

**MICROPROPAGATION OF  
*HYPOXIS COLCHICIFOLIA* BAKER,  
A VALUABLE MEDICINAL PLANT**

by

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## PREFACE

### DECLARATION

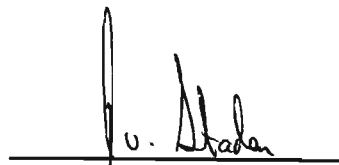
I hereby declare that this thesis, unless acknowledged to the contrary in the text, is the result of my own investigation under the supervision of Professor J. van Staden, Research Centre for Plant Growth and Development, School of Botany and Zoology, Faculty of Science and Agriculture, University of Natal, Pietermaritzburg, and has not been submitted, in part or in whole, to any other tertiary institution.

  
Margaret Rae Appleton

12-12-2003  
Date

### CONFIRMATION

As the candidate's supervisor I confirm that the above is correct and that I have approved this thesis for submission.

  
Professor J van Staden

23/12/2003  
Date

**“W**ithin the infant rind of this weak flower  
**P**oison hath residence and medicine power.”

(William Shakespeare, Friar Laurence in Romeo and Juliet, Act II, Scene III.)

## ABSTRACT

The large geophytic monocotyledon, *Hypoxis colchicifolia* Baker, has been identified for the importance of its corm extracts in the development of a potential non-toxic prodrug for the treatment of inflammation, certain malignancies and HIV-infection. The underground corms of this plant are also commonly used for therapeutic applications in traditional medicine in KwaZulu-Natal where it primarily occurs. A review of published literature revealed, however, that *H. colchicifolia* plants are currently harvested in an unsustainable manner from traditional collecting sites due largely to population growth, increased land use for urban development and agriculture, and the popularisation of *Hypoxis* plants for herbal remedies.

A further search of historical records established that *H. colchicifolia* plants were dominant in grassland vegetation prior to 1950, but had rapidly declined since then. Quantitative data subsequently gathered in this study from comparative surveys of both *H. colchicifolia* and *H. hemerocallidea* populations from sites with near-pristine, disturbed, burnt and mown grassland vegetation showed for the first time that exposure to human activity and the grassland management practices of mowing and burning incurred not only a 75% reduction in plant density of both these *Hypoxis* species, but also the total destruction of mature plants of *H. colchicifolia* in frequently mown and burnt areas. Flowering data recorded in these surveys, and confirmed by monitoring field performance of cultivated *H. colchicifolia* plants, showed that a contributing factor to the plant's inability to withstand these pressures was that juvenile forms only reached flowering maturity after three to four years growth, thus adversely affecting seedling recruitment. It was concluded therefore that, since *Hypoxis* species responded differently to mowing and burning, geophytic plants should be considered individually and not as "forbs" during the planning of grassland management programmes for natural conservation areas.

The need to cultivate *H. colchicifolia* to ensure its survival was also established using the new field data gathered in this study. Methods to propagate this species have, however, not been established. Data gathered on all the plants comprising a single population confirmed that mature plants survive to an estimated 20 years and longer in natural areas. Greatest hypoxoside yields were also obtained from corms with a fresh mass of 350g to 400g. Since these corms were estimated to be 10-years-old and older, propagation and cultivation methods that could sustain plant production and survival for long periods, and therefore increased hypoxoside yields, would have to be developed.

Several micropropagation systems suitable for the mass production of *H. colchicifolia* and from which phenotypically normal plantlets were recovered, were therefore established via organogenesis, embryo culture and somatic embryogenesis. The latter cultures have not been reported previously for *Hypoxis*. In the former culture the toxic effects of phenolic leachates and browning were controlled, and improved plantlet regeneration achieved, by adding polyvinyl pyrrolidone to the medium and introducing distinct sequential aseptic steps into the micropropagation procedure developed.

Defined protocols for the different phases of *in vitro* somatic embryogenesis are not readily available for monocotyledons, however, neither are the factors controlling embryogenesis and organ regeneration known. In this study the process of somatic embryogenesis from excised zygotic embryos of *H. colchicifolia* was shown to be complex and the resultant cultures very heterogeneous. Although the stage of development of the zygotic embryo explants was important at the time of inoculation, data showed that the induction and regulation of the processes of embryo culture and somatic embryogenesis were ultimately determined by the exogenously applied plant growth regulators.

By comparing the different pathways leading to plantlet regeneration, and the morphological stages of development of the structures produced both on solid and in liquid media, not only photographically, but also quantitatively and schematically, the repeated formation of pseudoembryonic structures and neomorphs confirmed that they form an integral part in the *in vitro* somatic embryogenic pathway of *H. colchicifolia*. Evidence suggested not only that two types of somatic embryos are produced in the embryogenic cultures of *H. colchicifolia*, but that the pseudoembryonic structures produced resemble the pseudobulbils produced in polyembryonic cultures of *Citrus*.

The success of the somatic embryogenic cultures was confirmed by the estimation that 28 112 somatic embryos and embryo clusters of *H. colchicifolia* could be obtained from 16 ml of somatic embryogenic liquid culture. Furthermore phenotypically normal plantlets regenerated from all of the micropropagation procedures developed were successfully transplanted from the laboratory, acclimatized under greenhouse conditions and their horticultural and field performances evaluated.

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## *Chapter 1*

# **A REVIEW OF THE MEDICINAL AND ECONOMIC POTENTIAL OF THE GENUS *HYPOXIS* L.**

## **1.1 INTRODUCTION**

*Hypoxis* is a genus of typical perennial geophytes which, in the USA, have been "little studied due to their inconspicuous and rather cryptic life cycle" (HERNDON, 1988). At the onset of a study on the species of *Hypoxis* from the Witwatersrand (now Gauteng) HEIDEMAN (1979; 1983) had also found that very little was known about these plants even though in spring they form a dominant component of the grassveld in the central and eastern regions of South Africa. Several taxonomic, physiological and chemical aspects of *Hypoxis* have since been researched and published providing valuable new data on these plants. The medicinal and economic potential of members of this genus, and indeed the family Hypoxidaceae, will be discussed in this review with particular emphasis on developments surrounding the treatment of viral diseases and those affecting the elderly.

## **1.2 CANCER, AIDS AND DRUG DEVELOPMENT**

Cancer has been recognized as a major global health problem for many years. The unexpected number of cancer cases recorded worldwide in 1996, however, indicated that significant new trends in both the occurrence and distribution of the disease were emerging. In that year alone six million people died from cancer while an additional ten million new cases were reported, 57% of which were from developing countries (KOTZENBERG, 1997). Supporting evidence showed that cancer was not only becoming a leading cause of death among the elderly, but also increasingly prevalent among young people and widespread in underdeveloped countries. Whilst the former trend was primarily due to increased life expectancy, the latter were attributed to rapid population growth, the swift spread of the human immunodeficiency virus (HIV) and the subsequent onset of acquired immunodeficiency syndrome (AIDS) and related opportunistic diseases.

Although cases of HIV infection had been detected in 74 countries by 1986 (QUINN *et al.*, 1986), accurate published data on its global status was still not available ten years later due largely to poor diagnoses and under-reporting of the disease. The extent to which the HIV epidemic had contributed to the increase in cancer in 1996 was therefore difficult to determine. To address this shortfall a joint co-sponsored United Nations Programme on HIV / AIDS was founded in 1996 to monitor the spread of HIV (TARANTOLA, 2001). In their first major report, UNAIDS stated that in 1997 an estimated 30.6 million people were HIV-positive worldwide (ZAREMBO, 1997). Since then the daily estimated global number of HIV infections has increased rapidly reaching 38.1 million in September 2000 (SAPA, 2000), 45.8 million in March 2002 (SAPA, 2002) and 53.9 million in September 2003 (SAPA, 2003).

The 1997 estimates also showed Africa to be the region most seriously affected by the HIV pandemic. Where only one to two million people were HIV-positive in Africa in 1986, this estimate had increased to 20.8 million by 1997 (ZAREMBO, 1997) and 25.3 million by 2001 (QUINN, 2001). It was also disclosed at the 1998 Geneva World Aids Conference that just under 6 million people contracted HIV in 1997 (COWLEY, 1998). With an estimated 2.9 million new infections that same year, it was suggested that South Africa had the fastest growing national HIV epidemic worldwide (MORTON, 1998). The scale of HIV / AIDS infection in South Africa has, however, remained largely unknown. In neighbouring Zimbabwe though, a survey of pregnant women led to the prediction that the HIV infection rate among rural women could rapidly increase from 14% to 50% if left untreated (GREGSON *et al.*, 1996). In fact the disease spread so swiftly that within four years of this prediction general health records indicated that 25% of 15 to 25 year-old Zimbabwean and South African females (MASLAND, 2000), and 36% of pregnant women in Botswana (QUINN, 2001) were living with HIV / AIDS.

