in order to harvest them. To try to mitigate this problem and save the plants, the corms were cut off only partially just below the base of the plants, while the rest of the corms and roots remained with the plants which were replanted in soil. This method worked very well as the plants continued to survive in the greenhouse and new corms were produced. This exercise proved that *Hypoxis* are resilient and can survive a lot of physical and physiological stresses. The method could also be tested in the field to see if it can be used to save the plants from the wild in the future when harvesting corms. To generate more *in vitro* materials, cormlets from regenerated shoots were cut into halves or quarters and used as explants for producing more shoots, and subsequently cormlets. After removing the shoots by cutting them off just above the point where they are attached to the cormlets, the cormlets were then cut vertically from the apex ensuring that there was a portion of shoot still attached. This also proved to work very well.

Difficulty was encountered with successful seed culture and regeneration as the seeds showed signs of deep dormancy. Several methods for breaking dormancy were tried without much success, these included chilling cultures at 5°C for 7 days before subjecting to normal culture conditions, soaking in concentrated sulphuric acid (95-97%) or diluted (50%) for 30 min prior to culture, soaking in 2% gibberellic acid for 16 h prior to culturing, or soaking in hot water with an initial temperature of 80°C for 45 min. All these methods failed to induce germination. However, when the seed coat was physically crushed with forceps germination occurred within two weeks.

5.1.1 Micropropagation on semi-solid medium (Paper I)

Out of the five types of explants tested for regeneration on semi-solid medium, only corms and seeds were regenerated. For the corms, the highest regeneration frequency of 100% was achieved in *H. filiformis* when cultured with a portion of shoot attached on the medium MS supplemented with 3 mg/l kinetin. The highest shoot regeneration rate (17 shoots/explant) was also obtained from the same species and on the same medium, but with the corms cultured without a portion of the shoot attached. *H. argentea* had the second highest shoot regeneration frequency of 70%, whilst both *H. acuminata* and *H. hemerocallidea* had only 20%. Seeds were only regenerated when the seed coats were crushed prior to culturing. The seed germination rate of 29%, 13.3%, and 12% were obtained from *H. argentea*, *H. hemerocallidea* and *H. filiformis*, respectively.

5.1.2 Micropropagation using bioreactors (Paper II)

H. filiformis had the highest regeneration frequency from the corm cultures, 85% of the cormlets regenerated in the PGR free MS medium and obtained the highest regeneration rate of 12 shoots/explant, while 75% regeneration was obtained from the shoot cultures in the MS medium supplemented with 1.1 mg/l TDZ. In *H. argentea*, the highest regeneration frequency (72%) was achieved from corm explants cultured in the PGR free MS medium, however, the highest shoot multiplication rate of 5 shoots/explant was achieved from the MS medium supplemented with 3 mg/l kinetin.

In both propagation methods, *H. filiformis* was the most responsive species *in vitro*. It regenerated faster and better compared to the other species used, and in both methods it formed a lot of friable calli that in most instances gave rise to shoots. Calli produced by *H. argentea* from the semi-solid medium was usually hard and often produced shoots.

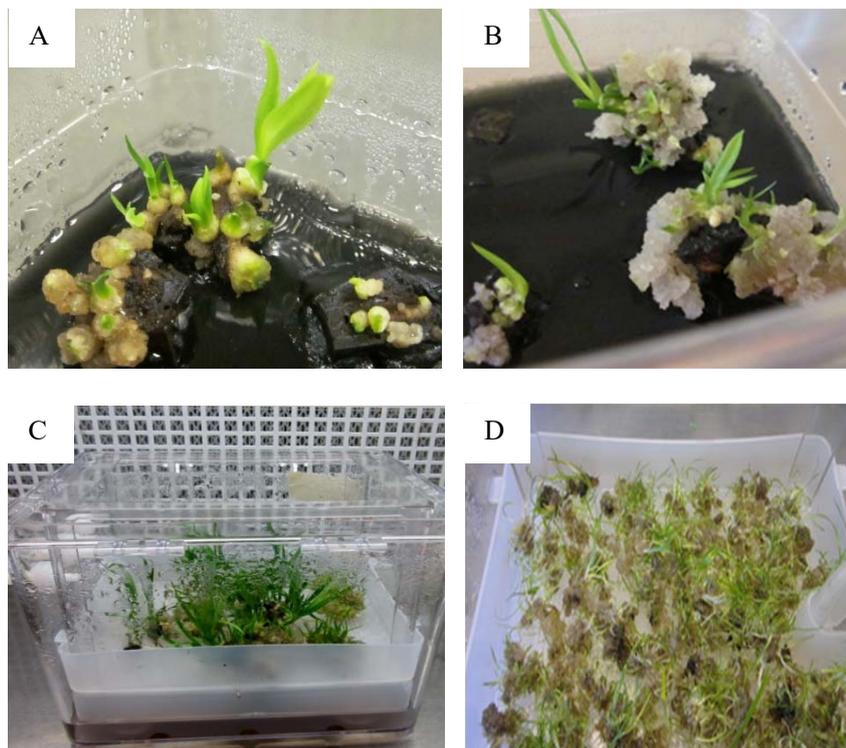


Figure 6. Regenerated shoots: Calli and shoots of *H. argentea* (A) and *H. filiformis* (B) on semi-solid medium and shoots of *H. filiformis* in bioreactor (C) and friable calli forming shoots (D).

