Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Landscape Planning, Horticulture and Agricultural Science

In vitro Regeneration of Four *Hypoxis* Species and Transformation of *Camelina sativa* and *Crambe abyssinica*

Master Thesis

By: Busie E. Nsibande

Department of Plant Breeding and Biotechnology
Alnarp
2012

In vitro Regeneration of Four *Hypoxis* Species and Transformation of *Camelina sativa* and *Crambe abyssinica*

Busie E. Nsibande

Supervisor: Professor Li-Hua Zhu

Examiner: Dr Per Hofvander

Credits: 45 HEC
Level: Advanced E
Course title: Research Project in Plant Breeding and Biotechnology, 45 credits
Course code: UU0069
Programme/education: Master education as Free Mover

Place of publication: Alnarp
Year of publication: 2012
Picture Cover: Flowering plant of *Hypoxis hemerocallidea*
Title of series: no: Självständigt arbete vid LTJ-fakulteten, SLU
ISSN:
ISBN:
Online publication: http://stud.epsilon.slu.se

Key Words: *Agrobacterium;* Biotechnology; *Camelina sativa*; Crambe *abyssinica*; *Hypoxis;*; *in vitro* propagation; regeneration; transformation, vacuum infiltration.

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Landscape Planning, Horticulture and Agricultural Sciences

Table of Contents

Abstract

The advance of science and biotechnology has made it possible to prevent the complete loss of plant species by providing propagation methods that are fast and can result in the mass production of exact copies of the targeted plant species. Genetic transformation has become a useful instrument in the introduction of new traits to plants such as increased yield and disease resistance.

In this study potential *in vitro* regeneration protocols for four *Hypoxis* species were successfully developed. Efficient indirect regeneration (100% with 8 shoots per explant) of *Hypoxis filiformis* was obtained when corm explants were directly cultured in MS supplemented 1 mg/l NAA. However, the highest mean number of shoots per explant (17) was obtained in basal MS supplemented with 3 mg/l kinetin. Efficient direct regeneration of *H. acuminata* (100% with 2 shoots per explant) was achieved in corm explants cultured with a piece of shoot attached in MS supplemented with 3 mg/l kinetin. Up to 30% and 21% seed germination was obtained in *H. argentea* and *H. filiformis* respectively, when seed coats were crushed before culturing in half-strength MS without PGRs. In addition, the use of activated charcoal and 0.1% mercuric chloride, respectively, helped in controlling the build-up of phenolic exudates in the medium and infection of explants and shoots by endogenous micro-organisms.

Agrobacterium-mediated transformation of *Camelina sativa* and *Crambe abyssinica* flowers by vacuum infiltration with three gene constructs: *AtHb2*. *BvHb2*, and v*hb* in *Camelina*; and the FWS 3-1 vector harbouring three genes: *FAR*, *WS*, and *DsRed* in *Crambe* failed to produce transgenic seeds.

Abbreviations

IAA : Indole-3-acetic acid
IBA : Indole-3-butyric acid
BAP : Benzyl-aminopurine
CW : Coconut water
GA_3 : Gibberellic acid
HIV/AIDS : Human imunodeficiency virus / Acquired immunodeficiency syndrome
Kin : Kinetin
Lep : Lepoivre
MS : Murashige and Skoog
NAA : Naphthalene-acetic acid
PGRs : Plant growth regulators

1.0 Introduction

Plants are the basis of human life as they provide 90% of human calorie intake and 80% of protein intake. However, out of three thousand plant species previously used by man as food, today the world relies on approximately twenty crop species for the provision of most of its calories. The ever increasing population versus the dwindling food supply has been clear indication that new and better technologies are urgently required to address the problem of food security. The past few years have seen a lot of advance on the use of new methods in plant sciences to develop new crops that involve the manipulation of plant cells, tissues, and organs. Genetic engineering has also played a major role in bringing about concise methods for breeding better crop varieties (Chawla, 2009).

The advance of science and biotechnology has made it possible to prevent the complete loss of plant species by providing propagation methods that are fast and can result in the mass production of exact copies of the targeted plant species. *In vitro* propagation is now widely used in plant biotechnology as an alternative technique of shortening the length of period taken by conventional breeding methods to develop new and better plant varieties. Plant tissue culture has become an important tool in the production of crops of economic value that are cultivated vegetatively such as fruits, vegetables and ornamental plants. On the other hand, the use of genetic engineering has played an important role in plant breeding and crop improvement by enabling the introduction of genes of interest into genomes of important crop species within a relatively short period of time.

1.1 Plant Biotechnology

1.1.1 Plant Tissue Culture

Plant tissue culture refers to the aseptic cultivation of plants, seeds, plant parts such as tissues, organs, embryos, single cells, protoplasts on nutrient media *in vitro* (Chawla, 2009). It is the science used to grow plant cells, tissues or organs that have been isolated from the mother plant on artificial medium under sterile conditions (George *et al*, 2008). Plant tissues have the ability to regenerate new complete plants, a feature called totipotency, which offers the possibility of producing new plants from organs or tissues of the mother plant. This usually involves culturing of a piece of differentiated tissue or organ called an explant that has been taken from the mother plant to initiate growth (George *et al*, 2008; Chawla 2009). Micropropagation is a term used to define the propagation of plantlets (microplants) by tissue culture, and is mainly aimed at

producing clonal plants that are true-to-type as the mother plants as well as avoiding pathogens and viruses (George *et al*, 2008).

Plants produce hormones that play an important regulatory role in their growth and development. Synthetic plant growth regulators (PGRs) with similar physiological activities and biological effects as natural phytohormones have been developed and are widely used in plant biotechnology to manipulate plant growth and development. Auxins and cytokinins are the most important groups of PGRs responsible for regulating and initiating growth in plant tissue culture and their combinations promote the growth of calli, cell suspensions and organs, and also regulate the direction of morphogenesis (George *et al*, 2008), or how the organs are shaped. Both auxins and cytokinins are needed for cell division, but auxins are mainly known to induce cell elongation and formation of callus, whereas cytokinins are responsible for stimulating growth and development, (Chawla, 2009); thus the correct balance of auxins and cytokinins is required to form either shoots or roots.

1.1.2 Agrobacterium – Mediated Gene Transfer

Genetic transformation has become a useful tool in the introduction of new traits to plants such as increased yield or disease resistance (Liu et al., 2008). The techniques involved are grouped into two: vector mediated gene transfer and direct DNA transfer – vectorless (Chawla, 2009). The vast majority of plant transformation is carried out by vector-mediated gene transfer. There are different genetic transformation methods for the vector-mediated gene transfer such as *Agrobacterium*-mediated gene transfer, and physical methods like micro-injection, polyethylene glycol-mediated transfer, electroporation, and microprojectile bombardment. *Agrobacterium* – mediated gene transfer can be further divided into in-planta and tissue culture-based methods. For most plant species tissue culture-based methods are the most efficient and commonly used. For tissue culture-based methods, three basic steps are involved: i) a system for introducing the target DNA into the target plant, ii) a system to enable selection of transgenic cells or plants from the non-transgenic ones, and iii) a protocol for regenerating the transgenic cells to give rise to shoots (Liu et al., 2008).

There are two types of naturally occurring soil bacteria that are known to transfer DNA to plant cells and are used for transformation, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. The former causes tumor formation on the infected plants while the latter results in hairy root formation on the infected plants. Although both types can be used for plant transformation, *A. tumefaciens* is the one commonly used. The first success and breakthrough in plant transformation by *Agrobacterium* was reported in 1983. This success was made possible because of characterization and full

exploitation of the plasmids carried by the two species of bacteria which provide natural gene transfer, gene expression and selection systems. Plasmids (Chawla, 2009).

1.2 The Genus Hypoxis and its Significance in Southern Africa

The genus *Hypoxis* belongs to the Hypoxidaceae family (Vinesi et al., 1990, Singh, 2004, Ndong et al., 2006, Singh, 2007, Sathekge et al., 2010, Appleton et al., 2012) and its species are easily recognizable as most of them have bright yellow star-shaped flowers and strap-like leaves. The Hypoxidaceae family has 55 genera and approximately 155 species with *Hypoxis* being the largest (Singh, 2007, Kocyan et al., 2011). The name *Hypoxis* comes from the Greek words "hypo" meaning below and "oxy" referring to the pointed base of the ovary or fruit. The genus has an estimate of 90 species world-wide (Singh, 2004, Kocyan et al., 2011). Species of this genus are perennial grasses that are widely distributed in sub-Saharan Africa, America, south-east Asia, Australia (Appleton and van Staden, 1995b, Singh, 2004), and Uganda (LPS, Undated). In Africa, the genus is widespread south of the Sahara, with a concentration of about 41 species in southern Africa (Singh, 2004). Most members of this genus produce attractive bright yellow flowers and thus are referred to as the yellow star plants (Appleton and van Staden, 1995b).

Hypoxis species have a long history of medicinal use on the African continent and mostly used in South Africa by the indigenous people (Boukes and van de Venter, 2011); and in primary health care as an immune booster for patients with HIV/AIDS (Singh, 2004). Eleven species are reported to be used in southern Africa for traditional purposes (Appleton et al, 2012). The corm is the underground part of the plant that it is usually sought as it is known to contain medicinal compounds. Two species, *H. hemerocallidea* (African potato) and *H. colchicifolia* are the most sought after for their use in African traditional remedies as well as for preparation of herbal teas and tinctures. They have been used by traditional healers for centuries in the treatment of urinary infections, heart weakness, internal tumours and nervous disorders and corms of *H. hemerocallidea* are being used to alleviate many immune related ailments such as the common cold, flu, arthritis, tumours, cancer and HIV/AIDS (Singh, 2004). The extracts of *H. hemerocallidea* are used in the treatment of benign prostate hyperplasia and the phytosterol extracts including their main constituents are now applied as anti-oxidants, anti-inflammatories, anti-diabetics, anti-convulsants, inhibitors of drug marker substances with new evidence of activity against cancerous and premalignant cancer cells (Drewes et al., 2008). In addition, African potato is also used to treat dizziness (Lake Product Services, undated), bladder disorder (Appleton and van Staden, 1995; Lake Product Services, undated), wounds, uterine and prostate gland disorders (Appleton and van Staden, 1995b), insanity and as immune boosters for patients with

HIV/AIDS (Mountain Herb Estate, 2008). *H. hemerocallidea* is counted amongst the special indigenous medicinal species of commercial importance in southern Africa (van Wyk, 2008).

African potato is used commonly as an immune booster to treat many ailments (Singh, 2004, Nair et al., 2007, Boukes et al., 2008), as well as for its nutritional value (Boukes et al., 2008) and is widely distributed in the savannah regions of South Africa, Swaziland and Zimbabwe (Katerere and Eloff, 2008). Biomedical evidence suggests that corm extracts of African potato possess anti-inflammatory, anti-neoplastic, anti-oxidant, anti-diabetic, and anti-infective properties thus making the plant a potential medicinal donor for modern and 21st century diseases of mankind (Owira and Ojewole, 2009). Van Wyk (2011) reported that millions of South African rands have been spent on research and development of *H. hemerocallidea* since 1967 resulting to the production of Harzol a brand formulated from *Hypoxis* phytosterols. The high medicinal value of the plant is due to hypoxoside, a sterol that is converted by the human body to rooperol, a biologically active compound that balances the human immune system thus offering great potential as a source of new drugs with immune-modulatory properties; and as such, is of economic value (Singh, 2004). However, there are several species that grow in the wild whose medicinal properties have not been ascertained and these can easily be confused with *H. hemerecollidea* or *H. colchicifolia* as some species are difficult to differentiate (ARC, 2010). Investigations done by Vinesi *et al* (1990) on the hypoxoside content of *H. obtusa*, also found in other species of *Hypoxis*, showed that the active ingredient of these compounds contained an uncommon aglycone structure consisting of C6(aromatic)-C3-C,-C6(aromatic), the main components of the glucoside fraction being hypoxoside and nyasoside.

Other species of *Hypoxis* have other uses other than medicinal, and these include the nursery industry and for making household products. *H. angustifolia* and *H. parvula* are good for ornamental use because of their small size and growth habits, the former being used in the nursery trade because of its spreading rootstock, and the latter is "excellent for alpine gardens as it prefers rocky terrain" (Singh, 2004). *H. rigidula* is used for making rope and hut trimmings because of its hard fibre, and the species *H. obtusa* is used by some South African tribes in the making of floor polish (Singh, 2004).

1.2.1 Micropropagation of *Hypoxis*

Despite that *Hypoxis* species have been used for many years in traditional medicine, and quite recently in the production of health supplements, seemingly not so many studies have been done on *in vitro* propagation as the information about it is very limited. Van Wyk (2008), when reviewing southern African medicinal plants of commercial importance noted that only a few species have been commercialized and that basic scientific information was often lacking. Appleton and van Staden (1995) reported that

there was a variation in the response of *H. acuminata, H. rigidula, H. obtusa* and *H. colchicifolia* when regenerated *in vitro*.