Although the HIV pandemic in Africa was considered to still be in a relatively early stage in 1997, the deaths of nearly 12 million people worldwide had already been attributed to AIDS (MORTON, 1998). First described in June 1981, AIDS has since become the most important infectious disease worldwide (ESPARZA, 2001). Recent reports on the South African situation have also concentrated less on the rates of HIV infection and more on AIDS related deaths as ever increasing numbers of HIV-positive infections have progressed from the asymptomatic to the symptomatic state of the disease. As a result the South African Medical Research Council was able to report that AIDS has become a major cause of death in South Africa and could be related to 40% of all adult deaths in 2000 / 2001. It is also claimed that these South African records are the first to clearly demonstrate

that the increase in the number of deaths of young adults has followed the pattern of, and can therefore be linked to, the spread of the AIDS epidemic in southern Africa (GALLOWAY, 2001).

Concurrently with the spread of AIDS, significant increases in opportunistic diseases and AIDS related cancers such as Kaposi's sarcoma have also been recorded annually from areas in Africa severely affected by HIV (KOTZENBERG, 1997; QUINN, 2001). This has resulted in a 15% increase in cancer and a decline in overall life expectancy of between 8 and 31 years in sub-Saharan Africa (ZAREMBO, 1997; SITAS, 1997; QUINN, 2001).

In this short overview, global estimates of HIV infection and changes recorded in the incidence of cancer have been presented to emphasize the magnitude of the HIV / AIDS pandemic, particularly in sub-Saharan Africa, and the impact it will have on future economies worldwide if it continues to spread unchecked. To this day, there are no reliable cures for these diseases. At present the best treatments available are anti-retroviral drugs such as retrovir (AZT), which can contribute to improving the quality of life of people living with HIV/AIDS, but cannot cure the disease, and secondly conventional clinical procedures which have been used to successfully treat many of the 100 known forms of cancer (SCHWARTZ, 1989; QUICK, 2001). However, few people in developing countries can afford these treatments. The enormous cost also makes it prohibitively expensive for the health departments of poor countries to administer anti-retroviral drugs to HIV patients from diagnosis to death, particularly in Africa where hospitals and health-care systems are often inadequate (FARMER *et al.*, 2001).

One of the greatest challenges facing the scientific and medical communities today, therefore, is to not only develop affordable cures and vaccines for cancer and HIV / AIDS, but also fully investigate and develop the use and effectiveness of alternative drugs and therapeutic treatments in the control of these diseases.

The discovery and synthesis of new drugs is not easily accomplished, however, with very few compounds ever reaching the stage of final clinical trials, a procedure known to span many years. The compound retrovir was first synthesised in 1964 yet its value as a drug (AZT) was only established on re-examination 20 years later (SCHWARTZ, 1989). A principal constraint in the development of drugs also recognised by the pharmaceutical industry, is that only one in every 10,000 synthesized (SCHWARTZ, 1989) and 40,000 natural compounds examined will ever prove useful (CRAGG *et al.*, 1996).

These obstacles were clearly demonstrated during the late 1950s and early 1960s when the United States National Cancer Institute (NCI) implemented a programme to identify potential new sources for anti-cancer drugs. Anti-cancer activity was detected in many of the 300,000 chemicals, both natural and synthetic, evaluated during the early stages of this programme. As screening methods were improved, however, the positive anti-cancer activity measured initially for many of the 114,000 plant-derived compounds screened was shown to be due to the presence of tannins and / or phytosterols in the extracts, the latter being an ubiquitous constituent of higher plants. It was concluded, therefore, that many of the early results had merely been an indication of the general biological activity of the plants tested and not their yields of anti-cancer activity as believed (BARCLAY and PERDUE, 1976; CRAGG *et al.*, 1996).

Between 1962 and 1982 thousands of the plant-derived constituents included in the NCI programme were therefore re-examined for anti-cancer activity using only two specific anti-tumour systems known at the time to be insensitive to tannins and phytosterols (CRAGG *et al.*, 1996). These were the cell cultures of human epidermoid carcinoma of the nasopharynx (KB) and mouse P388 lymphocytic leukaemia cells (P388). Only those compounds showing a positive response to these tests were monitored thereafter by measuring their activity against the tumour systems most likely to be predictive for clinical activity in man, namely P388 and cultures of mouse L1210 lymphoid leukaemia cells (L1210). Test results of the most promising groups of plants showed that 100% of the extracts taken from members of the Hypoxidaceae, including the genus *Hypoxis* L., were active against mouse P388. It was concluded therefore that this monocotyledonous family should be included among those plant groups to undergo further evaluation as potential anti-cancer agents (BARCLAY and PERDUE, 1976). Several of these early prototypes, compounds and plant extracts examined, including those common to the Hypoxidaceae, are also being extensively re-screened in the hope of finding novel compounds showing anti-HIV activity (CRAGG *et al.*, 1996).

The commercial value of plant or phyto-chemicals can, however, already be clearly illustrated by the often cited estimate that 25% of all modern drugs are plant-derived (KINGHORN and BALANDRIN, 1993). Many of these drugs have been developed from chemical compounds isolated from medicinal plants used in herbal remedies. Commonly referred to as active ingredients, these compounds act either directly or indirectly to prevent or treat disease. They also play an important role in maintaining the health of the patient (VAN WYK *et al.*, 1997). An example of a classical drug developed from a herbal remedy is that of opium extracted from the opium poppy, *Papaver somniferum* L., to control

pain more than 2000 years ago. Today its active analgesic ingredients, the alkaloids morphine and codeine, are routinely used as painkillers (SCHWARTZ, 1989).

The alternate practice of using natural resources in the therapeutic treatment of diseases and other ailments dates back to antiquity, however, the Ebers Papyrus ( $\pm 1550$  BC) from Egypt being one of the oldest surviving texts (HARTWELL, 1967). Remedies recorded in this early *materia medica* interestingly showed that northern Africa already had a greater variety of herbal tradition than any other continent at that time and that this formed the foundation for classical medicine in Greece and Rome (MNIMH, 1996). The African recipes listed in this old text were dominated by food plants in keeping with the belief that "every disease to which men are liable is occasioned by the substances whereon they feed" (IWU, 1993).

In Africa the use of herbs as a source of medication for health care is still widely practised. It is estimated that 82% of the populations of West African countries still use traditional medicines (ABBIW, 1990). While preparing herbal remedies, African healers attempt to first extract the active ingredients from the plant before administering the extract, infusion, decoction, or poultice to patients (KOKWARO, 1995). Similarly prepared herbal treatments have also been used for centuries throughout sub-Saharan Africa where an estimated 70% to 80% of African people continue to consult traditional healers today (MANDER, 1997).

The importance of plants in medicine to the people and tribes of sub-Saharan Africa is appropriately reflected in a widespread belief, still upheld by the Shona *n'anga* or traditional healer in Zimbabwe, that "*for every human illness there is a plant that possesses the property of neutralizing its effect*" (GELFAND *et al.*, 1985). These beliefs lend support to IWU's (1993) finding that in traditional African medicine as practised today there is no clear distinction as to when a "herb" ceases to be a health food and becomes a medicine.

Western medical and scientific opinion that "human health is directly dependant on a thriving natural environment" is also enjoying an increase in popularity globally, as has a return to the use of complementary and herbal medicine (CLARK, 1997). Renewed interest in the effect of plants on human health also led to several nutritional studies which showed that the risk of people developing various cancers and other common ailments can be significantly reduced by following diets rich in fruit and vegetables (PEGEL, 1997). Furthermore, research has demonstrated the important role of correct nutrition in the maintenance of the human immune system which, when functioning optimally, can effectively fight modern diseases where certain standard drug therapies have failed or only

been moderately successful (PEGEL, 1997; VANDERHAEGHE and BOUIC, 1999). Plant nutrients are also generally thought to be responsible for many of the “healing powers” attributed to natural remedies as outlined above. Meanwhile many cancer and HIV patients are therefore turning to herbal remedies and traditional healers for therapeutic treatment until cures for these diseases are found.

There has also been much speculation that a breakthrough in the treatment of AIDS would originate in Africa where the HIV pandemic is the most serious (VANDERHAEGHE and BOUIC, 1999). That science would turn to African plants for a solution is also not surprising bearing in mind the continent’s ancient and widespread herbal tradition. However, although pertinent data on the constituents and biological activity of many African medicinal plants has been widely published, the development of therapeutic agents from these plants has remained largely neglected (IWU, 1993).