5.2 Phylogenetic Analysis (Paper III)

Using the parsimony based methods, analysis of the sequences of the target chloroplast DNA regions, the *rbcL* gene, the *matK* gene, the *trnL-trnF* intergenic spacer and the *trnS-trnG* intergenic spacer of the eight known *Hypoxis* species resulted in the separation of the taxa into two main branches. *H. gerrardii* and *H. hemerocallidea* formed one branch and the other six taxa *H. acuminata*, *H. argentea*, *H. filiformis*, *H. iridifolia*, *H. parvifolia* and *H. rigidula* formed another branch. *H. gerrardii* and *H. hemerocallidea* were shown to be closely related to one another in relation to the other taxa. However, analysis of the unclassified taxa could not give conclusive results due to limitations emanating from insufficient information obtained from the sequenced target chloroplast DNA regions of the known taxa and that provided by the National Centre for Biotechnology Information.

Extraction of genomic DNA from the dried leaf tissues was a challenge as some of the primers that had worked previously with the fresh leaf tissues did not work with the dried samples. This resulted in different primer pairs being used for the same target region and obtaining shorter sequences from the dry plant tissues, thus providing limited information to properly classify the unknown taxa.

5.3 Analyses of some phytochemicals from corm extracts (Paper IV)

5.3.1 Ellagic acid

No ellagic acid was found in the corm extracts of all the analyzed species including *H. filiformis*, and thus basically confirming that it is not present in *Hypoxis* (Hillis, 2014) and nullifying the claim that *H. filiformis* is the only monocotyledonous member that contains ellagic acid (Yang *et al.*, 1998). It should, however, be elaborated that the investigation was done only on the corms and not on any other parts of the plant. Meanwhile, the investigation results, however, revealed the presence of an unknown compound that bears close resemblance to ellagic acid, but lacks a distinct feature (Paper IV, Figure 3).

5.3.2 Total phenolic content

The seven species: *H. acuminata*, *H. argentea*, *H. filiformis*, *H. gerrardii*, *H. hemerocallidea*, *H. iridifolia* and *H. parvifolia* showed significant differences in the content of total phenolics (Table 1). *H. iridifolia* and *H. gerrardii* had the highest total phenolic content of 369.64 µg/g and 318.21 µg/g, respectively, whereas *H. acuminata* had the lowest of 134.79 µg/g.

A general assessment using raw extracts from three of the species, *H. acuminata*, *H. gerrardii* and *H. iridifolia* revealed that some of the phenolic compounds contained in the corms include chlorogenic acid, coumaric acid and flavonoids. These were found in all three species and chlorogenic acid seemed to be the most abundant.

Table 1. Total phenolic content of seven Hypoxis species obtained from corm extracts in µg/g

Species	Total phenolic content (SE mean)
<i>H. iridifolia</i>	369.60 ± 28.0 a
<i>H. gerrardii</i>	318.20 ± 33.2 a
<i>H. parvifolia</i>	254.39 ± 6.21 b
<i>H. hemerocallidea</i>	204.56 ± 2.83 bc
<i>H. filiformis</i>	172.84 ± 9.84 cd
<i>H. argentea</i>	171.17 ± 5.10 cd
<i>H. acuminata</i>	134.79 ± 4.69 d

5.3.3 Hypoxoside

All the corm extracts of the seven species analyzed were found to contain hypoxoside in varying amounts, which differed significantly (Table 2). *H. gerrardii* (7.1%), *H. argentea* (6.6%) and *H. filiformis* (6.6%) had the highest hypoxoside contents compared to the other species. The lowest hypoxoside content of 1.5% was obtained from *H. parvifolia*.

Both epicatechin and the hypoxoside extracted from *H. filiformis* could not be used as a standard for quantification of the absolute hypoxoside content in the extracts, but were used to make a relative comparison confirming the presence of the compound in the corms of the different species. The results obtained by using either hypoxoside or epicatechin were the same. The purity of the extracted hypoxoside was estimated to be around 30%. This was mainly because the extraction method needed refining, which could not be conducted due to time limitation. Hypoxoside in the samples was identified by light spectrum(-) with two main peaks at 260 and 293 nm, respectively (Paper IV, Figure 5), using the mass spectra MS-ESI(-) with the main molecular ion, 605.1g/mol, and fragments 443.1 and 325.1 g/mol, respectively.