In vitro studies using corms as explants faced many challenges due to the presence of endogenous pathogens (Appleton and Van Staden, 1995; Appleton *et al*, 2012) and phenolic compounds that caused browning of the explants (Appleton *et al*, 2012). Ndong *et al* (2005) reported that when corm explants of *H. hemerocallidea* were cultured on medium they turned black rapidly resulting to the browning of the medium.
On their experimental work on *in vitro* regeneration of corm explants of *Hypoxis hemerocallidea*, Ndong *et al* (2006) found that agar-based medium comprising Murashige and Skoog (MS) (Murashige and Skoog, 1962) macroelements, microelements and vitamins of Nitsch and Nitsch supplemented with 3 mg/l of the cytokinin kinetin produced the best results on regeneration of multiple direct shoots. They also found that higher concentrations of cytokinins in corm cultures generally induced direct shoot formation compared to lower levels, and different combinations of plant growth regulators tested.
Vinesi *et al* (1990) conducted some regeneration tests on three species of *Hypoxis* but only managed to successfully culture explants from the rhizome of *H. obtusa* on MS medium supplemented with 1 mg/l 6-benzylaminopurine (BAP) while no regeneration from the other species tested, *H. nyasica* and *H. anguistifolia*. However, they were able to isolate and further characterize the active compound, hypoxoside, from the fresh rhizomes of the three species.
Appleton and Van Staden (1995) reported that "greatest growth response was obtained from the sterilized *H. hemerocallidea* corm explants placed on MS initiation medium supplemented with NAA and BA at a concentration of 1 mg/l each" (p 96).
Appleton and van Staden (1995a) reported obtaining the highest production of direct shoots from corm explants of *H. angustifolia* cultured on MS medium supplemented with 2 mg/l BA and indirect shoots on MS supplemented with 0.5 mg/l BA.
Page and Van Staden (1984) reported that 70% of corm explants from *H. rooperi* cultured on basal medium consisting MS, 30 g/l sucrose, 1000 mg/l vitamin free casein hydrolysate, 100 mg/l myo-inositol, 1 mg/l thiamine-HCl and supplemented with 1 mg/l BA produced shoots after five to six weeks, with root formation at about ten weeks after culturing. They were able to induce the remaining shoots by sub-culturing them into medium that contained no plant growth regulators, indicating the possibility of cultivating this species commercially.
Recently, Appleton *et al* (2012) reported that among all types of explants tested, flower buds of *H. colchicifolia* responded best in multiplication medium containing 2 mg/l BA compared to other types of explants tested.

1.2.2 *Hypoxis* in Swaziland

Swaziland, also known as the Kingdom of Swaziland, is a small landlocked, mountainous country in southern Africa with an area of 17 364 km^2 (Dlamini, 2009, Zwane et al., 2011) and located between latitudes 25^0 43' and 27^0 19' S longitudes 30^0 47' and 32^0 08' E with a population of 1 million, 126 thousand people (Zwane et al., 2011). It is situated in the south-eastern part of Africa sharing borders with the Peoples' Republic of Mozambique on the east and the Republic of South Africa which surrounds the rest of the country. The country has four major climatic regions: The Highveld, Middleveld, Lowveld and the Lubombo Plateau (Mzizi, 2002). *Hypoxis* species grow naturally in Swaziland and a number of them can be found in at least three regions. There are twelve species that are recorded by the Swaziland National Trust Commission (SNTC, 2011).

According to Owira and Ojewole (2009) about 80% of the population in sub-Saharan Africa, and about 85% in Swaziland (Amusan et al., 2007), use traditional medicine. *Hypoxis* species, especially *H. hemerocallidea* or African potato are counted amongst the common plants used in traditional medicine in Swaziland. Amusan *et al* (2007), reported that the corm infusion of *H. hypoxis* is used as an all-purpose remedy, hence the local name "zifozonke", including HIV/AIDS related illnesses. Additionally, it is listed amongst the indigenous medicinal plants commonly collected for income generation and home use (Manyatsi et al., 2010). The popularity of the use of African potato has placed it amongst threatened species due to over-harvesting (Katerere and Eloff, 2008). Despite the popularity African potato and other species of this genus in traditional medicine, so far no efforts have been made to cultivate any of them.

1.3 Biotechnology Application in the Improvement of Camelina sativa

In European history, *Camelina sativa* L. Crantz (Zubr, 1997, Ryhanen et al., 2007, Büchsenschütz-Nothdurft et al., 1998, Lu and Kang, 2008a) is known to be one of the oldest crops (Grauda et al., 2007, Ryhanen et al., 2007) that was cultivated in the 19^{th} century in countries like France, Holland, Belgium and Russia (Fröhlich and Rice, 2005); and has recently generated interest to scientists and oil processors as a crop for diversification of agriculture, and as a source for production of high quantity and quality oil for biofuel, feeding, food, and pharmacy (Grauda et al., 2007). It is commonly known as Gold Pleasure or false flax (Zubr, 1997, Ryhanen et al., 2007, Terpinc et al., 2012, Kuvshinov et al., 2011, Lu and Kang, 2008a) and belongs to the genus *Crucifarae* (Fröhlich and Rice, 2005), family Brassicaceae (Ryhanen et al., 2007, Liu et al., 2008, Kuvshinov et al., 2011), tribe Sisymbrieae (Kuvshinov et al., 2011).

Camelina sativa, a low input crop (Zubr, 1997), falls amongst the important emerging vegetable oilseed groups and is a potential crop in the production of non-food industrial oils (Büchsenschütz-Nothdurft et al., 1998, Liu et al., 2008, Lu and Kang, 2008a) as well as a source of omega-3 fatty acids in the health sector (Lu and Kang, 2008a). It is one of several oilseed crops that are being studied because of the potential it has in the production of biofuel (Frame et al., 2007, Liu et al., 2008). *Camelina* seeds contain about 43% oil in dry matter, 90% unsaturated fatty acids in the oil of which about 50% are polyunsaturated-linoleic acid (18:2n− 6) and α-linolenic acid (18:3n− 3), and about 3% erucic acid (22:1n − 9) in the oil (Zubr, 1997). According to Lu (2011) the biggest challenge in oilseed biotechnology is to be able to produce large quantities of vegetable oil to meet the global demand at cheaper prices; a challenge that could be addressed by increasing the oil yield and expanding production to marginal land. Efforts made earlier, to increase oil and fatty acid content faced difficulties due to insufficient knowledge of the enzymes and genes involved in the biosynthesis processes (Lu et al., 2011). Oil from *Camelina sativa* is a good source of α-linolenic acid, a fatty acid that can influence cardiovascular risk factors such as serum lipids, blood pressure, and hemostatic factors thus reducing the risk of coronary heart diseases (Karvonen et al., 2002). It has many uses ranging from being used as an edible oil to being used as a traditional remedy for treating stomach and duodenal ulcers, wound, burns, and eye inflammations (Terpinc et al., 2012).

The points discussed above are justification enough for the need to develop transformation systems for *Camelina sativa* so that the full agronomic characteristics are exploited. Liu *et al.* (2008), describe a transformation method for *C. sativa* that is an *Agrobacterium*-mediated transformation system via tissue regeneration. The method involves dipping the plants in an *Agrobacterium* solution that contains the vector to be expressed, incubating them and later selecting for transformed plants. Lu and Kang (2008a) in their quest to improve oil quality and other agronomic characters developed an *Agrobacterium*-mediated in planta gene transfer method through vacuum infiltration for generating transgenic *Camelina* plants. The main feature of this method is that plants, at the early flowering stage, are inoculated with the *Agrobacterium* solution during a vacuum infiltration process. With the help of the fluorescent protein DsRed as a visual selection marker, transgenic seeds of over 1% have been obtained (Lu and Kang, 2008a).

1.4 Biotechnology Application in the Improvement of Crambe abyssinica

Crambe abyssinica Hoscht. Ex R.E. Fries (Mastebroek et al., 1994, Wang et al., 2000, Muuse et al., 1992), common names Abyssinian mustard or Abyssinian kale (Chhikara et al., 2012), is an annual herb that belongs to the genus *Crambe* and family Crucifer or Brassicaceae (Li et al., 2010, Li et al., 2011, Chhikara et al., 2012) Its origins are believed to be in the Mediterranean region. It derives its usefulness from the seed, which has an oil content of 35% (Wang et al., 2000, Bruun and Matchett, 1963) of which 50 to 60% (Laghetti et al., 1995); 55 to 60% (Wang et al., 2000, Bruun and Matchett, 1963, Mastebroek et al., 1994, Chhikara et al., 2012, Li et al., 2011, Li et al., 2010) is erucic acid. It is resistant to insect feeding, tolerant to abiotic stresses like salinity, cold temperature, heat and drought, and exposure to heavy metal. Furthermore, the siliques of *Crambe* do not shatter or discharge seeds when ripe thus preventing seed loss. These characteristics make the crop a potential gene donor for modification of other oilseed brassica species to enhance erucic acid content, yield including genetic manipulation to improve tolerance to biotic and abiotic stress, and for production of proteins of importance in the pharmaceutical industry (Chhikara et al., 2012).

The high erucic acid content (about 55%) in seeds makes crambe a promising industrial oilseed crop (Lazzeri et al., 1994, Daubos et al., 1998, Li et al., 2010) compared to other oilseed crops. Fatty acids from crambe can be turned into various products in the pharmaceutical and cosmetic industries whilst the oil derivatives has other industrial uses such as in lubricants, rubber additives, new type of nylon, base for paints and coatings, high temperature hydraulic fluids, dielectric fluid, waxes, etc. (Wang et al., 2000). Erucic-based materials are environmentally friendly, which explains why they are increasingly used as lubricants (Leonard, 1992).

To be competitive with the petroleum industry, it is important that the content of a specific fatty acid must be high enough in order to considerably reduce the purification costs (Mietkiewska et al., 2007). Oil modification by conventional breeding has been a difficult task due to the complexity of oil biosynthesis. Genetic engineering offers a more precise and efficient method for increasing fatty acid contents. For this purpose, an efficient regeneration and transformation method is prerequisite as long as tissue culture-based transformation is concerned. Genetic modification of crambe has not been available until very recently when the first efficient regeneration and transformation protocol was reported by Li *et al* (2010) using kanamycin as a selection agent in which a regeneration frequency of 60% and transformation efficiency up to 2.1% were obtained. Subsequently Li *et al.* (2011), were successful in developing a highly efficient regeneration protocol for *Crambe* using hypocotyl explants with a

regeneration frequency exceeding 95%. The success was credited to the inclusion of various factors in their investigation such as the effects of N-source, C-source, AgNO3, cultural conditions as well as the concentration and combination of PGRs on the regeneration frequency. Chikara *et al.* (2012) have also been able to develop an efficient *Agrobacterium*-mediated transformation system for *C. abyssinica* by infecting hypocotyl explants with *Agrobacterium tumefasciens* using hygromycin as the selective agent and they achieved a regeneration frequency of 50 to 70% and a transformation frequency of 6.7 to 8.3%.

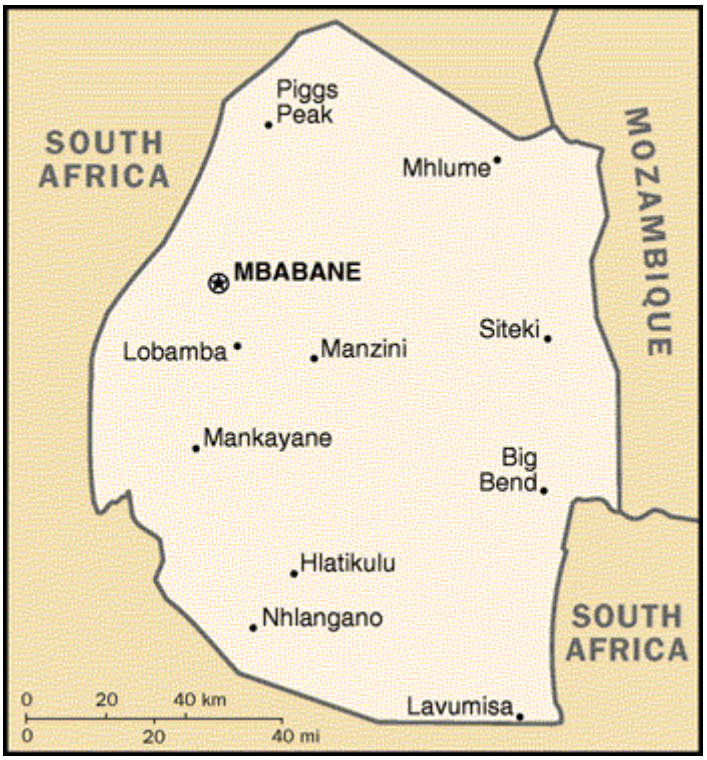

Figure 1: Map of the Kingdom of Swaziland

1.5 Objectives

The overall goal of the study was to apply biotechnology in some important plant species with special characteristics aiming at developing suitable protocols for their future improvement and enhancement.