Research conducted on the South African Hypoxidaceae used in traditional medicine is of particular interest, therefore, as it has shown that the sterols and sterolins present in the corms of *Hypoxis* plants are beneficial to human health. The importance of these phytosterols in both human and animal nutrition has been comprehensively reviewed by PEGEL (1997). Results from this research showed that the human immune system could be boosted if these plant nutrients were administered to patients in the precise ratio found in *Hypoxis* plants. It was argued that these nutrients could potentially stimulate the immune system via selective modulation, to a level where it could effectively fight cancer, AIDS, bacteria, viruses and tuberculosis, as well as slow down the aging process. Controlled clinical trials to determine not only the medical efficacy of *Hypoxis* corm extracts as daily supplements to stabilize the immune systems of HIV-positive patients (BOUIC *et al.*, 1996), but also their potential as non-toxic prodrugs for the treatment of inflammation and certain malignancies (ALBRECHT, 1996) continue.

To properly assess the medicinal and commercial potential of the genus *Hypoxis*, however, pertinent literature relating to the traditional uses of members of the family Hypoxidaceae, for both medicinal and daily purposes, should be reviewed.

### **1.3 THE TRADITIONAL USES OF THE HYPOXIDACEAE**

A cosmopolitan family, the Hypoxidaceae is represented by several genera worldwide with the exception of Europe and Asia (WILLIS, 1966). In all the literature reviewed, however, only two genera, *Curculigo* Gaertn. (= *Empodium* Salisb. = *Forbesia* Eckl.) and *Hypoxis* L.,

are mentioned as being utilized for traditional medicinal purposes. Both genera are used in herbal remedies in Asia. *Hypoxis* is also used in the Americas and sub-Saharan Africa where the genus is well represented. Only some of the African species of *Hypoxis* are reportedly used in traditional healing (WATT and BREYER-BRANDWIJK, 1962).

Recently specific terminology with respect to traditional or ethno-medicine was introduced in an attempt to standardise ethnobotanical reports on traditional herbal medicine. The following terms will therefore be used in the present study. The term "traditional usage / use" includes all plant usage by local peoples whether for daily living, traditional rituals and customs, or for medicinal, agricultural or veterinary purposes. Following CUNNINGHAM (1988 a), "medicinal" plant or "traditional medicine" will replace the terms "muthi", "magico-medicine" or "ethno-medicine" regardless of whether the plant material is used for physiological, psychological, religious or magical purposes.

### **1.3.1 TRADITIONAL USES OF THE NON-AFRICAN SPECIES OF *CURCULIGO* AND *HYPOXIS***

A brief survey of some available literature on medicinal plants in which the properties and uses attributed to non-African *Curculigo* and *Hypoxis* species were given, revealed that many of the published records of reported medicinal usage by herbalists are not original, but have simply been cited and re-cited from other publications. No attempt was made to validate these uses by tracing the original publications and pharmacopoeia, however, as this was beyond the scope of the present study. Comprehensive literature reviews referred to included those by CHOPRA *et al.* (1958), HARTWELL (1967), and PERRY and METZGER (1980).

Several Asian species of *Curculigo* are used for traditional purposes. In India the leaves of *C. orchoides* Gaertn., originally cited as *Hypoxis orchoides* Kurtz. and commonly known as the "poison bulb", are used on whitlows (HARTWELL, 1967). The rhizomes of this species are used to treat piles, diarrhoea (RAO and BERI, 1951), jaundice, gonorrhoea and skin diseases (CHOPRA *et al.*, 1958). They are also ground and eaten by tribal people (RAO *et al.*, 1978). A common bazaar medicine, *C. orchoides* plant parts are prescribed in the above instances because of their soothing properties and potency as a tonic, aphrodisiac or diuretic (CHOPRA *et al.*, 1958).

The juice extracted from stems of *C. brevipedunculata* Elm. is used as a topical skin application by people in the Philippines to stop the pain caused by centipede bites. In both

India and China decoctions prepared from the roots of *C. disticha* Gagnep are taken to soothe sore throats (PERRY and METZGER, 1980). Many medicinal properties are attributed to *C. latifolia* Dryand throughout India, China and Sumatra. A decoction prepared from its roots and flowers is prescribed as a diuretic to treat genito-urinary complaints, while its fruits are eaten to stimulate the appetite. The rhizome is used either as a decoction to relieve menorrhagia, an infusion to reduce fever or a lotion to treat eyes. These remedies are sometimes accompanied by magic (PERRY and METZGER, 1980).

Only three non-African species of *Hypoxis* are used for traditional purposes. The roots of the first, *H. decumbens* L. (originally cited as *H. scorzonera* L.), are used in the West Indies in ointment form to treat testicular tumours (HARTWELL, 1967). In Brazil the edible sweet "tuber" of *H. braziliensis* is used as cattle fodder (GUIMARAES, 1926; HARTWELL, 1967). The third, *H. aurea* Lour., is used in both India and China not only as a tonic during convalescence, but also as a treatment for a wide range of complaints including wasting diseases, impotence, indigestion, eye and ear infections, and wounds. It is the only *Hypoxis* species recorded from India (SANTAPAU and HENRY, 1973) and was at one stage apparently nearly as popular as ginseng, *Panax ginseng* Linn., because of its rejuvenating, reconstructive and aphrodisiacal properties (LEWIS and ELVIN-LEWIS, 1977; STUART, 1977).

PERRY and METZGER (1980) questioned the identity of the third species, however, as the latter authors had referred to it by the common Chinese name ascribed to *Curculigo orchioides* and not that of *H. aurea*. It is well known that the vernacular or common name given to specific medicinal plants by herbalists can be an unreliable source of identification as the same name is often used to describe many species, and even genera, which have similar medicinal uses. Even the spelling of vernacular names in the literature reviewed was found misleading at times as shown by references to *C. orchioides* as both "Kalimusli" (RAO and BERI, 1951) and "kala musli" (CHOPRA *et al.*, 1958). Inexact use of names was in fact apparent throughout the literature reviewed.

This brief overview of the Asian and American medicinal species of *Curculigo* and *Hypoxis* has, however, served to highlight similarities between the herbal remedies and medicinal properties of both these genera. Overall, plant parts of both *Curculigo* and *Hypoxis* species are used in herbal remedies not only because of their soothing / healing properties when applied to wounds and infections as a skin balm, but their potency when prescribed as a tonic, appetite stimulant, aphrodisiac or diuretic.

### 1.3.2 TRADITIONAL USES OF THE AFRICAN HYPOXIDACEAE

The documented uses of the African Hypoxidaceae with traditional value are presented in Table 1. As in Asia and America, *Empodium* Salisb. (= *Forbesia* Eckl. = *Curculigo* Gaertn.) and *Hypoxis* L. are again the only medicinally important genera recorded in the literature reviewed on the Hypoxidaceae from sub-Saharan Africa. It was found that in this region only one of the seven (REID and ARCHER, 1993) to nine (SNIJMAN, 2000) species of *Empodium*, a genus endemic to southern Africa, thirteen of the approximately 50 species / varieties of *Hypoxis* (HILLIARD and BURTT, 1987), and ten unidentified *Hypoxis* plants have been recorded as useful medicinal plants.

The widespread use of the same common and / or vernacular name to describe several different medicinal plants at both the generic and specific levels was often encountered in both popular and scientific publications on traditional plant usage in sub-Saharan Africa leading to confusion. This was also found to apply to the non-African Hypoxidaceae as outlined above. Because of the uncertainty surrounding the correct scientific identity of many African medicinal plants both the common and vernacular names given to the various species of *Empodium* and *Hypoxis* with traditional value have been included in Table 1, wherever available. The accuracy of these names has, however, not been verified and the list is by no means complete. As a result of this confusion many *Hypoxis* species, and their medicinal applications, may have been incorrectly identified perhaps even deliberately. It has also been shown that the availability and supply of correctly identified medicinal plants severely restricts the pharmaceutical industry in the development of plant-derived drugs (TYLER, 1986).

It also became obvious during the compilation of Table 1, that many of the traditional medicinal applications and cures attributed to the Hypoxidaceae have been cited and recited in many books, scientific publications and popular articles, but without any supporting evidence as to their validity and efficacy. It should also be noted at the outset that the accuracy of much of the information published on the traditional medicinal uses of the Hypoxidaceae has been neither scientifically confirmed nor proven in clinical trials. An attempt was therefore made to trace as many original reports as possible in which the remedies and methods of administration were described. The references in Table 1 indicate, therefore, whether the plant uses described are from either an original source, a review or of uncertain origin. If the original description or record was found then later references have not been included in the table. As a result the majority of reports, publications and books on the subject could be eliminated. It was concluded, therefore, that

very few publications contain authentic, novel information on the traditional uses of the Hypoxidaceae and that much of the literature is repetitive and anecdotal.

An explanation for this could be that, although the earliest *materia medica* originated in Egypt, remedies used in traditional medicine as practised in sub-Saharan Africa have always been safeguarded by traditional healers and passed down orally from generation to generation (HUTCHINGS, 1996; MAHLABA, 1999). Local pharmacopoeia are, therefore, not available for the southern regions of Africa and few publications appear to contain original, authentic descriptions of either the actual plants harvested, including the Hypoxidaceae, or the methods and recipes used by traditional healers.