Table 1. Relative hypoxoside content of seven Hypoxis species obtained from corm extracts

Species	Chromatograph area (SE mean)	Percent
<i>H. gerrardii</i>	12634.0 ± 706	7.08 a
<i>H. argentea</i>	11864.0 ± 85.5	6.64 a
<i>H. filiformis</i>	11861.0 ± 68.3	6.64 a
<i>H. acuminata</i>	8579.0 ± 189	4.81 b
<i>H. hemerocallidea</i>	6044.0 ± 349	3.39 c
<i>H. iridifolia</i>	5893.0 ± 206	3.30 c
<i>H. parvifolia</i>	2643.4 ± 9.76	1.48 d

6 Conclusions and future perspectives

The work contained in this thesis can be summarized with the following highlights:

- ✓ *In vitro* regeneration protocols for four *Hypoxis* species: *H. acuminata*, *H. argentea*, *H. filiformis* and *H. hemerocallidea* using corms as explants were successfully established on semi-solid medium.
- ✓ An *in vitro* regeneration protocol of corm explants of *H. filiformis* with 100% regeneration efficiency and high shoot multiplication rate with the shoot number 17 per regenerated explant was successfully achieved on semi-solid medium.
- ✓ An *in vitro* seed culture protocol successfully developed using semi-solid medium.
- ✓ An effective *in vitro* propagation method for the mass production of *Hypoxis* using the temporary immersion system in bioreactors has been developed.
- ✓ Phylogenetic analysis of eight species: *H. acuminata*, *H. argentea*, *H. filiformis*, *H. gerrardii*, *H. hemerocallidea*, *H. parvifolia* and *H. rigidula* showed that *H. gerrardii* and *H. hemerocallidea* were more related to each other than to the rest of the taxa.
- ✓ It has been shown that the corms of seven species of *Hypoxis*: *H. acuminata*, *H. argentea*, *H. filiformis*, *H. gerrardii*, *H. hemerocallidea* and *H. parvifolia* do not contain ellagic acid
- ✓ The content of phenolic compounds differed and among seven *Hypoxis* species: *H. acuminata*, *H. argentea*, *H. filiformis*, *H. gerrardii*, *H. hemerocallidea* *H. parvifolia*; and the highest amount found in *H. iridifolia* and *H. gerrardii*.
- ✓ The important medicinal compound hypoxoside was found to be present in corm extracts of the seven species analyzed: *H. acuminata*,

H. argentea, *H. filiformis*, *H. gerrardii*, *H. hemerocallidea* and *H. parvifolia*.

- ✓ *H. hemerocallidea* popularly known to contain hypoxoside and whose corm extracts are mainly used in the pharmaceutical industry was found to have a relatively low content of the compound compared to *H. gerrardii*, *H. argentea* or *H. filiformis*.

Future considerations include further improvement of the *in vitro* protocols for *Hypoxis* species on both semi-solid medium and in bioreactors with the temporary immersion system to increase the proliferation frequency for mass production. The former is important for obtaining the start-up materials to be used in the latter if the intention is to produce *Hypoxis* in a large scale. It has been shown that the dormancy of the seeds is partially due to the hard seed coat and breaking of the seed coats mechanically resulted in some seeds germinating. However, the germination frequency was not high implying that there are other underlying factors hindering germinability. An in-depth study of these underlying factors is necessary as they can provide the basis for finding solutions to the problem.

The growth pattern of polymorphism displayed by *Hypoxis* has emphasized the need to use molecular methods in the identification of taxa and to efficiently distinguish different taxa to prevent wrong identification. Since not all the species may contain compounds with medicinal value, some may even have health hazardous compounds, it is thus important that taxa are correctly classified. The use of molecular analyses such as chloroplast markers, as well as other molecular markers, can be a straightforward method for efficient breeding and commercialization of the species in the future.

The results from the analyzed species of *Hypoxis* have shown that besides hypoxoside, the species contain phenolic compounds which are associated with health. The same phenolic compounds may not be found in different species, and their content may vary among species. It would be interesting to evaluate the types and amount of phenols that are present in the different *Hypoxis* species, as well as their functions in relation to health or medicinal uses.

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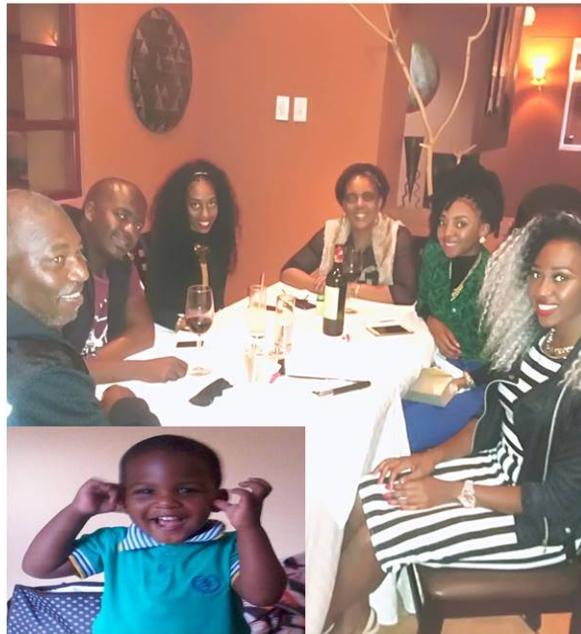
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