The specific objectives were:

1.5.1 To regenerate and establish *Hypoxis* species collected in different agro-ecological regions of Swaziland *in vitro*.

1.5.2 To study the effects of plant growth regulators on the performance of different explants from *Hypoxis* species during *in vitro* regeneration and establishment.

1.5.3 To introduce target genes into *Camelina sativa* and *Crambe abyssinica* through *Agrobacterium* vacuum infiltration.

2.0 Materials and Methods

2.1 Regeneration of Hypoxis

2.1.1 Mother Plants

Four species of *Hypoxis* obtained from three Agro-ecological regions of Swaziland were used in this study: *Hypoxis acuminata* from Malkerns (Middleveld) in the Manzini region, *Hypoxis argentea* from Ntfonjeni (Highveld) in the Hhohho region, *Hypoxis filiformis* from Tjaneni (Lubombo Plateau) and Nhlangano (Highveld) in the Lubombo and Shiselweni regions respectively, and *Hypoxis hemerocallidea* from Bethany (Middleveld) in the Manzini region. The corms were planted in the greenhouse in 2:1 compost to sand mixture in Alnarp, Swedish University of Agricultural Sciences. Leaves, shoot apexes, roots, flower buds, basal part of inflorescence peduncles, corms, and seeds were harvested and used for explants.

2.1.2 Preparation and Sterilization of Explants

All explants were surface-sterilized in 6% calcium hypochlorite solution ($Ca_2Cl\ O_2$). Unless stated otherwise, seeds, leaves, shoot apexes, basal part of inflorescence peduncles, and flower buds, were washed in the solution with one or two drops Tween 20 with shaking for fifteen minutes before washing three times in sterile distilled water and subsequently plating on culture medium. Roots were first washed at length in running tap water, washed in 70% alcohol and rinsed once with distilled water before washing with 6% calcium hypochlorite solution and rinsing with sterile distilled water

as stated above. For corms, a modification of the protocol described by Ndong *et al.* (2006) was used. The corms were first washed at length in running tap water with a detergent, followed by washing in 70% alcohol for five minutes, washing in 6% calcium hypochlorite solution with one or two drops Tween 20 with shaking for thirty minutes. The outer epidermal tissue was removed with a knife and the corms cut into large pieces, approximately 3 cm thick, washed in 6% Calcium hypochlorite solution with one or two drops Tween 20 for fifteen minutes followed by rinsing in sterile distilled water three times. They were then cut into smaller pieces of approximately 0.5 – 1 cm^2 thickness before placing on the culture medium.

2.1.3 Culture Media

MS (Murashige and Skoog, 1962) salts and vitamins were used as the basal medium for most cultures, where basal medium 1 (BM1) was MS with 30 g/l sucrose and 8 g/l Bacto agar; and basal medium 2 (BM2) was the same as BM1 but with 0.1 g/l myo-inositol and 1 g/l casein hydrolysate. Lepoivre medium, which contained Lepoivre macronutrients, MS micronutrients and vitamins as well as 30 g/l sucrose and 8 g/l Bacto agar, was used in other cultures. Various combinations and concentrations of plant growth regulators (PGRs), mainly auxins and cytokinins, were added in the basal culture media. Auxins used included indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA). Cytokinins were benzylaminopurine (BAP) and kinetin (Kin); gibberellic acid (GA_3) was only used in meristem and seed cultures. Medium was changed regularly and the frequency depended on the requirement such as browning, contamination, or infection of cultures. Explants were either maintained in the same culture medium for the duration of the investigation or transferred to other types of media (see the results section for details).

2.1.4 Culture Conditions

All explants were cultured in a growth chamber with day and night temperature $25/18^0$C and the photoperiod of 16 hours, and light intensity of 40 µm mol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes.

2.1.5 Treatments

Due to contamination, phenolic exudates, and endogenous infections it was not possible to maintain the same number of explants in each treatment. In order to avoid death of explants due to phenolic exudation 0.5 – 1.0% activated charcoal was added in the culture medium and according to Birmeta (2004) this amount is effective in reducing the browning of medium resulting from phenolic exudates. Polyvinyl pyrrolidone (PVP) at 0.5 g/l and 5 – 15% coconut water were added in some treatments. Coconut water was derived by filtering a commercial blend of coconut milk and coconut pulp. Insufficient plant material and the destructive nature of deriving particular explants

prevented having the same treatments conducted in all four *Hypoxis* species. The media used in the treatments for the different types of explant are given in Table 1.

Table 1: List of Culture Media Used in Regeneration and Establishment of Four *Hypoxis* Species

Code	Composition	Explant Type
BM1	MS salts, 30 g/l sucrose, 8 g/l agar, pH 5.8	All
BM2	MS salts, 30 g/l sucrose, 0.1 g/l myo-inositol, 1 g/l casein hydrolysate, 8 g/l sucrose, pH 5.8	Corms, flower buds, seeds
$BM1^{+}$	BM1, 0.5 mg/l BAP	Corms, roots, seeds
$BM1^{++}$	BM1, 1 mg/l BAP, 0.5 mg/l IBA	Corms,seeds
$BM1^{+++}$	BM1, 1 mg/l kinetin, 0.5 mg/l IBA	Corms, seeds
BM1BAP1	BM1, 1 mg/l BAP	Corms, seeds
BM1BAP2	BM1, 2 mg/l BAP	Corms, seeds, roots
BM1BAP3	BM1, 3 mg/l BAP	Corms, seeds
BM1BAP5	BM1, 5 mg/l BAP	Corms
BM1Kin1	BM1, 1 mg/l kinetin	Corms, seeds, roots
BM1Kin2	BM1, 2 mg/l kinetin	Corms, seeds
BM1Kin3	BM1, 3 mg/l kinetin	Corms, seeds, roots
BM1Kin5	BM1, 5 mg/l kinetin	Corms
BM1NAA1	BM1, 1 mg/l kinetin	Corms
BM1IAA5	BM1, 5 mg/l kinetin	Corms
BM1CW1	BM1, 5% (v/v) coconut water	Seeds
BM1CW2	BM1, 10% (v/v) coconut water	Seeds
KinCW1	BM1, 2.4 mg/l kinetin, 5% v/v coconut water	Corms
BAPIAA5	BM1, 0.5 mg/l BAP, 5 mg/l IAA	Corms
$BAP^{+}NAA$	BM1, 1.5 mg/l BAP, 0.5 mg/l NAA	Corms, shoot apexes
BAP3NAA1	BM1, 3 mg/l BAP, 1 mg/l NAA	Corms, inflorescence peduncles
BM2NAA1	BM2, 1 mg/l NAA	Corms
BM2BAP3	BM2, 3 mg/l BAP	Corms
BM2Kin3	BM2, 3 mg/l kinetin	Corms
Kin3KCl	BM1, 3 mg/l kinetin, 1.5 g/l potassium chloride (KCl)	Corms
$Kin3KCl^{+}$	BM1 with double-strength MS, 3 mg/l kinetin, 1.5 g/l KCl	Corms
Lep1	Lepoivre salts (Lepoivre macronutrients, MS micronutrients, MS vitamins), 0.2 mg/l BAP, 30 g/l sucrose, 8g/l agar, pH 5.8	Leaves

2.1.5.1 Culture of Shoot Apexes

Only shoot apexess of *H. hemerocallidea* were cultured due to insufficient number of plants. For this purpose, four corms directly gathered from the wild in Bethany, in the Manzini region (Middleveld), were used. The corms were surface sterilized as described in 2.1.2, while the roots and leaves were removed leaving a portion of shoot attached to the corm. The base of the shoot with a piece of shoot and corm attached were aseptically removed with the aid of a microscope and cultured in the initiation medium comprising full-strength MS salts and vitamins, 1.5 mg/l BAP (benzylaminopurine), 0.5 mg/l NAA (naphthaleneacetic acid), and 20 g/l sucrose. The pH was adjusted to 5.8 with NaOH and HCl before adding 8 g/l Bacto agar; 1% activated charcoal was also added.

2.1.5.2 Culture of Basal Parts of Inflorescence Peduncles

Explants from the basal part of the inflorescence peduncles of *H. hemerocallidea* from Bethany in the Manzini region (Middleveld) and *H. filiformis* from Tjaneni in the Lubombo region (Lubombo Plateau) were cultured in different media. The explants were aseptically removed from the base of the inflorescence peduncle attached to the corm under a stereo-binocular microscope. Five explants of *H. hemerocallidea* were culture in liquid medium comprising MS salts, 1 mg/l BAP, 0.1 mg/l IBA , 0.05 mg/l GA_3, 1 g/l casein hydrolysate, 30 g/l sucrose, and pH 5.8. Six meristems from *H. filiformis* were cultured in MS medium containing 1 mg/ l NAA and 3 mg/l BAP with activated charcoal. *H. hemerocallidea* explants were removed from the liquid medium after two weeks and were cultured in the same solid medium as *H. filiformis*.

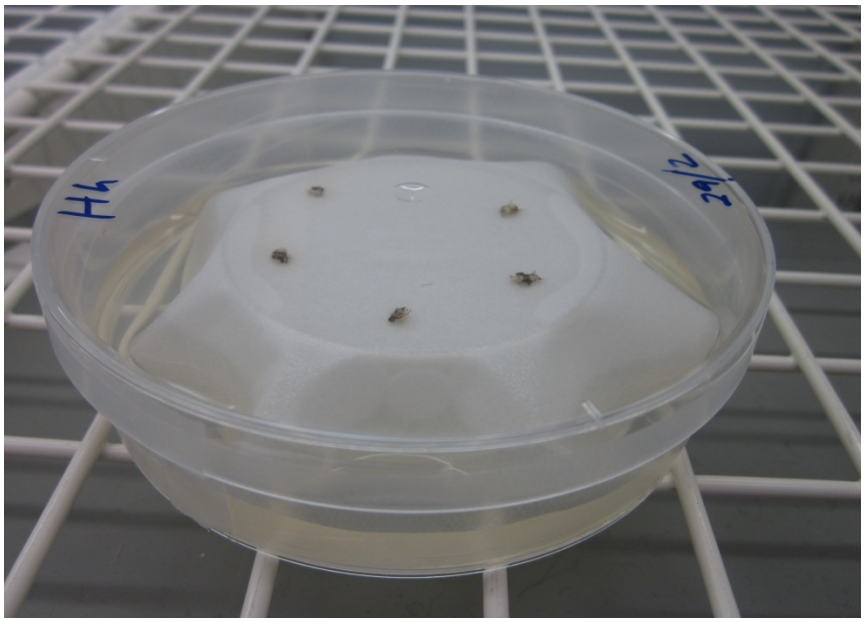

Figure 2: Inflorescence peduncle of *H. hemerocallidea* in liquid culture

2.1.5.3 Leaf Culture

Young leaves of *H. acuminata, H. filiformis* and *H. hemerocallidea* were harvested, washed then cut into small pieces before being surface sterilized. Following sterilization, leaves were further cut into approximately 0.5 cm^2 pieces before being placed on the culture medium. Explants were cultured in BM1, BM1 supplemented with 0.5 mg/l, and Lepoivre salts with 30 g/l sucrose and 0.2 mg/l BAP. The pH was adjusted to 5.8 before 8 g/l Bacto agar was added. Ten explants were cultured for each treatment.

2.1.5.4 Culture of Flower Buds

Unopened flower buds of *H. argentea, H. filiformis* and *H. hemerocallidea* were first washed and surface-sterilized. The buds were cut into half below the perianth segment (Appleton et al., 2012). The top part was removed and the lower part cultured in double-strength MS containing 30g/l sucrose, 0.1 g/l myo-inositol, 1 g/l casein hydrolysate, 5% (v/v) coconut water and 8 g/l Bacto agar, a modification of the protocol used by Appleton *et al.*(2012). Agar was added after adjusting the pH to 5.8. Six explants were cultured for each species.