An exception, however, is Smith's 1888 publication in which he describes his informants as mainly "the ladies of the mission, among others, who have access to information from the Native professionals, where others find a sealed book" (SMITH, 1895). The author noted that the "herb-doctors" used about three hundred indigenous plants, including *Hypoxis*, in their practices and that the medicinal knowledge of these plants was kept secret thus lending support to the above suggestion. To maintain this secrecy the "herb-doctors" would mislead interested parties, other than the ladies of the mission, by either ascribing a use to a plant very different from its own or showing them another, totally incorrect, plant or plant species to harvest.

To summarise, important contributions containing firsthand accounts of traditional uses of the Hypoxidaceae are by SMITH (1888), SMITH (1895), GERSTNER (1938, 1939), HULME (1954) and BRYANT (1966). The latter author's work was first published in the *Annals of the Natal Museum* in 1909 but later lost in a fire (HUTCHINGS, 1989 a). Recent publications with original descriptions of medicinal plant usage are GELFAND *et al.* (1985), HEDBERG and STAUGÅRD (1989), HUTCHINGS (1992, 1996), PUJOL (1993), POOLEY (1998) and VAN DER MERWE (2000).

The two most important comprehensive reviews examined were those by WATT and BREYER-BRANDWIJK (1962) and HUTCHINGS (1996). Both deal with African medicinal plants, including the Hypoxidaceae. HUTCHINGS (1996) not only reviews the medicinal value of 1032 plant species used by traditional Zulu healers, but also includes some firsthand accounts of remedies currently practised in traditional medicine. Two earlier publications which initially appeared to include some novel information on the medicinal properties of African plants are those by GITHENS (1949) and JACOT-GUILLARMOD (1971). On closer examination, however, it became clear that the uses attributed to the

Hypoxidaceae by these authors were not new but cited from other sources e.g. SMITH (1895) and HULME (1954), and without providing the references.

It also became apparent, as mentioned above, that some of the medicinal properties attributed to *Hypoxis*, particularly those documented by WATT and BREYER-BRANDWIJK (1962) and HUTCHINGS (1996), have been repeatedly cited in scientific and popular articles, but without the present authors referring back to the original records. Since the actual methods of treatment were not always included in the review by WATT and BREYER-BRANDWIJK (1962), this appears to have led, in later publications, to the assumption that *Hypoxis* plants can be used to cure all sorts of medical conditions for which there is no real evidence.

For example, *Hypoxis* is listed in WATT and BREYER-BRANDWIJK (1962) as a parasiticide. This was based on a detailed account describing the use of a species of *Hypoxis*, referred to as *inKomfe*, as an ingredient in a tea taken to expel "intestinal beetles", which were identified as a species of dung-beetle (BRYANT, 1909, 1966). Although the tea is obviously an effective purgative, the validity of the treatment had in fact been questioned by BRYANT (1909, 1966) himself because, as he noted, winged beetles cannot survive in the human intestine and the condition is unknown to medical science. No other descriptions of this treatment were found in the literature. As there was, and still is, no evidence proving the effectiveness of the *Hypoxis* tea as a parasiticide, it should perhaps be concluded, firstly, that this traditional custom might be more aptly interpreted as having a magical rather than a medicinal application and secondly, that by blindly citing WATT and BREYER-BRANDWIJK (1962), researchers might have incorrectly credited *Hypoxis* plant extracts as having effective parasitocidal properties.

Another misleading example is that in which *Hypoxis colchicifolia* Baker (synonym: *H. latifolia* Hook) (HILLIARD and BURTT, 1986) has often been cited, as per WATT and BREYER-BRANDWIJK (1962), as very poisonous due to the presence of the alkaloid haemanthine, a compound effective against roundworms. The earliest record found in which *H. colchicifolia* plants were reported to contain haemanthine was in GITHENS (1949). This was, however, recorded without citing a reference and contained no evidence to support the claim. The author also claimed that, due to the presence of the alkaloid haemanthine, several species of *Hypoxis* are useful expectorants and vermifuges, and that *H. colchicifolia* is specifically used as a purgative and to expel roundworms, but again without supporting evidence or references. The latter claim may also have contributed to WATT and BREYER-BRANDWIJK (1962) listing *Hypoxis* plants as effective paracitocides.

With respect to *Hypoxis* plants being poisonous, however, BRYANT (1966) was the first to state, in 1909, that the Zulu people considered *H. colchicifolia* to be very poisonous but he did not attribute this to the presence of haemanthine. The level of toxicity was apparently dependent on the plant part used, the season and the method of preparation of the medicine. The corms of *H. colchicifolia* were therefore used by the Zulu people to kill small vermin and to trap snakes. *H. colchicifolia* plants are apparently also considered to be very potent medicinally as they are referred to as “-emnyama” or “black” medicine which is the term applied to special medicinal plants which are the first to be administered to a patient to expel evil (BRYANT, 1966). The poisonous nature of *Hypoxis* plants has, however, not been recorded in traditional medicine, neither have any deaths. Healers continue today to administer extracts orally to patients to treat various illnesses without apparent side effects (HUTCHINGS, 1996). Scientific evidence from both clinical and pharmacological trials has in fact indicated that *Hypoxis* extracts are not toxic if used correctly (PEGEL, 1979; 1980).

*Hypoxis* plants are also said to be most effective when used in conjunction with other medicinal plants (MAHLABA, 1999). This fact has generally been overlooked, however, even though reported by BRYANT in 1909 (BRYANT, 1966). Recently the corms of both *H. colchicifolia* and *H. hemerocallidea* Fischer and Meyer (synonym: *H. rooperi* S. Moore) (HILLIARD and BURTT, 1986) are described as frequently used in *intelezi*, a mixture used to treat psychological disorders (PUJOL, 1993). Another unpublished example told to the author while researching the present study is the use by Zulu traditional healers of a poultice made from a mixture of *H. colchicifolia* corm slices and portions of a secret bulb. The poultice is placed on a woman's back to correct or dispel marital problems.

Although it is often stated that plants have been used for centuries by herbalists and traditional healers worldwide to treat various cancers and urogenital diseases (BAYLEY and VAN STADEN, 1988), reports of the use of *Hypoxis* corm extracts by traditional healers to treat and prevent cancer have only been published recently (KAMWENDO *et al.*, 1985; HUTCHINGS, 1986a, b, 1989; HEDBERG and STAUGÅRD, 1989). Similarly reports on traditional treatments for urogenital diseases could not be found other than that *Hypoxis* corm extracts are used by Zulu healers to correct impotency and sterility (BRYANT, 1966) and as diuretics (HUTCHINGS, 1992, 1996). Recently, however, GELFAND *et al.*, (1985) reported that the powdered corms of *H. obtusa* Burch are taken orally to treat gonorrhoea and infertility in women, but there is no medical proof to validate any of these claims.

Although fully trained traditional healers consult widely and are confident about their treatments and the future of their careers, they refer patients they cannot treat to hospitals.

Durban herbalist, Protus Cele, also believes that traditional healers in South Africa must have a cure for all diseases, even AIDS and cancer, but that these are as yet unknown to him (MUNNIK, 1994). As personally observed by the author, the most popular plant currently sold at the eThekweni Municipality's large Warwick Avenue Triangle Medicinal Plant Market, Durban, KwaZulu-Natal as a cure for Aids is *H. hemerocallidea*, commonly known today by the popularised name of "African potato".

Finally geophytes have been used by archaeologists to establish the role that plant collecting played in the foodways of early hunter-gatherer communities (HALL, 1996). At an archaeological site at Melkhoutboom in the Eastern Cape Province, South Africa, the best preserved plant remains were frequently cormous geophytes, the most abundant being *Watsonia*, *Hypoxis* and *Moraea* species. From this evidence it was concluded that these corms were the major plant foodstuffs of the community and were widely distributed and seasonally available in spring (DEACON, 1976). The use of these plants, particularly the *Hypoxis* species, for medicinal purposes as well as a food source is a real possibility, however, and does not appear to have been considered.

To summarise, overall *Empodium* and *Hypoxis* plants are used traditionally in sub-Saharan Africa as medicines, magical charms, a food source and for veterinary applications. The rootstocks or corms are used for the former while leaves are plaited to make string and fibres from which strong ropes are made. Their medicinal properties are very similar to those outlined above for the non-African genera, *Curculigo* and *Hypoxis*. These can be summarised as being calming, cleansing and healing. Extracts are also used to induce sleep or an appetite, to reduce inflammation, and for reproductive purposes. *Empodium* and *Hypoxis* plants are therefore most successfully used as tonics, purgatives, expectorants, diuretics, skin balms or ointments, anti-inflammatories or aphrodisiacs. The main difference between traditional Asian and African applications is the widespread use in southern Africa of *Hypoxis* plants in the veterinary field to treat complaints such as infertility, gallsickness, and heartwater, and as an ointment to heal sores and cracked hooves of sheep and cattle.