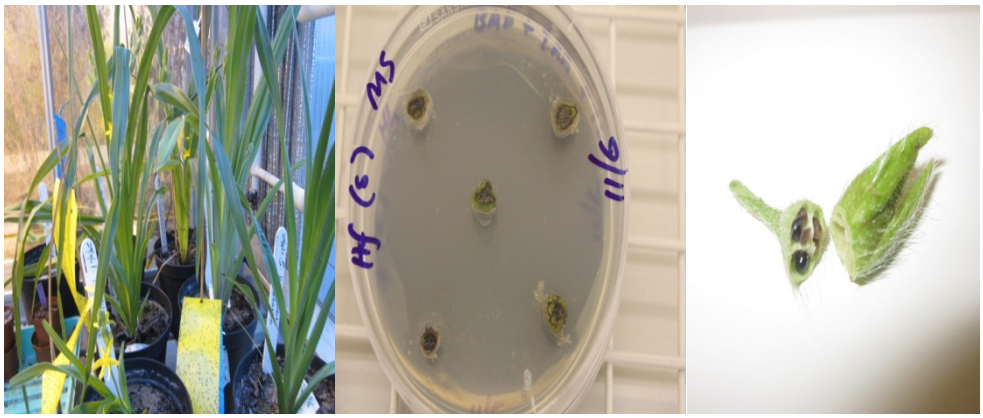

Figure 3: *Hypoxis* plants in greenhouse (left); flower bud culture of *H. filiformis* in PGR-free MS (middle); Open seed capsule with black shiny seeds (right)

2.1.5.5 Culture of Seeds

Seeds were subjected to various treatments including dormancy breaking methods. The treatments depended on the availability of seeds for each species. The seeds were harvested from the plants in the greenhouse and surface sterilized by washing in a 6% calcium hypochlorite solution for 15 minutes with shaking and rinsed three times in sterile distilled water.

Treatments for *H. hemerocallidea* seeds:

i) Direct culture in BM1
ii) Culture in BM1 supplemented with 0.5 mg/l BAP
iii) Culture in Lepoivre medium

iv) Cultureoin Lepoivre medium with cold treatment at 4^0C in darkness for six days.

v) Pretreated in 0.2% potassium nitrate (KNO_3) for one hour with shaking followed by culturing in Lepoivre medium.

vi) Culture on BM1 supplemented with 0.5 mg/l BAP and cold treated at 4^0C in darkness for six days prior to exposure to normal growing conditions.

vii) Pretreated in 0.2% potassium nitrate for one hour with shaking followed by culturing in BM1 with 0.5 mg/l BAP.

viii) Culture in BM1 and cold treatment at 4^0C in darkness for six days before subjecting to normal growth conditions.

ix) Soaking in concentrated sulphuric acid for 10 minutes, followed by washing in 70% alcohol and rinsing three times with sterile distilled water and culturing in BM1, $BM1^+$, BM1Kin1, BM1Kin2, BM1Kin3 (Table 1).

x) Soaking in hot water with an initial temperature of approximately 80^0 C until the temperature dropped to about 30^0 C, duration close to 45 mintutes, and subsequently culturing in the same media as in (ix) above.

xi) Sterilized seeds placed between two wet pieces of sterile filter paper, chilled for six days, soaked in concentrated sulphuric acid for ten minutes before rinsing three times with sterile distilled water and subsequently culturing in BM1Kin3.

xii) Sterilized seeds soaked in 2% gibberellic acid (GA_3) for 16 hours and immediately cultured in BM1, $BM1^+$, $BM1^{+++}$, BM1 with 5% (v/v) coconut water (BM1CW1), and BM1 supplemented with 2.4 mg/l kinetin and 5% (v/v) coconut water (KinCW1).

xiii) Seeds surface sterilized and the seed coat crushed with forceps before culturing in BM1 with half-strength MS.

xiv) Seeds surface sterilized in 6% calcium hypochlorite solution for 25 minutes, washed in sterile distilled water three times before crushing the seed coats with forceps and culturing in BM2 with double-strength MS.

Treatments for *H. filiformis* seeds:

i) Seeds soaked in hot water as in x above followed by sterilization in 6% calcium hypochlorite solution for twenty minutes, rinsing three times in sterile distilled water and culturing in BM1, $BM1^+$, $BM1^{++}$, $BM1^{+++}$, BM1Kin1, BM1Kin2, BM1Kin3, BM1BAP1, BM1BAP2, BM1BAP3, and Lep1 (Table 1).

ii) Seeds surface sterilized and the seed coat crushed with forceps before culturing in nutrient broth consisting MS salts, 1 mg/l BAP, 0.1 mg/l IBA, and 0.05 mg/l GA_3, BM1 supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA, and BM1 with half-strength MS.

iii) Seeds surface sterilized and the seed coat crushed with forceps before culturing in BM1 with half-strength MS.

iv) Seeds surface sterilized and the seed coat crushed with forceps before culturing in darkness in BM1 with half-strength MS.

v) Seeds surface sterilized in 6% calcium hypochlorite solution for 25 minutes, washed in sterile distilled water three times before crushing the seed coats with forceps and culturing in BM2 with double-strength MS.

Treatments for *H. argentea* seeds:

Seeds were surface sterilized before crushing the seed coats with forceps and culturing in BM2, BM2 with half-strength MS, BM2 with half-strength MS and 5% (v/v) coconut water, and BM2 supplemented with 1 mg/l BAP and 0.5 mg/l NAA. The seeds were cultured in light or darkness.

2.1.5.6 Root Culture

Roots for *H. acuminata, H. filiformis,* and *H. hemerocallidea* were cleaned and sterilized using the protocol described in section 2.1.2. The roots were then cut into pieces of 0.5 – 1 cm and cultured in BM1, $BM1^{+}$, BM1BAP2, BM1Kin1, and BM1Kin3 media (Table 1).

2.1.5.7 Culture of Corms

Corm explants were cultured for all four *Hypoxis* species but not always in the same type of medium. To save on plant material, a non-destructive strategy was devised where only part of the corm was used whilst the rest remained attached to the plant in the green house and the plant survived and continued growing. Corms were cleaned and surface sterilized using the protocol described in section 2.1.2. The number of explants used varied depending on the availability of material, and in most instances the total number of explants was greatly reduced due to contamination, phenol exudates or infection by endogenous microbial agents. The number of explants that remained for the duration of the culture period ranged between 2 and 8.

i) Culture of *H. acuminata* corms

The corms were divided into three parts: the uppermost part of the corm with a bit of shoot attached; the top part of the corm below the shoot; and the middle part of the corm. The various corm divisions were cultured in one or more of the following media: BM1, $BM1^{++}$, $BM1^{+++}$, BM1BAP1, BM1BAP2, BM1BAP3, BM1BAP5, BM1Kin1, BM1Kin2, BM1Kin3, BM1Kin5, and BM1NAA (table 1).

ii) Culture of *H. argentea* corms

Only the top part of the corm was used for explants. The corms were divided into two parts, the uppermost part with a portion of shoot attached and the part immediately below without shoot. Explants were cultured in BM1, BM1^{++}, BM1$^{+++,}$ BM2, BM2NAA, BM2BAP3, BM2Kin3 BAP3NAA, BM1CW1, BM1CW2, KinCW1, and LEP1 (table 1).

iii) Culture of *H. filiformis* - Lubombo corms

Corm explants were either taken indiscriminately from any part of the corm or at the uppermost part of the corm without a portion of shoot attached. Explants were cultured in BM1BAP3, BM1Kin3, BM1NAA1, BM1IAA5, BAP^{+}NAA, BAPIAA5, BM1NAA1, and BAP3NAA1 (table 1).

iv) Culture of *H. hemerocallidea* corms

Corm explants of *H. hemerocallidea* were derived as in iii) above and cultured in BM1, BM1Kin1, BM1Kin2, BM1Kin3, BM1CW1, BM1CW2, KinCW1, BM1^{++}, BM^{+++}, LEP1, BM1NAA1, BM1IAA5, Kin3KCl, Kin3KCl^{+}, BM2, BM2NAA1, BM2BAP3, and BM2Kin3 (table 1).

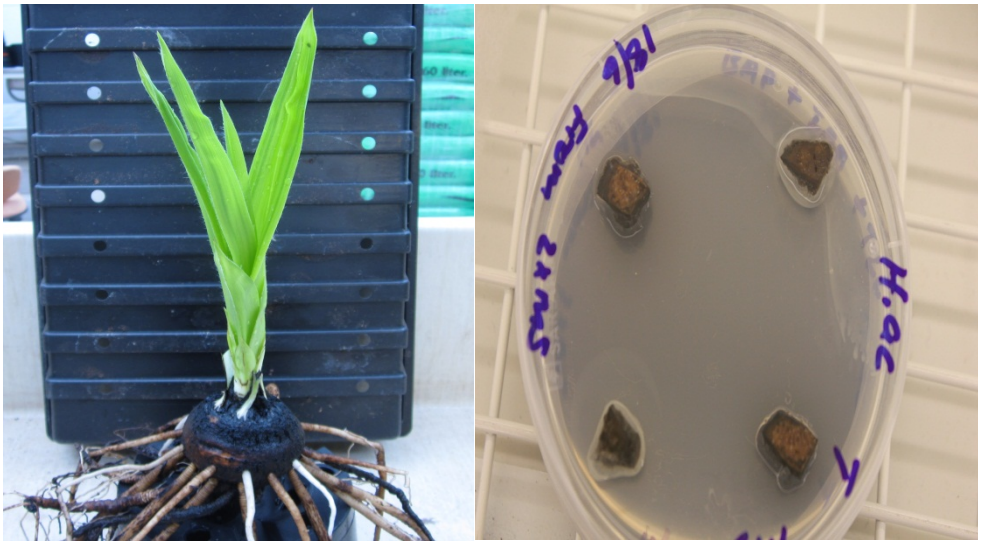

Figure 4: ***Hypoxis*** plant with corm and roots (left) and corm explants in culture (right)

2.2 Transformation of Camelina sativa

2.2.1 Testing of *Camelina* Seeds for Kanamycin Tolerance

Seeds were tested for tolerance of the antibiotic kanamycin before vacuum infiltration was done by growing on germination medium of half-strength MS with 10 g/l sucrose, and 7 g/l Bacto agar supplemented with 0, 50, 100, 150, 200, 250, 300, and 350 mg/l kanamycin. Two petri dishes with 25 seeds each were used for each treatment. The results were recorded at 7 and 14 weeks.

2.2.2 Gene Construct and Activation of *Agrobacterium* Strain

The *Agrobacterium tumefaciens* strain AGL1, that has successfully been used in floral dip transformation of radish (Curtis, 2004) and wheat (Agarwal et al., 2008), harboring three separate gene constructs: *AtHb2* a haemoglobin gene from Agrabidopsis (Jokipii-Lukkari et al., 2009), *BvHb2* a haemoglobin gene from sugar beet (Salort et al., 2010), and *vhb* a homologous bacterial gene (Dikshit and Webster, 1988) was used in the study.

Prior to infiltration, the bacteria kept at $^{-}85^{0}$ C were activated by culturing on the solid LB medium containing, 50 mg/l of the antibiotics kanamycin and rifamycin respectively, overnight at 28^{0} C. This was followed by growing the bacterial cells in liquid LB supplemented with the same concentration of the antibiotics at 28^{0} C overnight with shaking. Bacterial cells were harvested by centrifugation at 4400 rpm for 15 minutes and the pellets were suspended in a solution consisting MS salts, 50 g/l sucrose and 0.05% (v/v) silwet (Lu and Kang, 2008a). The optical density (OD_{600}) of the bacterial cell suspension was maintained between 0.5 and 1.

2.2.3 Inoculation of Plants

Flowering plants of *Camelina sativa*, cultivar Celine, grown under controlled conditions with day and night temperatures of 21^{0} C and 18^{0} C, respectively, photoperiod of 16 hours and 70% relative humidity were vacuum infiltrated according to Lu and Kang (2008b) with the *Agrobacterium* strain AGL1 harboring the three gene constructs. Between three and six plants were used for each gene construct depending on the flowering stage. Unopened flower buds and old flowers were removed and only freshly opened flowers were inoculated by placing in a vacuum desiccator with the inflorescence dipped in the bacterial solution contained in a beaker (Figure 6A). A pressure of 85 kPa was applied to the desiccator for 10 minutes before the plants were removed and covered with a plastic bag for approximately 24 hours before they were taken back to the normal growing conditions described above. The plants were allowed to grow (Figure 6B) until the seeds reached maturity and were harvested. The treatment was repeated two times.

2.2.4 Screening for Transgenic Seeds

Seeds (Table 2) were planted *in vitro* in the selection medium consisting of half-strength MS, 10 g/l sucrose, 50 mg/l kanamycin, 7 g/l Bacto agar, and the pH was adjusted to 5.7 with before adding agar.

Table 2: Number of *C. sativa* seeds harvested and screened in each gene construct

Gene Construct	Replicate 1	Replicate 2	Replicate 3	Total
AtHb2	400	186	324	910
BvHb2	359	105	156	620
vhb	200	111	312	623

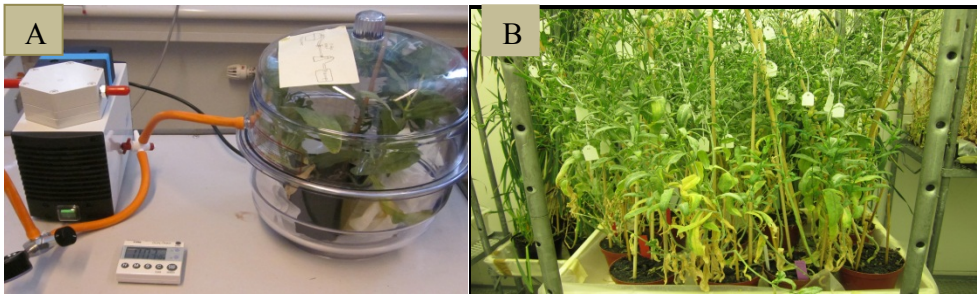

Figure 5: Vacuum infiltration of *Camelina sativa* and inoculated plants. A – Vacuum infiltration; B – Inoculated plants in growth chamber

2.3 Transformation of Crambe abyssinica

2.3.1 Gene Construct and Activation of *Agrobacterium* Strain

The vector FWS 3-1 containing three genes: The fatty acid reductase gene (*FAR*), the wax synthase gene (*WS*), and the marker *DsRed* (a fluorescent protein) carried by the *Agrobacterium tumefaciens* strain AGL1 was used in this study. The bacteria were activated, grown and harvested as described in 2.2.2. Bacterial concentration was maintained between OD_{600} 0.5 and 0.6.

2.3.2 Inoculation of Plants

Early flowering plants of *Crambe abyssinica,* variety Galactica were infiltrated with the strain AGL1 and inoculated according to the protocol described for *Camelin sativa* in 2.2.3. The treatment was replicated two times; flowers from two and three plants were inoculated respectively.

2.3.3 Screening for Transgenic Seeds

Mature seeds were harvested and screened (Table 3) for the presence of the *DsRed* marker using a double protein fluorescent light before culturing on selection medium consisting half-strength MS, 10 g/l sucrose, 50 mg/l kanamycin, and 7 g/l Bacto agar with pH 5.7.

Table 3: Number of *C. abyssinica* seeds harvested and screened

Gene Construct	Replicate 1	Replicate 2	Total
FWS 3-1	30	86	116

3.0 Results

3.1 In vitro Establishment and Regeneration of Hypoxis

Adding 0.5 – 1.0% activated charcoal was effective in reducing the build-up of phenolic exudation in the culture media, in addition, 5 – 15% (v/v) coconut water added in the culture medium also reduced the build-up of phenolic exudates in the medium. However, 0.5 g/l polyvinyl pyrrolidone (PVP) added in the medium failed to alleviate this problem. Washing infected explants and shoots with a solution of 0.1% mercuric chloride ($HgCl_2$) for five minutes helped in clearing almost all noticeable infections. Activated charcoal was added in most culture media, whereas mercuric chloride was only used when the need arose.

Explants of leaves, flower buds, roots and basal part of inflorescence peduncles failed to regenerate in any of the media (Table 1) in which they were cultured in all four *Hypoxis* species. Explant cultures of the basal part of the inflorescence peduncles were only done on *H. filiformins* and *H. hemerocallidea*. Explants of inflorescence peduncles of *H. filiformis* failed to regenerate after 18 weeks whereas those of *H. hemerocallidea* were overcome by microbial infections 3 weeks in culture and did not survive. Leaf explants turned brown and had to be sub-cultured regularly but when 1-1.5% activated charcoal or 5-15% coconut water were added in the media browning was notably reduced. Despite these measures no callus, shoots or roots were produced during the culture period.

3.1.1 Culture of Shoot Apexes

Two (50%) of the shoot apexes of *H. hemerocallidea* were able to grow into shoots when cultured in solid MS medium supplemented with 1.5 mg/l BAP, 0.5 mg/l NAA, 20 g/l sucrose and 1.5% activated charcoal with pH 5.8 (Figure 10A). However, the shoots were lost later due to fungal infection at a very early stage.

3.1.2 Culture of Seeds

Seeds only germinated when the seed coats were crushed with forceps before culturing (Figure 6), all other seed treatments did not respond to the culture media. All seeds were initially cultured in half-strength PGR free MS medium; once germinated, the young seedlings were transferred to full-strength MS without PGRs. Germination commenced about two weeks after culture with *H. argentea* obtaining the highest germination percentage of 29.9 in half-strength MS (Table 4).

Table 4: *In vitro* seed germination of *Hypoxis* species with crushed seed coats

Culture Medium	Species	No. of Seeds	Germinated Seeds	Germination %
Half-strength MS (BM1)	*H. argentea*	67	20	29.9
	*H. filiformis** - Lubombo	19	4	21.1
	H. filiformis - Shiselweni	35	5	14.3
BM2 with double-strength MS and 5% (v/v) coconut water	*H. filiformis* - Shiselweni	10	1	10
	H. hemerocallidea	5	0	0

*1 seed produced callus and 6 shoots; this was counted as one germinated seed

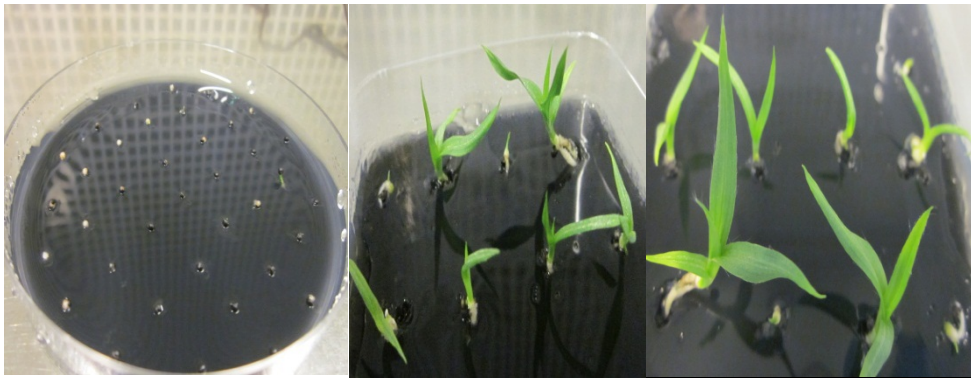

Figure 6: Germinating seeds on half-strength MS (left) of *H. argentea* with crushed seed coats and 5-week old seedlings on full-strength MS (middle and right)

3.1.3 Culture of Corms

3.1.3.1 *Hypoxis acuminata*

Corm explants of *H. acuminata* only responded to *in vitro* when cultured with a piece of shoot attached. Explants cultured without the shoot attached did not produce callus, shoots or roots during the culture period (Table 5). Direct shoots were produced in MS without PGRs, however most media supplemented with PGRs had indirect shoots via callus. Explants initially cultured in PGR free MS medium for 11 weeks before culturing in MS supplemented with 3 mg/l kinetin produced up to 2 shoots per explant On the other hand, explants transferred from MS without PGRs to MS supplemented with 5 mg/l also produced up to 2 shoots per explant and in some instances with roots but in other cases thick roots with no shoots were produced by the same explants (Figure 8).

Table 5: Response of *H. acuminata* corm explants cultured with a portion of shoot to different media after 21 weeks of *in vitro* culture

PGR Concentration	No. of Explants	Callus (%)	Shoots (%)	No. of shoots/explant (Mean ± SE)	Roots (%)
PGR free	6	0.0	16.7	1.0	0.0
3 mg/l BAP	2	100.0	50.0	1.0	0.0
3 mg/l kinetin	2	0.0	50.0	1.0	0.0
PGR free to 5 mg/l kinetin	2	100.0	100.0	1.5 ± 0.5	100.0
PGR free to 3 mg/l kinetin	2	50.0	100.0	2.0 ± 1.0	0.0

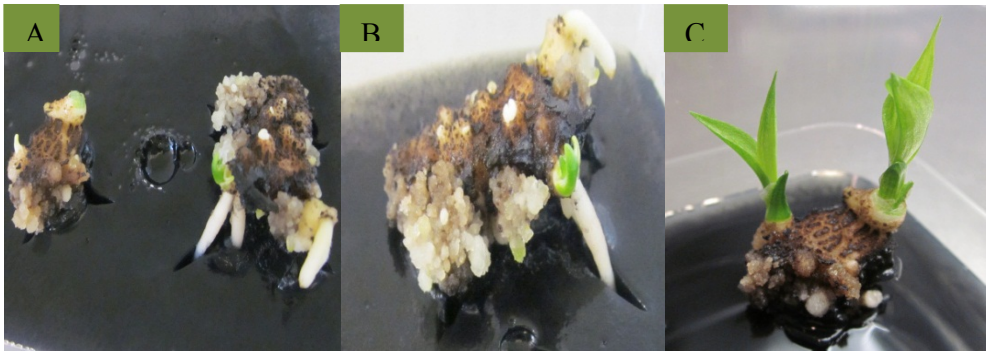

Figure 7: Corm explants of *H. acuminata* at different stages of development. A – Callus and roots of *H. acuminata* cultured on MS with 5 mg/l kinetin; B – Callus, root and emerging shoot in MS supplemented with 5 mg/l kinetin; C – Indirect regeneration via callus, 9 week old shoots.

3.1.3.2 *Hypoxis argentea*

Corm explants were cultured with or without a piece of shoot attached. Corm explants with a piece of shoot attached were only cultured in MS medium without PGRs and did not produce any callus, shoots or roots during the culture period (Table 6). However, corm explants that were cultured without shoot attachment produced callus, shoots, or roots on MS media with or without PGRs (Figure 8). Seventy-five percent of corm explants initially cultured in MS with 1 mg/l NAA before transferring to MS medium with 3 mg/l kinetin produced shoots via callus; however the highest mean number of indirect shoots per explant (8) was obtained when explants were directly cultured in MS medium with 3 mg/l BAP. Explants that were directly cultured in MS with 3 mg/l kinetin produced only callus (100%) as indicated in Table 6.

Table 6: Response of *H. argentea* corm explants to different media after 9 weeks *in vitro* culture

PGR Concentration	No. of Explants	Callus (%)	Shoots (%)	No. of shoots/explant (Mean ± SE)	Shoots with Roots
PGR free	7	14.3	0.0	-	-
3 mg/l BAP	8	37.5	37.5	7.7 ± 0.9	0
3 mg/l BAP from 1 mg/l NAA*	4	75.0	50.0	4.5 ± 2.5	0
3 mg/l kinetin	6	100.0	0.0	-	-
3 mg/l kinetin from 1 mg/l NAA*	4	75.0	75.0	3.0 ± 1.0	1

*Explants transferred from MS with 1 mg/l NAA to MS with 3 mg/l BAP or kinetin after 5 weeks.

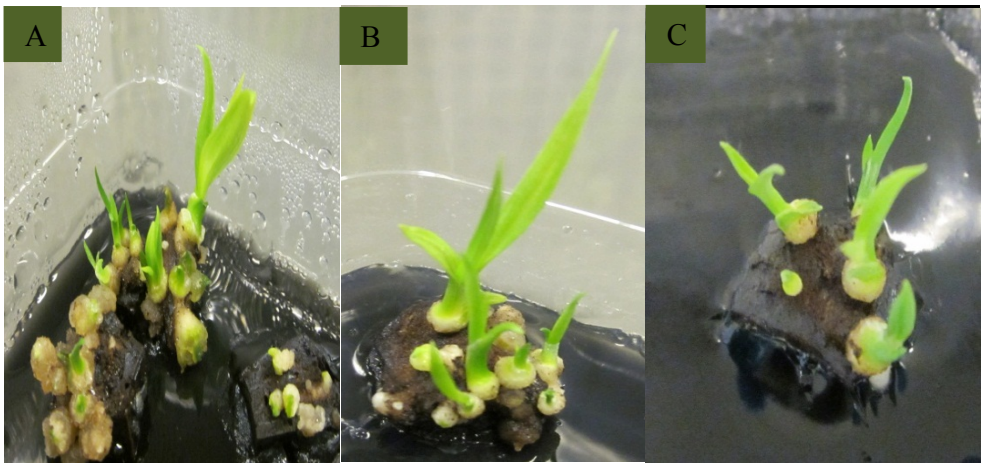

Figure 8: Shoots of *H. argentea* from corm explants**.** A – Callus and indirect shoots from corm explants cultured in MS with 3 mg/l BAP; B – Shoots arising from corm explants first cultured in MS with 1mg/l NAA for 5 weeks and transferred to MS with 3 mg/l BAP; C – Direct shoots from corm explant cultured in MS supplemented with 1 mg/l NAA before culturing in MS with 3 mg/l kinetin.