The three *Hypoxis* species most commonly used in southern Africa are *H. hemerocallidea*, *H. colchicifolia* and *H. rigidula* (Table 1). The belief in, and popularity of, these plant species and traditional treatments in South Africa has even resulted in plants of *H. hemerocallidea* and *H. colchicifolia* becoming "uncommon" in areas where they are normally harvested (NAIDOO, 1998) regardless of whether they are considered to be poisonous or not.

**Table 1** Documented traditional usage of the Hypoxidaceae from sub-Saharan Africa. Recognized common and vernacular names have been included. Unidentified *Hypoxis* species reported as used for traditional purposes have been placed at the end of the table. Abbreviations: Afrikaans (A), Ndebele (Nd), Sesotho (S), Shona (Sh), Setswana (=Tswana) (T), Xhosa (X) and Zulu (Z) –speaking peoples; species singular (sp), species plural (spp).

GENUS & SPECIES Synonyms Country	REFERENCES * original reference # literature review ◇ references uncertain	COMMON and VERNACULAR NAMES	PLANT PARTS USED	PREPARATION AND METHOD OF ADMINISTRATION	TRADITIONAL USES Medicinal, food, agricultural, veterinary, magical and craft
<b>EMPODIUM SALISB.</b>					
<p><i>Empodium plicatum</i> (Thunb.) Garside Syn: <i>Forbesia plicata</i> (Thunb.) Nel <i>Curculigo plicata</i> Dryand</p> <ul style="list-style-type: none"> <li>• South Africa</li> <li>• Lesotho</li> </ul>	<ol style="list-style-type: none"> <li>1. HULME, 1954 *</li> <li>2. HUTCHINGS, 1996 #</li> <li>3. JACOT-GUILLARMOD, 1971<sup>◇</sup></li> </ol>	<p><i>isidwa esincane senkagala</i> (Z)</p> <p>(Autumn) golden star Ploegtydblommetjie Sterretjie (A)</p>	<ul style="list-style-type: none"> <li>• Corm</li> <li>• Corm</li> </ul>	<ul style="list-style-type: none"> <li>• A decoction prepared from the pounded corm, is sipped slowly</li> <li>• Eaten</li> </ul>	<ul style="list-style-type: none"> <li>• Taken to loosen and relieve chest trouble believed to be caused by charms or poison in one's food. Then an emetic made from an infusion of <i>Gladiolus dalenii</i> Geel, is given to excise the evil in the chest</li> <li>• Food source</li> </ul>
<b>HYPOXIS L.</b>					
<p><i>H. angustifolia</i> Lam.</p> <ul style="list-style-type: none"> <li>• Zimbabwe</li> <li>• Lesotho</li> </ul>	<ol style="list-style-type: none"> <li>1. TREDGOLD, 1986<sup>◇</sup></li> <li>2. POOLEY, 1998<sup>◇</sup></li> </ol>	<p>Small yellow star <i>hodo</i> (Sh)</p> <p><i>molinyana</i> (S)</p>	<ul style="list-style-type: none"> <li>• Corm (tuber)</li> </ul>	<ul style="list-style-type: none"> <li>• Scraped clean, washed, and eaten raw (grated), boiled or roasted</li> </ul>	<ul style="list-style-type: none"> <li>• Food source when edible during the months November to March</li> </ul>
<p><i>H. argentea</i> Harv. ex Baker</p> <ul style="list-style-type: none"> <li>• South Africa</li> <li>• Lesotho (2 &amp; 3)</li> <li>• Congo</li> </ul>	<ol style="list-style-type: none"> <li>1. POOLEY, 1998<sup>◇</sup></li> <li>2. JACOT-GUILLARMOD, 1971<sup>◇</sup></li> <li>3. WATT, BREYER-BRANDWIJK, 1962 #</li> </ol>	<p><i>isinana</i> (Z)</p> <p><i>leihlo-khoma-le-leholo</i> (S)</p> <p><i>lesikitlane</i> (S)</p>	<ul style="list-style-type: none"> <li>• Corm or rootstock</li> <li>• Corm or rootstock</li> <li>• Plant</li> </ul>	<ul style="list-style-type: none"> <li>• As per WATT <i>et. al.</i>, 1962, but no references</li> <li>• Eaten</li> <li>• Ointment from crushed dried corms mixed with fat is smeared on cow's teats</li> <li>• Not given</li> </ul>	<ul style="list-style-type: none"> <li>• Food source</li> <li>• To treat and sooth cow's teats when cracked (from PHILLIPS, 1917)</li> <li>• An African medicine</li> </ul>

Table 1 Cont.

GENUS & SPECIES Synonyms Country	REFERENCES * original reference # literature review ◇ references uncertain	COMMON and VERNACULAR NAMES	PLANT PARTS USED	PREPARATION AND METHOD OF ADMINISTRATION	TRADITIONAL USES Medicinal, food, agricultural, veterinary, magical and craft
<p><b><i>H. argentea</i> Harv ex Baker var. <i>sericea</i> Bak.</b> = <i>H. sericea</i> (SMITH, 1895)</p> <ul style="list-style-type: none"> <li>• South Africa</li> </ul>	<ol style="list-style-type: none"> <li>1. SMITH, 1895 *</li> <li>2. GERSTNER, 1938 *</li> <li>3. BATTEN and BOKELMANN, 1966 ◇</li> <li>4. SMITH, 1966 ◇</li> </ol>	<p>Famine root i-Xalanxa (Z)</p> <p><i>inKomfe</i> (Z) <i>inKofe</i> (Z) Star of Bethlehem Small yellow star <i>iNongwe</i> (X) Kaffertulp (A)</p>	<ul style="list-style-type: none"> <li>• Corm (small)</li> <li>• Leaves</li> <li>• Corm or rootstock</li> </ul>	<ul style="list-style-type: none"> <li>• Usually roasted, sometimes boiled</li> <li>▫ An oil is obtained by roasting the corm</li> <li>• Yields good fibre</li> <li>• Dried and powdered</li> <li>▫ Not given</li> </ul>	<ul style="list-style-type: none"> <li>• Food source in times of famine</li> <li>▫ Oil is used to treat animal sores, eg. fresh wounds and skin chafes on horses</li> <li>• For ropes and plaited cords</li> <li>• Edible</li> <li>▫ A remedy for stomach trouble</li> </ul>
<p><b><i>H. colchicifolia</i> Baker</b> Syn: <i>H. latifolia</i> Hook <i>H. oligotricha</i> Baker</p> <ul style="list-style-type: none"> <li>• South Africa</li> </ul>	<ol style="list-style-type: none"> <li>1. BRYANT, 1966 (reprint of 1909) *</li> <li>2. GERSTNER, 1939 * (HUTCHINGS, 1996 #)</li> <li>3. GITHENS, 1949 #</li> </ol>	<p><i>iLabatheca</i> (Z)</p> <p>-<i>emnyama</i> (= black medicine - the first to be administered to a patient to expel evil)</p>	<ul style="list-style-type: none"> <li>• Plant</li> <li>• Corm (bulb)</li> <li>• Corm</li> <li>• Corm (root)</li> </ul>	<ul style="list-style-type: none"> <li>• Dependant on part used, season and method of preparation</li> <li>• Hot infusion taken as an emetic</li> <li>▫ Not given</li> <li>▪ Not given</li> <li>• Bulb is ground and placed in food</li> <li>• Infusion</li> <li>▫ Infusion as an emetic</li> <li>▪ Placed whole in snake holes</li> <li>• Used as a purgative</li> </ul>	<ul style="list-style-type: none"> <li>• Reputed by the Zulu to be very poisonous</li> <li>• To dispel fearful dreams symptomatic of a weak heart</li> <li>▫ For impotency and barrenness</li> <li>▪ To induce temporary insanity or delirium in a healthy person, but not used as a cure for the same</li> <li>◆ To kill all small vermin</li> <li>• Taken for hysterical fits</li> <li>▫ Used as a love charm</li> <li>▪ To trap snakes</li> <li>• "Contains haemanthine" which is effective against roundworm</li> </ul>

Table 1 Cont.