3.1.3.3 *Hypoxis filiformis*

One hundred percent regeneration was achieved in *H. filiformis* corm explants cultured in MS supplemented with 1 mg/l NAA for 18 weeks, MS supplemented with 3 mg/l BAP after first culturing in MS with 1 mg/l NAA for 5 weeks, and MS supplemented with 3 mg/l BAP following an initial culture in MS with 1 mg/l NAA and 3 mg/l BAP for 9 weeks (Table 7). The highest mean number (17) of shoots per explant was obtained when corm explants were cultured in MS supplemented with 3 mg/l kinetin, from the onset. Well-developed shoots were then transferred to MS medium supplemented with 2 mg/l BAP and 5 mg/l IAA for rooting. In this medium about 50% shoots produced roots within two weeks. Figure 9 illustrates the response of *H. filiformis* corm explants to the different media.

Table 7: Response of *H. filiformis* corm explants to different media after 18 weeks *in vitro* culture

PGR Concentration	No. of Explants	Callus (%)	Shoots (%)	Mean no. of shoots/explant (Mean ± SE)	Shoots with Roots
PGR free	4	0.0	50.0	1.0 ± 0.0	0
1 mg/l NAA	3	100.0	100.0	7.7 ± 2.3	0
3 mg/l BAP from 1 mg/l NAA	5	100.0	100.0	5.6 ± 1.6	5
3 mg/l BAP from 1 mg/l NAA + 3 mg/l BAP	7	100.0	100.0	10 ± 3.7	1
3 mg/l kinetin	4	50.0	50.0	17.0 ± 14.0	0
3 mg/l kinetin from 1 mg/l NAA	8	75.0	12.5	10.0	0
5 mg/l BAP from 1 mg/l kinetin	4	75.0	75.0	14.7 ± 2.0	1

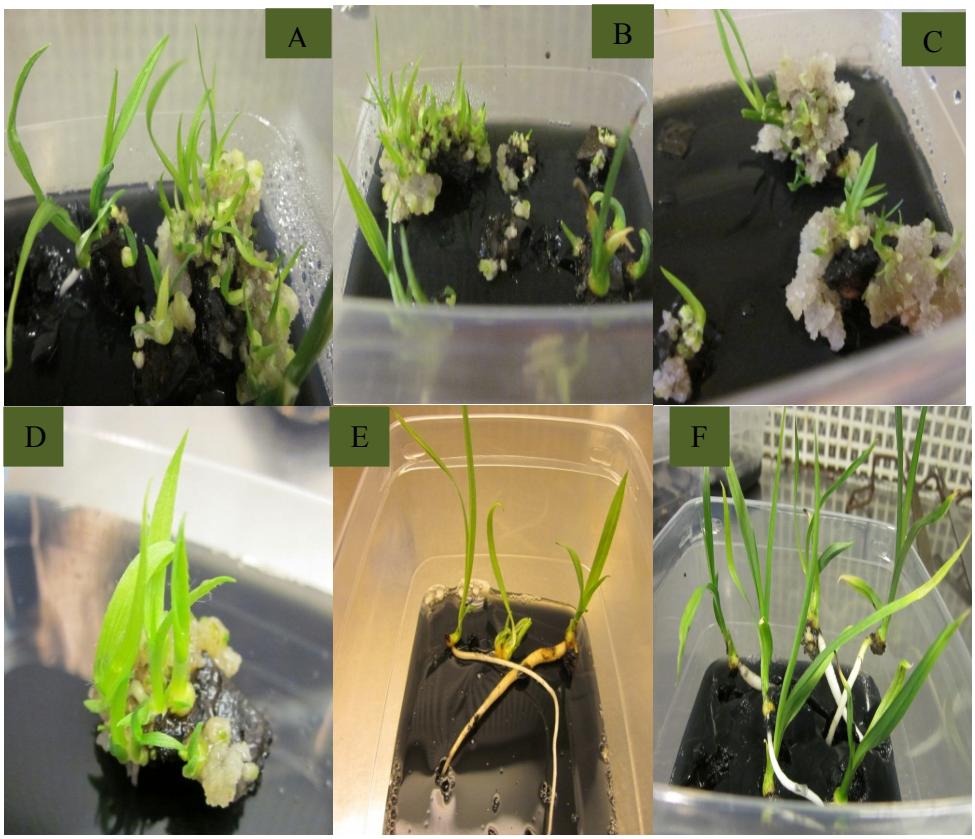

Figure 9: Shoots of *H. filiformis* from corm explants cultured in different media. A – callus and multiple shoots produced in MS with 1 mg/l NAA; B – Shoots in MS with 3 mg/l BAP (after culturing in MS with 1 mg/l NAA); C – Callus and shoots in MS with 5 mg/l BAP (after culturing in MS with 1 mg/l NAA); D – corm explant with callus and multiple shoots in MS with 3 mg/l kinetin; E - 17-week-old shoots with long roots in MS with 3 mg/l BAP initially cultured in MS with supplemented 1 mg/l NAA for 5 weeks; F - shoots rooting in MS supplemented with 2 mg/l BAP and 5 mg/l IAA.

3.1.3.4 *Hypoxis hemerocallidea*

Corm explants of *H. hemerocallidea* took a very long time to respond to *in vitro* culture compared to the other three species, and the response was relatively poor (Figure 10B). Shoots were only produced when the explants were first cultured in MS medium supplemented with 1 mg/l NAA for 9 weeks before culturing in MS medium supplemented with 3 mg/l BAP, 3 mg/l kinetin, or 5 mg/l kinetin (Table 8). Direct shoots started to develop 10 weeks after transfer to the MS medium supplemented with cytokinins, and growth of the shoots was very slow and retarded.

Table 8: Response of *H. hemerocallidea* corm explants to different media after 31 weeks *in vitro* culture

PGR Concentration	No. of Explants	Callus (%)	Shoots (%) (Mean ± SE)	Mean no. of shoots/explant	Roots (%)
3 mg/l BAP*	2	0.0	50.0 ± 0.5	2.0	0.0
3 mg/l kinetin*	3	0.0	33.3 ± 0.3	1.0	0.0
5 mg/l kinetin*	5	0.0	40.0 ± 0.2	1.0	0.0

*Initially cultured on MS with 1mg/l NAA for 9 weeks

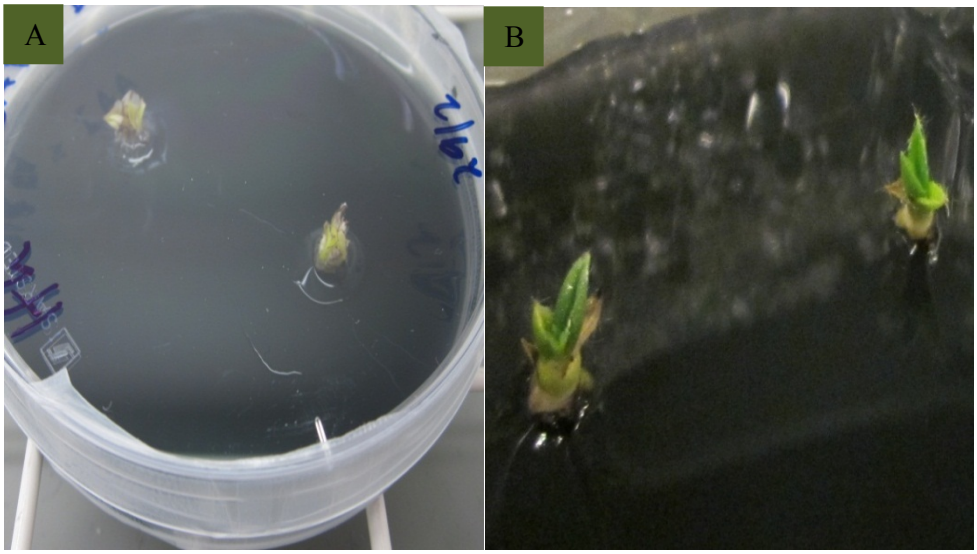

Figure 10: Shoots of *H. hemerocallidea* from shoot meristems and corm explants. A – Shoots from shoot meristem explants cultured on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA, four weeks in culture; B – Shoots from corm explants on MS supplemented with 3 mg/l BAP.

3.2 Transformation of Camelina sativa

3.2.1 Testing of *Camelina* Seeds for Kanamycin Tolerance

At 50 mg/l kanamycin, germination was vigorous and the germinated seedlings looked green and healthy but subsequent emerging true leaves were all pale. With a higher dose of kanamycin seedlings were less vigorous, appeared paler, retarded and exhibited slow growth as illustrated in Figure 11.

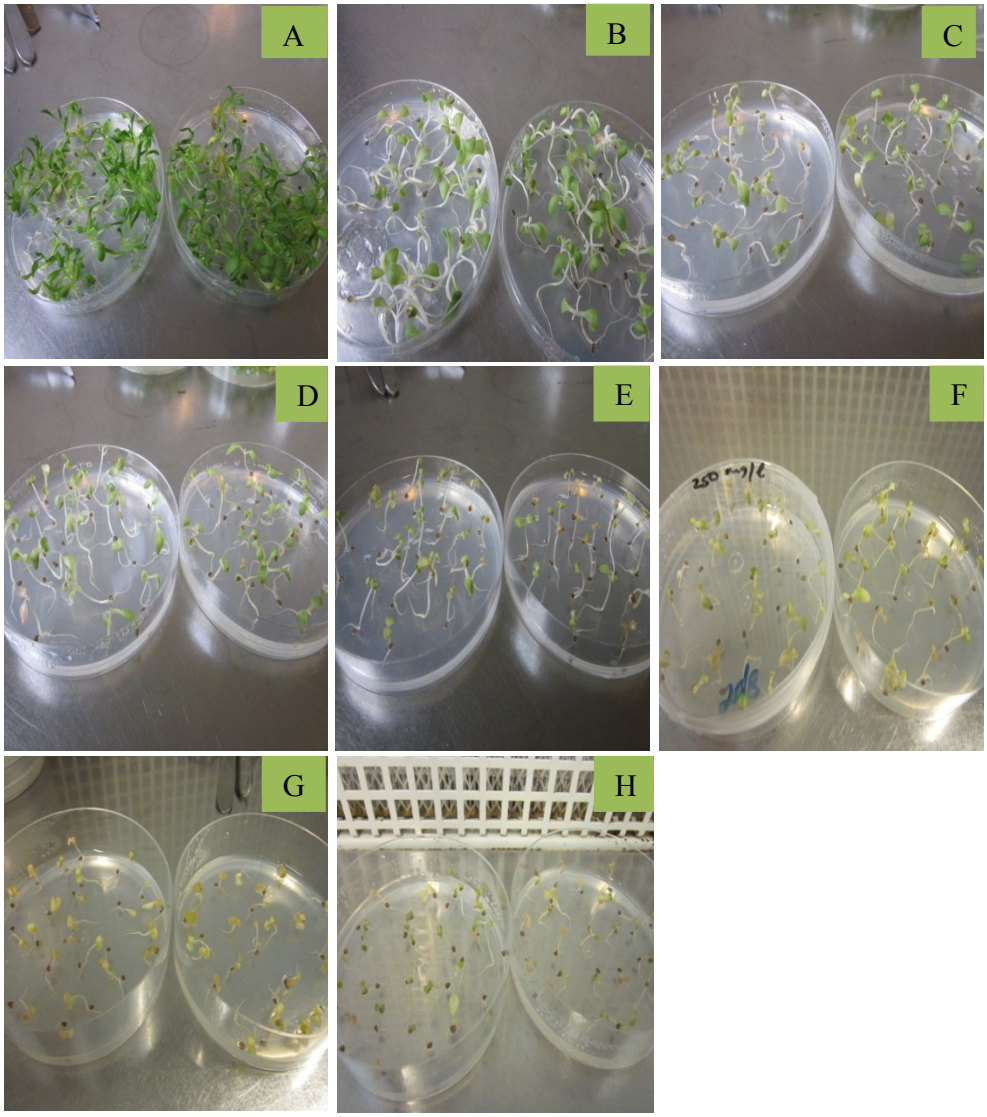

Figure 11: Seedlings of *Camelina sativa* on germination medium of various kanamycin concentrations. A – kanamycin free; B - 50 mg/l kanamycin; C - 100 mg/l; D - 150; E - 200; F 250; G - 300; H - 350.

3.2.2 Inoculated Plants

Inoculation of open flowers of *Camelina sativa* with *Agrobacterium* harboring the constructs *AtHb2*, *BvHb2*, or *vhb* by vacuum infiltration failed to produce transgenic seeds. In the selection medium containing 50 mg/l kanamycin, true leaves of seedlings arising from the first generation of the inoculated inflorescence were pale and within a few days the whole seedling would turn pale and die (Figure 12). In some instances chimeric-looking seedlings were observed but these later turned pale and subsequently died.

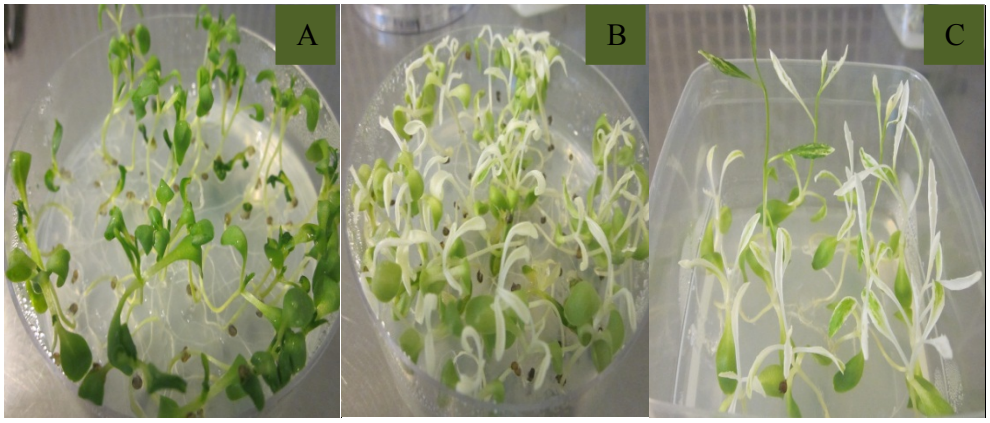

Figure 12: Seedlings of *C. sativa* from non-inoculated and inoculated plants. A – Negative control, kanamycin-free medium; B – Positive control, 50 mg/l kanamycin; C – Seedlings from inoculated plants

3.3 Transformation of Crambe abyssinica

No transgenic seeds were obtained from plants of *C. abyssinica* inoculated with *Agrobacterium* harbouring the FSW 3-1 vector with the *FAR*, *WS*, and *DsRed* genes. When first generation seeds harvested from the inoculated plants were viewed under fluorescent light no fluorescent protein was detected; in addition, seeds cultured on the selection germination medium containing 50 mg/l kanamycin produced seedlings that had yellow leaves which later became pale, an indication that they were non-transgenic (Figure 13).

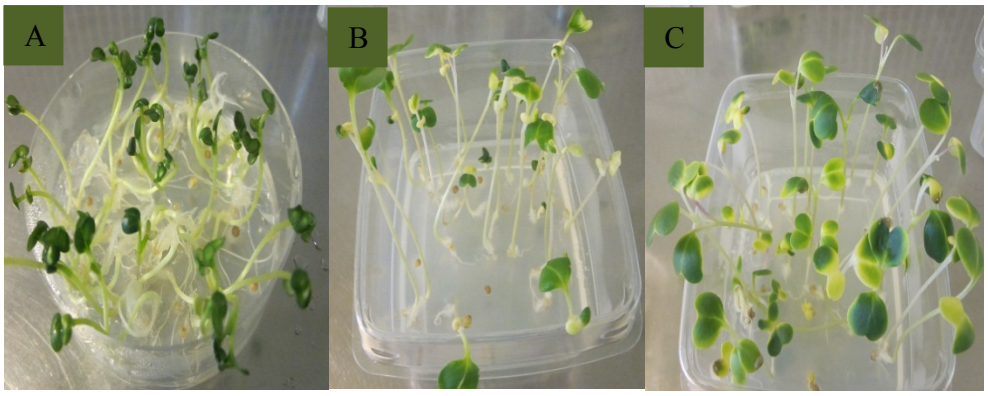

Figure 13: Seedlings of *C. abyssinica* from non-inoculated and inoculated plants. A – Normal non-inoculated seedlings; B - Control, non-inoculated seedlings on 50 mg/l kanamycin; C – Seedlings from inoculated plants in 50 mg/l kanamycin.

4.0 Discussion

4.1 Regeneration of Hypoxis

It is well known that *in vitro* establishment and regeneration is affected by many factors, such as explant type, the physiological status of mother plants, genotype, species and medium composition, type and combination of PGRs, and culturing conditions. In this study, among all the explant types tested, *in vitro* regeneration was only obtained from shoot meristems and corms. Seeds only responded when the seed coats were crushed. The results obtained from corm explants of the four *Hypoxis* species cultured in the same type of media were not similar. Corm explants of some species produced shoots in MS without PGRs while others did not; others produced one or more shoots when cultured in MS supplemented with a particular type of PGR while others only produced callus and no shoots in the same medium supplemented with equally the same PGR concentration. The presence and concentrations of PGRs in the media were at most influential in the initiation and establishment of the regeneration process but in some instances, like in the case of *H. acuminata* it was necessary to first culture corm explants on PGR-free MS before transferring to MS with PGRs. Regeneration of corm explants in medium without PGRs was only obtained in *H. filiformis*; and in *H. acuminata* when the corms were cultured with a portion of shoot attached. Almost all corm explants responded fairly well to *in vitro* regeneration when first cultured in medium containing 1 mg/l of the auxin NAA before transferring to media with higher concentrations of cytokinins (BAP or kinetin).

As already noted, the response of corm explants to *in vitro* regeneration in the four *Hypoxis species* varied considerably. A similar observation was made by Appleton and van Staden (1995b) when conducting regeneration studies on four *Hypoxis* species that included *H. acuminata* and *H. hemerocallidea*, they found that the response varied with respect to growth regulation requirement, shoot multiplication and callus production. In this study, *Hypoxis filiformis* was the most responsive species to *in vitro* as direct shoots were produced from corm explants only 3 weeks after culture on MS medium without PGRs (50% regeneration) and within 5 weeks when 1 mg/l NAA (100% regeneration) was added. Corm explants of this species produced a lot of friable callus in MS medium supplemented with PGRs, which at some instances covered the whole explant and spilt over to the medium; most of these gave rise to shoots. *H. hemerocallidea* was very slow in responding to *in vitro* regeneration, shoots were only obtained 19 weeks after culture, 9 weeks in 1 mg/l NAA and 10 weeks in 3 mg/l BAP or kinetin; even when shoots were produced they were pale and stunted. No shoots were produced when *H. hemerocallidea* corm explants were directly cultured in either 3 mg/l BAP or kinetin. Contrary to this, Ndong *et al* (2006) were able to achieve 100% regeneration efficiency in corm explants of *H. hemerocallidea* with 5-8 direct shoots in basal MS medium

supplemented with 3 mg/l kinetin. This variation may be caused by the physiological condition of the plants when the explants were taken, and growth conditions. Appleton and van Staden (1995b) stated that the greatest growth response was achieved when *H. hemerocallidea* corm explants were cultured in initiation medium comprising MS supplemented with the same proportion of 1 mg/l NAA and BA.

Corm explants of *H. acuminata* produced direct shoots within 11 weeks when cultured with a piece of shoot attached in MS medium without PGRs, while corm explants of *H. argentea* and *H. hemerocallidea* did not produce any shoots when cultured with a portion of shoot attached in PGR-free MS. However, corm explants of *H. argentea* produced callus and shoots when supplemented with 3 mg/l BAP or kinetin with or without prior culture in 1 mg/l NAA. On the other hand, corm explants of *H. acuminata* responded positively (100% regeneration) when first cultured in MS medium without PGRs before culturing in MS supplemented with 3 mg/l or 5 mg/l kinetin. These results indicate that *in vitro* regeneration is a complex process that is affected by many factors.

In vitro germination of *Hypoxis* seeds seem to be strongly blocked by their hard seed coats, evidenced by the study conducted by Appleton *et al* (2012) who reported that seeds of *H. colchicifolia* failed to germinate *in vitro*. Similarly, in this study seeds did not germinate and all dormancy breaking efforts applied failed to induce germination of the four *Hypoxis* species. Germination was obtained only when the seed coat was broken with forceps before placing seeds on culture medium. With this method up to 30% germination was obtained in *H. argentea* seeds, and 21% in *H. filiformis*.

4.2 Transformation of Camelina sativa and Crambe abyssinica

Lu and Kang (2008a) found that by using *Agrobacterium*-mediated transformation with the fluorescent protein *DsRed* over 1% transgenic seeds of *Camelina sativa* could be obtained through vacuum infiltration. The success of the investigation resulted to an increase in oil seed content, particularly fatty acids. Successful floral dip methods for transforming *Camelina* plants, without the need for vacuum filtration have been developed (Liu et al., 2008, Kuvshinov et al., 2011). However, in this study no transgenic seeds were obtained using the vacuum infiltration method for *Camelina* transformation. This is probably due to a very limited number of plants and flowers used as a result of time limitation.

Li *et al.* (2010) reported developing efficient protocols for regenerating and transforming *Crambe*, and a highly regeneration protocol (Li et al., 2011) that involve the use of hypocotyls as explants. They were able to obtain a transformation efficiency

of 2.1% using tissue culture-based methods. On the other hand, tissue culture-based transformation methods are time consuming, thus it would be worthwhile to develop an in-planta method for the transformation of crambe hence the reason for conducting this study using the *Agrobacterium* vacuum infiltration method. The results obtained were not positive, and this could be due to a number of factors including insufficient number of plants and flowers used in the test.

5.0 Conclusion

Highly potential *in vitro* regeneration protocols for the four *Hypoxis* species used in this study have been successfully developed where efficient indirect regeneration (100% with 8 shoots per explant) of *Hypoxis filiformis* was obtained when corm explants were cultured in MS medium containing 1 mg/l NAA. However, the highest mean number of shoots per explant (17) was obtained in the same species, on basal MS supplemented with 3 mg/l kinetin. Efficient direct regeneration of *H. acuminata* (100% with 2 shoots per explant) was achieved when corm explants were cultured with a piece of shoot attached in MS supplemented with 3 mg/l kinetin following initial culture on PGR free MS for 11 weeks. Seventy-five percent (3 shoots per explants) regeneration was obtained with corm explants of *H. argentea* cultured in MS supplemented with 3 mg/l kinetin following an initial culture in MS supplemented with 1 mg/l NAA for 5 weeks. Direct regeneration of 50% with 2 shoots per explant was achieved in corm explants of *H. hemerocallidea* initially cultured in MS containing 1 mg/l NAA for 9 weeks before transfer to MS supplemented with 3 mg/l BAP. Seed germination of up to 30% was achieved when seed coats of *Hypoxis argentea* were cracked before growing in half-strength MS basal medium.

The results clearly indicate that the explant type, and the type, concentration, and combination of plant growth regulators play a major role in directing the responsiveness of each *Hypoxis* species to *in vitro* culture. They also indicate the potential of the protocols presented in the regeneration, establishment, and mass production of the four *Hypoxis* species.

Transformation of *Camelina sativa* and *Crambe abyssinica* by *Agrobacteium* vacuum infiltration was unsuccessful even though the same protocol has been successfully used in the transformation of *C. sativa* resulting in the production of transgenic seeds. Repeated use of the protocol and inoculating a relatively large number of flowers may increase the chances of yielding transgenic seeds in both *C. sativa* and *C. abyssinica*.

Acknowledgements

The author is grateful to the following: Professor Li-Hua Zhu, Swedish University of Agricultural Sciences for her guidance, supervision, critical reading and constructive comments on the thesis; the help provided by Annelie Ahlman and Emelie Ivarson in the laboratory; assistance from the staff of the Mushroom Development Unit and the National Genetic Resources Centre, Ministry of Agriculture, Swaziland in collecting plant material; assistance provided by Mr Zacharia Dlamini, Assistant National Herbarium Curator, Ministry of Agriculture, Swaziland, in the identification of *Hypoxis* species; the coordinator of NORDIC-SADC Plant Genetic Resources Centre Dr Moneim Fatih and the NORDIC-SADC Plant Genetic Resources Centre, Lusaka, Zambia for financial support.

References

AGARWAL, S., LOAR, S., STEBER, C. & ZALE*, J. 2008. Floral Transformation of Wheat.

AMUSAN, O. O. G., SUKATI, N. A., DLAMINI, P. S. & SIBANDZE, F. G. 2007. Some Swazi phytomedicines and their constituents. *African Journal of Biotechnology,* 6**,** 267-272.

APPLETON, M. R., G.D.ASCOUGH & STADEN, J. V. 2012. *In vitro* regeneration of *Hypoxis colchicifolia* plantlets. *South African Journal of Botany***,** 10.

APPLETON, M. R. & VAN STADEN, J. 1995a. In vitro propagation of a miniature Hypoxis, H-angustifolia. *Ornamental Plant Improvement***,** 95-97.