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<p><i>H. colchicifolia</i> cont.</p> <ul style="list-style-type: none"> <li>Congo</li> </ul>	<p>2. PUJOL, 1993 (revised edition) *</p> <p>3. CUNNINGHAM, 1988a</p> <p>4. HUTCHINGS, 1996 ◇</p> <p>5. POOLEY, 1998 ◇</p> <p>6. WATT, BREYER-BRANDWIJK, 1962 #</p>	<p><i>iLabatheca-emnyama</i> (Z)</p> <p><i>igudu, ingcobo</i> (Z)</p> <p>Broad-leaved <i>Hypoxis</i></p>	<ul style="list-style-type: none"> <li>Corm</li> <li>Plant</li> </ul>	<ul style="list-style-type: none"> <li>Infusion: boil 2 or 3 small pieces of corm in H<sub>2</sub>O and drink as tea, but only 2 cup at a time (Z)</li> <li>Solution as above drunk in larger portions (X) and</li> <li>Used as an enema (X)</li> <li>Frequently used in the making of <i>Intelezi</i> (Z+X)</li> <li>◇ Piece of corm placed in person's pocket</li> <li>Not given</li> </ul>	<ul style="list-style-type: none"> <li>To stop nausea / vomiting</li> <li>To calm the heart / anxiety</li> <li>To induce a good feeling, sleep and an appetite (Z)</li> <li>◇ To induce vomiting (X)</li> <li>To enema children (X)</li> <li>A magical plant used for its psychoactive virtues</li> <li>◇ Acts as a magical charm to calm an emotionally upset person</li> <li>◇ Effective during court cases</li> <li>Used as an African medicine</li> </ul>
<p><i>Hypoxis</i> sp. Known as <i>ilabatheca</i> – reported to be <i>H. colchicifolia</i>.</p> <ul style="list-style-type: none"> <li>South Africa</li> </ul>	<p>1. HUTCHINGS, 1992, 1996 * (Valley Trust healers, but plant not seen by author)</p>	<p><i>ilabatheca</i> (Z)</p>	<ul style="list-style-type: none"> <li>Corm or rhizomes</li> </ul>	<ul style="list-style-type: none"> <li>Not given</li> </ul>	<ul style="list-style-type: none"> <li>Used as diuretics</li> <li>To treat patients with psychiatric disturbances</li> </ul>
<p><i>H. coriacea</i> (=?)</p> <ul style="list-style-type: none"> <li>South Africa</li> </ul>	<p>1. GERSTNER, 1938 *</p>	<p><i>inKomfe</i> (Z)</p>	<ul style="list-style-type: none"> <li>Leaves</li> </ul>	<ul style="list-style-type: none"> <li>Yields good fibre</li> </ul>	<ul style="list-style-type: none"> <li>To make ropes</li> </ul>
<p><i>H. gerrardii</i> Baker</p> <ul style="list-style-type: none"> <li>South Africa</li> </ul>	<p>1. HULME, 1954 *</p>	<p>Small yellow star <i>inkomfe</i> (Z)</p>	<ul style="list-style-type: none"> <li>Corm</li> </ul>	<ul style="list-style-type: none"> <li>Corms are pounded and boiled to prepare a decoction to drink</li> </ul>	<ul style="list-style-type: none"> <li>To treat stomach ailments such as gripes (colic) and dysentery</li> </ul>

Table 1 Cont.

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<b><i>H. hemerocallidea</i></b> <b>Fischer and Mey.</b> Syn: <i>H. rooperi</i> S. Moore • South Africa	1. GERSTNER, 1938 * 2. SMITH, 1966 ◇ 3. PUJOL, 1993 (revised ed.) * 4. HUTCHINGS, 1992, 1996 * 5. POOLEY, 1998 ◇	<i>inKomfe</i> (Z) <i>inKofe</i> (Z) Gifbol (=poison) (A) Kaffertulp (A) <i>incomfe</i> (Z) (X) (PUJOL, 1990)      Star flower	<ul style="list-style-type: none"> <li>• Leaves</li> <li>• Not specified</li> <li>• Corm or tuberous bulb</li> <li>• Stems + leaves</li> <li>• Leaves</li> <li>• Root and leaves</li> <li>• Corm</li> <li>• Corm (bulb)</li> </ul>	<ul style="list-style-type: none"> <li>• Yield good fibre</li> <li>• Not given</li> <li>• Sundried, powdered corm is given in ½ cup doses twice daily               <ul style="list-style-type: none"> <li>▫ One teaspoon powdered corm is boiled in 500 ml H<sub>2</sub>O and one tablespoon is drunk twice daily</li> <li>▫ As a magical plant in the making of Zulu <i>Intelezi</i></li> </ul> </li> <li>• Mixed with roots and leaves of other plants               <ul style="list-style-type: none"> <li>▫ Not given</li> </ul> </li> <li>• Not given</li> <li>• Not given</li> <li>• Decoction prepared from ground corm, is given orally or as an enema               <ul style="list-style-type: none"> <li>▫ Infusion administered as an emetic</li> </ul> </li> <li>• Not given</li> </ul>	<ul style="list-style-type: none"> <li>• For ropes and plaited cords</li> <li>• Used as a purgative</li> <li>• To treat dizziness and apoplexy (fits) (Z remedy)               <ul style="list-style-type: none"> <li>▫ To calm and sedate (Z)</li> </ul> </li> <li>• Used for its psychoactive virtues               <ul style="list-style-type: none"> <li>▫ Specifically for prostate gland complaints (Z)</li> <li>▫ To wean children (Z)</li> </ul> </li> <li>• To stop children's coughs and fever – Xhosa remedy</li> <li>• To treat bladder disorders (X)</li> <li>• Alleviate madness (X)</li> <li>• To treat patients who cannot speak perhaps as a result of shock (X)               <ul style="list-style-type: none"> <li>▫ To treat dizziness (Z)</li> <li>▫ To treat mental disorders (Z)</li> </ul> </li> <li>• Used to blacken floors</li> </ul>

Table 1 Cont.

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<p><i>H. hemerocallidea</i> cont.</p> <ul style="list-style-type: none"> <li>• South Africa : Northwest Province (Setswana people)</li>   <li>• Lesotho</li>   <li>• Lesotho</li>           <li>• Botswana</li>         <li>• Congo</li> </ul>	<p>2. VAN DER MERWE, 2000 *</p> <p>3. JACOT-GUILLARMOD, 1971<sup>◇</sup></p> <p>4. WATT, BREYER-BRANDWIJK, 1962 #</p>	<p><i>tsuku-ya-poo</i> (T)</p> <p><i>kharatsa, moli-</i> (S) <i>lotsane, moli-</i> (S)</p>	<ul style="list-style-type: none"> <li>• Corm</li>                   <li>• Leaves</li> <li>• Not specified</li> <li>• Not specified</li> <li>• Corm</li>           <li>• Leaves</li>           <li>• Corm</li>           <li>• Not specified</li> </ul>	<ul style="list-style-type: none"> <li>• Decoction. Corm shavings or pieces boiled in water for a few minutes, cooled and dosed. Dosage not fixed. Treatments repeated as needed</li>   <li>▫ Mixed with bulb scales of <i>Boophane disticha</i>, roots of <i>Pouzolzia mixta</i>, tubers of <i>Rhoicissus tridentata</i> as a decoction or infusion</li>   <li>• Not given</li> <li>• As a magical plant</li>   <li>• One of the ingredients of a magical medicine</li> <li>• A drop of blood from patient's forehead is collected in hollowed out corm which is buried</li>   <li>• Not given</li>           <li>• Decoction</li>   <li>▫ Juice from corms</li> <li>• Not given</li> </ul>	<ul style="list-style-type: none"> <li>• To treat infertility in cows. Described as a "female" <i>tsuku-ya-poo</i></li> <li>• Used as a panacea to treat general ailments in cattle. No reports of side effects or toxicity</li> <li>▫ To treat cows after an abortion (<i>pholotso</i>) and to prevent further abortions in the herd</li>   <li>• To make strong, lasting ropes</li> <li>• Used as a charm to cure headaches</li> <li>• Used as a charm against thunder and storms</li> <li>• To cure headaches (from PHILLIPS, 1917)</li>   <li>• For ropes used to build huts and reed enclosures, and for sewing grain baskets</li> <li>• A tonic for weak children</li> <li>• Used as a purgative</li> <li>▫ Applied to burns</li> <li>• Used as an African medicine</li> </ul>

Table 1 Cont.

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<b><i>H. multiceps</i> Buchinger ex Baker</b> <ul style="list-style-type: none"> <li>• South Africa</li> <li>• Lesotho</li> </ul>	1. GERSTNER, 1938 * 2. POOLEY, 1998 ◇ 3. WATT, BREYER-BRANDWIJK, 1962 # 4. JACOT-GUILLARMOD, 1971 ◇	<i>inKomfe</i> (Z) <i>inKofe</i> (Z) Winter star flower  <i>moli-motsane</i> (S) <i>morethetho</i> (S)	<ul style="list-style-type: none"> <li>• Leaves</li> <li>• Not specified</li> </ul>	<ul style="list-style-type: none"> <li>• Yield good fibre</li> <li>• An ingredient in a mixture smeared on pegs which are placed in the ground around the homestead</li> </ul>	<ul style="list-style-type: none"> <li>• For making ropes</li> <li>• Used as a charm against lightning (PHILLIPS, 1917)</li> </ul>
<b><i>H. nyasica</i> Baker</b> <ul style="list-style-type: none"> <li>• Eastern Africa</li> <li>• Congo</li> <li>• Zomba, Malawi</li> </ul>	1. GITHENS, 1949 # 2. WATT, BREYER-BRANDWIJK, 1962 # 3. KAMWENDO <i>et al.</i> , 1985 ◇	kamba (M) kale (M) chikasu (M)	<ul style="list-style-type: none"> <li>• Corm (root)</li> <li>• Not specified</li> <li>• Plant</li> <li>• Corm</li> </ul>	<ul style="list-style-type: none"> <li>• Contains an alkaloid used as an expectorant</li> <li>• A decoction taken orally to induce perspiration</li> <li>• Not given</li> <li>• Not given</li> </ul>	<ul style="list-style-type: none"> <li>• To treat coughs</li> <li>• Used as a cough remedy</li> <li>• Used as an African medicine</li> <li>• Uterine cancer</li> </ul>
<b><i>H. obtusa</i> Burch</b> <ul style="list-style-type: none"> <li>• South Africa</li> <li>• Zimbabwe</li> <li>• Malawi</li> </ul>	1. GERSTNER, 1938 * 2. GELFAND <i>et al.</i> , 1985 *	<i>inKomfe</i> (Z) <i>inKofe</i> (Z) Yellow star <i>hodzori</i> (Sh)	<ul style="list-style-type: none"> <li>• Leaves</li> <li>• Corm (tuber)</li> <li>• Corm</li> </ul>	<ul style="list-style-type: none"> <li>• Yields good fibre</li> <li>• Infusion taken orally</li> <li>▫ Powder taken orally in porridge</li> <li>▪ Powder taken orally</li> <li>◆ Decoction taken orally</li> <li>• Powder taken orally</li> <li>▫ Infusion taken orally</li> </ul>	<ul style="list-style-type: none"> <li>• Used to make ropes</li> <li>• Commonly used to treat bile emesis (Sh)</li> <li>▫ To treat inflammatory discharge, interpreted as gonorrhoea (Sh)</li> <li>▪ Infertility in women (Nd)</li> <li>◆ As an aphrodisiac (Nd)</li> <li>• To treat heart pains</li> <li>▫ To treat abdominal pains</li> </ul>



Table 1 Cont.

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<p><i>H. villosa</i> L.f. var. <i>obliqua</i> (Jacq.) Baker = <i>H. sericea</i> (SMITH, 1895)?</p> <ul style="list-style-type: none"> <li>• South Africa</li> </ul> <ul style="list-style-type: none"> <li>• Congo</li> <li>• The Lobedu</li> </ul>	<ol style="list-style-type: none"> <li>1. SMITH, 1895 *</li> <li>2. GERSTNER, 1938 *</li> <li>3. SMITH, 1966 ◇</li> <li>4. BATTEN and BOKELMANN, 1966 ◇</li> <li>5. WATT, BREYER-BRANDWIJK, 1962 #</li> </ol>	<p><i>i-Xalanxa</i> <i>inKomfe</i> (Z)</p> <p>Inkbol (A) Golden winter star Sterretjies (A)</p>	<ul style="list-style-type: none"> <li>• Corm (large)</li> </ul> <ul style="list-style-type: none"> <li>• Leaves</li> </ul> <ul style="list-style-type: none"> <li>• Plant</li> <li>• Corm</li> </ul> <ul style="list-style-type: none"> <li>• Corm, leaf and flower</li> </ul>	<ul style="list-style-type: none"> <li>• Water is boiled in a hollowed out corm</li> <li>◦ Corms are roasted to get resinous exudate which is dried and powdered</li> <li>• Yields good fibre</li> </ul> <ul style="list-style-type: none"> <li>• Not given</li> <li>• Corms are planted on mounds dedicated to the gods</li> <li>◦ Corms are impaled on sticks planted outside the homestead</li> <li>• Eaten</li> </ul>	<ul style="list-style-type: none"> <li>• Boiled water is used to wash bad (infected, septic) animal sores and wounds</li> <li>◦ Powdered resin is used to attach assegai head onto shaft of spear</li> <li>• To make ropes</li> </ul> <ul style="list-style-type: none"> <li>• Used as an African medicine</li> <li>• Magical intentions</li> </ul> <ul style="list-style-type: none"> <li>◦ To repel witches</li> </ul> <ul style="list-style-type: none"> <li>• Produces diarrhoea in domestic stock</li> </ul>
<p><i>H. villosa</i> L.f. var. <i>scabra</i> Baker</p> <ul style="list-style-type: none"> <li>• Lesotho</li> </ul>	<ol style="list-style-type: none"> <li>1. WATT, BREYER-BRANDWIJK, 1962 #</li> <li>2. JACOT-GUILLARMOD, 1971 ◇</li> </ol>	<p><i>moli-letaha</i> (S) <i>molinyana</i> (S) <i>khuoa-kemaoatle</i> (S)</p>	<ul style="list-style-type: none"> <li>• Leaves</li> <li>• Corm</li> </ul>	<ul style="list-style-type: none"> <li>• Not given</li> <li>• A magical plant or charm</li> </ul>	<ul style="list-style-type: none"> <li>• To make ropes which soon rot as of poor quality</li> <li>• Used against thunder</li> </ul>
<p><i>Hypoxis</i> sp.</p> <ul style="list-style-type: none"> <li>• South Africa</li> </ul>	<ol style="list-style-type: none"> <li>1. HUTCHINGS, 1986, 1989b *</li> </ol>		<ul style="list-style-type: none"> <li>• Corm or rootstock</li> </ul>	<ul style="list-style-type: none"> <li>• Juices are extracted</li> </ul>	<ul style="list-style-type: none"> <li>• Used for cancer</li> <li>• To treat the complexion</li> </ul>
<p><i>Hypoxis</i> sp.</p> <ul style="list-style-type: none"> <li>• South Africa</li> </ul>	<ol style="list-style-type: none"> <li>1. HUTCHINGS, 1992 *</li> </ol>		<ul style="list-style-type: none"> <li>• Corm</li> </ul>	<ul style="list-style-type: none"> <li>• Infusions are taken orally</li> </ul>	<ul style="list-style-type: none"> <li>• For palpitations</li> </ul>

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<b>Hypoxis spp.</b> • South Africa	1. BRYANT, (1966) (reprint, 1909) *  2. HUTCHINGS, 1996 * (Valley Trust Healers)	<i>inKomfe</i> (Z) <i>inKomfe enkula</i> (Z)  <i>inkomfe</i> also <i>ilabatheca</i> (Z)	• Plant  • Corm (roots)  • Corm	• Not given – depends on part used, season and method of preparation  • Used with other plants. A small handful of each is pounded and then infused together like tea. A cupful of the infusion is drunk when cool.  • Pounded and mixed with pork fat to make an ointment	• Reputed by the Zulu to be very poisonous  • Used to expel intestinal beetles whose presence is indicated by nervous irritation, pains and fits. Unknown to medical science, this has been interpreted as a treatment for internal parasites  • Used as a purgative • To treat ulcers and impetigo • Used as an anthelmintic
<b>Hypoxis sp.</b> • Botswana	1. HEDBERG and STAUGÅRD, 1989	<i>tshuko ya poo</i> (T)	• Corm (bulb)	• Fresh corms are placed in milk and fed to bulls	• To increase fertility of bulls
<b>Hypoxis sp.</b> • Botswana	1. HEDBERG and STAUGÅRD, 1989 ◇	<i>tshukuyapona</i> (T)	• Corm (bulb)	• Boiled in water. One teaspoon of solution is then taken 3 times a day	• To treat skin, lung, stomach and liver cancer • To prevent cancer in children
<b>Hypoxis sp.</b> • The Manyika	1. WATT, BREYER- BRANDWIJK, 1962 *		• Corm or rootstock	• Burnt to ash ▫ Decoction is taken daily for two weeks	• Ash is applied to wounds ▫ To wean a child if the mother falls pregnant
<b>Hypoxis sp.</b> • The Karanga	1. WATT, BREYER- BRANDWIJK, 1962 *		• Corm or rootstock	• Bitter juice, formed by sprinkling salt on the cut surface of corm, is licked off and swallowed ▫ Juice taken in large doses	• A remedy for bilious vomiting • Loss of appetite • Abdominal pains • Fever ▫ Used to induce vomiting
<b>Hypoxis spp.</b> (unidentified) • Lesotho	1. JACOT- GUILLARMOD, 1971 ◇		• Corm • Leaves	• Eaten raw • Not given	• As a food source • To make ropes which are used to tie up corpses
<b>Hypoxis spp.</b> (±6) • Zimbabwe	1. TREDGOLD, 1986 ◇		• Corm (tuber)	• Not given, presumed eaten but not used as food ▫ Magical plants	• Mildly purgative and therefore prized medicinally ▫ Bring good luck

This review of both the non-African and African genera of the Hypoxidaceae has, however, shown that the traditional medicinal properties and uses of *Empodium* (= *Curculigo*) and *Hypoxis* are very similar. Also common to both these genera is the yellow pigmentation of their slow growing, perennial rootstocks or corms (HILLIARD and BURTT, 1978), yet this fact was not alluded to with respect to traditional healing in any of the literature examined.