APPLETON, M. R. & VAN STADEN, J. 1995b. Micropropagation of some South African Hypoxis species with medicinal and horticultural potential. *Ornamental Plant Improvement***,** 75-77.

ARC. 2010. *Crop Science: Medicinal plants* [Online]. Pretoria: Agricultural Research Council. Available: www.arc.agric.za [Accessed 3 Februay 2011 2011].

BIRMETA, G. 2004. *Genetic Variability and Biotechnological Studies for the Conservation and Improvement of Ensente ventricosum.* PhD Doctoral thesis, Swedish University of Agricultural Sciences.

BOUKES, G. J. & VAN DE VENTER, M. 2011. Cytotoxicity and mechanism(s) of action of Hypoxis spp. (African potato) against HeLa, HT-29 and MCF-7 cancer cell lines. *Journal of Medicinal Plants Research,* 5**,** 2766-2774.

BOUKES, G. J., VAN DE VENTER, M. & OOSTHUIZEN, V. 2008. Quantitative and qualitative analysis of sterols/sterolins and hypoxoside contents of three Hypoxis (African potato) spp. *African Journal of Biotechnology,* 7**,** 1624-1629.

BRUUN, J. & MATCHETT, J. 1963. Utilization potential of Crambe abyssinica. *Journal of the American Oil Chemists' Society,* 40**,** 1-5.

BÜCHSENSCHÜTZ-NOTHDURFT, A., SCHUSTER, A. & FRIEDT, W. 1998. Breeding for modified fatty acid composition via experimental mutagenesis in Camelina sativa (L.) Crtz. *Industrial Crops and Products,* 7**,** 291-295.

CHAWLA, H. S. 2009. *Introduction to Plant Biotechnology,* New Hampshire, Science Publishers.

CHHIKARA, S., DUTTA, I., PAULOSE, B., JAIWAL, P. K. & DHANKHER, O. P. 2012. Development of an Agrobacterium-mediated stable transformation method for industrial oilseed crop *Crambe abyssinica* 'BelAnn'. *Industrial Crops and Products,* 37**,** 457-465.

CURTIS, I. S. 2004. Production of Transgenic Crops by the Floral-Dip Method.

DAUBOS, P., GRUMEL, V., IORI, R., LEONI, O., PALMIERI, S. & ROLLIN, P. 1998. Crambe abyssinica meal as starting material for the production of enantiomerically pure fine chemicals. *Industrial Crops and Products,* 7**,** 187-193.

DIKSHIT, K. L. & WEBSTER, D. A. 1988. Cloning, characterization and expression of the bacterial globin gene from Vitreoscilla in Escherichia coli. *Gene,* 70**,** 377-386.

DLAMINI, W. M. 2009. Characterization of the July 2007 Swaziland fire disaster using satellite remote sensing and GIS. *Applied Geography,* 29**,** 299-307.

DREWES, S. E., ELLIOT, E., KHAN, F., DHLAMINI, J. T. B. & GCUMISA, M. S. S. 2008. Hypoxis hemerocallidea-Not merely a cure for benign prostate hyperplasia. *Journal of Ethnopharmacology,* 119**,** 593-598.

FRAME, D. D., PALMER, M. & PETERSON, B. 2007. Use of Camelina sativa in the diets of young turkeys. *Journal of Applied Poultry Research,* 16**,** 381-386.

FRÖHLICH, A. & RICE, B. 2005. Evaluation of Camelina sativa oil as a feedstock for biodiesel production. *Industrial Crops and Products,* 21**,** 25-31.

GRAUDA, D., LAPINA, L., STRAMKALE, V. & RASHAL, I. 2007. Camelina Sativa as a Crop for Diversification of Agriculture and as a Producer of High Quality Oil. *Environment, Technology, Resources, Proceedings***,** 262-265.

JOKIPII-LUKKARI, S., FREY, A. D., KALLIO, P. T. & HÄGGMAN, H. 2009. Intrinsic non-symbiotic and truncated haemoglobins and heterologous Vitreoscilla haemoglobin expression in plants. *Journal of Experimental Botany,* 60**,** 409-422.

KARVONEN, H. M., ARO, A., TAPOLA, N. S., SALMINEN, I., UUSITUPA, M. I. J. & SARKKINEN, E. S. 2002. Effect of [alpha]-linolenic acid[ndash]rich Camelina sativa oil on serum fatty acid composition and serum lipids in hypercholesterolemic subjects. *Metabolism,* 51**,** 1253-1260.

KATERERE, D. R. & ELOFF, J. N. 2008. Anti-bacterial and anti-oxidant activity of Hypoxis hemerocallidea (Hypoxidaceae): Can leaves be substituted for corms as a conservation strategy? *South African Journal of Botany,* 74**,** 613-616.

KOCYAN, A., SNIJMAN, D. A., FOREST, F., DEVEY, D. S., FREUDENSTEIN, J. V., WILAND-SZYMANSKA, J., CHASE, M. W. & RUDALL, P. J. 2011. Molecular phylogenetics of Hypoxidaceae - Evidence from plastid DNA data and inferences on morphology and biogeography. *Molecular Phylogenetics and Evolution,* 60**,** 122-136.

KUVSHINOV, V., KANERVA, A., KOIVU, K. & KUVSHINOVA, S. 2011. *Transformation in Camelina sativa*. United States of America patent application 12/288,791. 22 March 2011.

LAGHETTI, G., PIERGIOVANNI, A. R. & PERRINO, P. 1995. Yield and oil quality in selected lines of Crambe abyssinica Hochst. ex R.E. Fries and C. hispanica L. grown in Italy. *Industrial Crops and Products,* 4**,** 203-212.

LAZZERI, L., LEONI, O., CONTE, L. S. & PALMIERI, S. 1994. Some technological characteristics and potential uses of Crambe abyssinica products. *Industrial Crops and Products,* 3**,** 103-112.

LEONARD, E. C. 1992. High-erucic vegetable oils. *Industrial Crops and Products,* 1**,** 119-123.

LI, X., AHLMAN, A., LINDGREN, H. & ZHU, L.-H. 2011. Highly efficient in vitro regeneration of the industrial oilseed crop Crambe abyssinica. *Industrial Crops and Products,* 33**,** 170-175.

LI, X. Y., AHLMAN, A., YAN, X. F., LINDGREN, H. & ZHU, L. H. 2010. Genetic transformation of the oilseed crop Crambe abyssinica. *Plant Cell Tissue and Organ Culture,* 100**,** 149-156.

LIU, X. J., LEUNG, S., BROST, J., ROOKE, S. & NGUYEN, T. 2008. Camelina sativa transformation by floral dip and simple large-scale screening of markerless transformants. *In Vitro Cellular & Developmental Biology-Animal,* 44**,** S40-S41.

LPS. Undated. Product Name: African Potato. Available: www.lps-misa.com [Accessed 1 March 2011].

LU, C. & KANG, J. 2008a. Generation of transgenic plants of a potential oilseed crop Camelina sativa by Agrobacterium-mediated transformation. *Plant Cell Reports,* 27**,** 273-278.

LU, C. F. & KANG, J. L. 2008b. Generation of transgenic plants of a potential oilseed crop Camelina sativa by Agrobacterium-mediated transformation. *Plant Cell Reports,* 27**,** 273-278.

LU, C. F., NAPIER, J. A., CLEMENTE, T. E. & CAHOON, E. B. 2011. New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. *Current Opinion in Biotechnology,* 22**,** 252-259.

MANYATSI, A. M., MHAZO, N. & MASARIRAMBI, M. T. 2010. Utilisation of Wetland Plant Resources for Livelihood in Swaziland: The Case of Lobamba Lomdzala Area. *Current Research Journal of Social Sciences,* 2**,** 282-268.

MASTEBROEK, H. D., WALLENBURG, S. C. & VAN SOEST, L. J. M. 1994. Variation for agronomic characteristics in crambe (Crambe abyssinica Hochst. ex Fries). *Industrial Crops and Products,* 2**,** 129-136.

MIETKIEWSKA, E., BROST, J. M., GIBLIN, E. M., BARTON, D. L. & TAYLOR, D. C. 2007. Cloning and functional characterization of the fatty acid elongase 1 (FAE1) gene from high erucic Crambe abyssinica cv. Prophet. *Plant Biotechnology Journal,* 5**,** 636-645.

MURASHIGE, T. & SKOOG, F. 1962. A REVISED MEDIUM FOR RAPID GROWTH AND BIO ASSAYS WITH TOBACCO TISSUE CULTURES. *Physiologia Plantarum,* 15**,** 473-497.

MUUSE, B. G., PETRUS CUPERUS, F. & DERKSEN, J. T. P. 1992. Composition and physical properties of oils from new oilseed crops. *Industrial Crops and Products,* 1**,** 57-65.

MZIZI, J. B. 2002. Leadership, Civil Society and Democratisation in Swaziland. *In:* DEVELOPMENT POLICY MANAGEMENT FORUM, U. N. E. C. F. A. U. (ed.) *No.9.* Adis Ababa: Development Policy Management Forum (DPMF).

NAIR, V. D. P., DAIRAM, A., AGBONON, A., ARNASON, J. T., FOSTER, B. C. & KANFER, I. 2007. Investigation of the antioxidant activity of African potato (Hypoxis hemerocallidea). *Journal of Agricultural and Food Chemistry,* 55**,** 1707-1711.

NDONG, Y. A., WADOUACHI, A., SANGWAN-NORREEL, B. S. & SANGWAN, R. S. 2006. Efficient in vitro regeneration of fertile plants from corm explants of Hypoxis hemerocallidea landrace Gaza - The "African Potato". *Plant Cell Reports,* 25**,** 265-273.

OWIRA, P. M. O. & OJEWOLE, J. A. O. 2009. 'African Potato' (Hypoxis hemerocallidea corm): A Plant-Medicine for Modern and 21st Century Diseases of Mankind? - A Review. *Phytotherapy Research,* 23**,** 147-152.

PAGE, Y. M. & VANSTADEN, J. 1984. Invitro-Propagation of Hypoxis-Rooperi. *Plant Cell Tissue and Organ Culture,* 3**,** 359-362.

RYHANEN, E. L., PERTTILA, S., TUPASELA, T., VALAJA, J. N., ERIKSSON, C. & LARKKA, K. 2007. Effect of Camelina sativa expeller cake on performance and meat quality of broilers. *Journal of the Science of Food and Agriculture,* 87**,** 1489-1494.

SALORT, J. M. M., MOLINERO, A. I. S. & SALOM, R. S. 2010. *Method for producing transgenic plants with increased yield, comprising expressing of haemogrobin from Arabidopsis*. USA patent application 7674953. 9 March 2010.

SATHEKGE, N. R., KRITZINGER, Q. & PRINSLOO, G. 2010. Comparison of secondary metabolite content and antimicrobial activity of four Hypoxis species used in traditional medicine. *South African Journal of Botany,* 76**,** 403-403.

SINGH, Y. 2004. The Genus *Hypoxis*. Available: www.plantzafrica.com [Accessed 10 May 2012].

SINGH, Y. 2007. Hypoxis (Hypoxidaceae) in southern Africa: Taxonomic notes. *South African Journal of Botany,* 73**,** 360-365.

SNTC 2011. Swaziland's Flora Database. July 2011 ed.: Swaziland National Trust Commission.

TERPINC, P., POLAK, T., MAKUC, D., ULRIH, N. P. & ABRAMOVIČ, H. 2012. The occurrence and characterisation of phenolic compounds in Camelina sativa seed, cake and oil. *Food Chemistry,* 131**,** 580-589.

VAN WYK, B. E. 2008. A broad review of commercially important southern African medicinal plants. *Journal of Ethnopharmacology,* 119**,** 342-355.

VAN WYK, B. E. 2011. The potential of South African plants in the development of new medicinal products. *South African Journal of Botany,* 77**,** 812-829.

WANG, Y. P., TANG, J. S., CHU, C. Q. & TIAN, J. 2000. A preliminary study on the introduction and cultivation of Crambe abyssinica in China, an oil plant for industrial uses. *Industrial Crops and Products,* 12**,** 47-52.

VINESI, P., SERAFINI, M., NICOLETTI, M., SPANO, L. & BETTO, P. 1990. Plant-Regeneration and Hypoxoside Content in Hypoxis-Obtusa. *Journal of Natural Products,* 53**,** 196-199.

ZUBR, J. 1997. Oil-seed crop: Camelina sativa. *Industrial Crops and Products,* 6**,** 113-119.

ZWANE, P. E., MASARIRAMBI, M. T., MAGAGULA, N. T., DLAMINI, A. M. & BHEMBE, E. 2011. Exploitation of *Agave americana* L plant food security in Swaziland. *American Journal of Food and Nutrition*.