A yellow colour in plants is usually indicative of the presence of polyphenolic constituents (MARSTON *et al.*, 1996). It is interesting that the yellow stem bark of *Brackenridgea zanguebarica* Oliv. (Ochnaceae) is used traditionally to heal wounds and sores (WATT and BREYER-BRANDWIJK, 1962) as are the corms of *Curculigo* and *Hypoxis*. More recently the polyphenols extracted from the yellow bark of *B. zanguebarica* collected in Malawi and Tanzania, were shown to have antifungal properties. It was concluded that the polyphenols are therefore responsible for the wound healing properties of the stem bark (MARSTON *et al.*, 1996). Since visual attributes play a major role in the initial selection of plant material for medicinal purposes by traditional herbalists, the yellow colouration probably largely influenced their choice of bark. No reference could be found with respect to either the function or significance of the yellow pigment in the corms of *Curculigo* and *Hypoxis*, or to the role it played in their selection for use in herbal remedies.

Herbal remedies and cures have played a significant role in the development of modern drugs. The traditional importance as well as the potential medicinal and economic value of the Hypoxidaceae was also recognised by the South African pharmaceutical industry several decades ago. Continued interest in the commercial development of phytopharmaceuticals therefore inevitably resulted in a thorough assessment of the medicinal properties attributed to *Hypoxis* corm extracts. These aspects will be discussed in the following section.

#### **1.4 THE COMMERCIALISATION OF THE GENUS *HYPOXIS***

Traditional African medicine has contributed greatly to the current commercial status of *Hypoxis*. Of particular importance has been the gradual development of the immune-enhancing product, Moducare™ Sterinol™, from a traditional herbal farm remedy as first mentioned by VAN STADEN (1981) and later described in detail by VANDERHAEGHE and BOUIC (1999). The latter authors did not specify *Hypoxis* as the medicinal plant which had originally been studied to obtain the correct formulation for the products, however, and only referred to the use of a “wild plant from the veldt” in the herbal remedy. This was done

deliberately to protect the species and prevent *Hypoxis* plants from being indiscriminately harvested from the wild (VANDERHAEGHE, pers. comm.).

#### **1.4.1 THE HYPOXIS STORY**

Known since the early 1900s that aqueous extracts obtained from the corms of *Hypoxis* plants are used by Zulu healers to correct impotency and sterility (BRYANT, 1966), it has recently been reported that cancer is also being treated using these traditional methods (KAMWENDO *et al.*, 1985; HUTCHINGS, 1986, 1989). Local healers in Botswana now use *Hypoxis* extracts to not only treat various cancers but also prevent cancer from developing in children (HEDBERG and STAUGÅRD, 1989). Realising the value of the original traditional cures, settlers and farmers in South Africa experimented with and successfully tried some of these herbal remedies in their homes (VAN STADEN, 1981).

According to VANDERHAEGHE and BOUIC (1999), a significant milestone was reached in 1958 when an elderly man, critically ill and dying from prostate cancer, was treated with a traditional herbal remedy which had previously been successfully used by a farmer's wife. Boiled aqueous extracts from the corms of *H. hemerocallidea* were given to the patient to drink (VAN STADEN, 1981). The boiled extract was administered as a cold tea in 100 ml portions five times daily (PEGEL, 1984). The remedy was so effective that the patient not only recovered, but survived for another ten years.

A relative, R.W. Liebenberg, witnessed this remarkable recovery and, realising that this plant might hold the key to a cure for cancer, particularly prostate cancer, devoted his life to examining the chemical and medicinal properties of this and other *Hypoxis* plants. His theory was also supported in the 1960s by the findings of the American National Cancer Institute (NCI). The NCI identified members of the Hypoxidaceae as potential anti-cancer agents and recommended that the anti-cancer activity shown by this family should be thoroughly investigated (refer page 4). Events, people and research steered by R.W. Liebenberg and which led to the development of a "potent immune system cure", are outlined in detail by VANDERHAEGHE and BOUIC (1999) and will only be referred to in passing for the purposes of this review.

#### **1.4.2 THE POTENTIAL OF HYPOXIS IN MODERN HEALTH CARE**

Today, however, although many clinical procedures used to treat cancers are successful, a cure remains elusive and the incidence of cancer continues to escalate. Cancers

affecting the elderly are also becoming increasingly important as are those developing as a result of AIDS as outlined above. Survival rates for liver, bone, lung pancreas and prostate cancer, all common among the elderly, have remained unchanged since the late 1960s because they are difficult to treat (VANDERHAEGHE and BOUIC, 1999). Cancer of the prostate gland, for example, affects older men. It accounted for 25% of all malignancies diagnosed among American men in 1993 and was ranked by the American Cancer Society as the second prime cause of cancer mortality among men in the USA after lung cancer at the time (DAWSON and VOGELZANG, 1994). In South Africa the lifetime risk of men developing prostate cancer is reported to be amongst the highest in the world (KOTZENBERG, 1997) thus further emphasising its importance in southern Africa.

Benign prostatic hypertrophy (BPH), a non-malignant enlargement of the fibromuscular and epithelial structures within the prostate gland, is also very prevalent amongst ageing men (BERGES *et al.*, 1995). This common ailment affects more than 25 million men in North America alone (VANDERHAEGHE and BOUIC, 1999). BPH does not necessarily develop into prostate cancer, but can either contribute to incorrect diagnoses or result in increased risk of malignancy if treated surgically (DAWSON and VOGELZANG, 1994).

Recently finasteride, a 5-alpha-reductase inhibitor used to treat and alleviate the symptoms of BPH, became available in the USA. The suggestion has since been made in the USA that, because of its non-surgical mode of action, this or other similar therapeutic agents such as the alpha-receptor blocking agent, alfuzosine (BERGES *et al.*, 1995), could be effectively used to delay or even prevent the clinical onset of prostate cancer in high-risk men by initially targeting and reducing BPH. It was therefore suggested that the application of these or other therapeutic agents as a method to control the onset of prostate cancer merited further investigation (DAWSON and VOGELZANG, 1994). Plant products sold for this purpose in Germany are not available in the USA, however, as their effectiveness and standard concentrations have not been proven (TYLER, 1994; BERGES *et al.*, 1995). To determine the effect of medications on BPH, however, the mechanism that causes BPH should be understood. This is clearly described by TYLER (1994), and DAWSON and VOGELZANG (1994).

#### **1.4.2.1 Sterol and $\beta$ -Sitosterolin Extracts from *Hypoxis* Corms**

Meanwhile independent research to develop a cure for prostate cancer continued in South Africa. According to VANDERHAEGHE and BOUIC (1999), R.W. Liebenberg initially persuaded Dr. Ebbinghaus from Germany to test the *Hypoxis* corm extract on his patients

suffering from prostate problems. Results showed a reduction in the symptoms of BPH, but not prostate cancer. He concluded that the plant extract could be most effectively used in the treatment of the prostate gland by alleviating the symptoms of BPH, but that more research would be required to prove this.

In South Africa, therefore, the main direction of scientific research on the *Hypoxis* corm extracts was to initially isolate and identify the constituents of value in the treatment of BPH rather than concentrate on any potential anti-cancer compounds. These constituents were identified as the plant sterols and  $\beta$ -sitosterolins. The significance of these compounds and methods for their extraction were presented in the United Kingdom Patent Specification No. 1,417,272 of 10 December 1975 (PEGEL and LIEBENBERG, 1975). Sterolins (phytosterol glucosides) are sterol molecules bound to a glucose molecule. They are more soluble than the sterols and therefore more readily absorbed by the body. Sterolins can also be inadvertently removed from the corm extracts, however, as the glucose molecules are easily broken off by hydrolysis leaving only sterols in the extract.

Commercial interest by the pharmaceutical industry, therefore, initially resulted in the aqueous *Hypoxis* corm extracts being tested in clinical trials to determine their potency as a treatment for BPH. Of the 738 patients treated, 96% showed significant BPH regression. Results were reported in the United Kingdom Patent Specification No. 1,259,503 originally lodged in 1969 by K.S. Warren, but proceeded with in the name of R.W. Liebenberg in March 1970 and finally published on 5 January 1972. The claim that the corm extracts exhibited antibiotic, anti-inflammatory and diuretic properties, and relieved the conditions of cholesterosis and arthritis during treatments was made in the same (LIEBENBERG, 1972) and Federal Republic of Germany No 2,015,877 (LIEBENBERG, 1970) patent.

During this initial research it was discovered, however, that sterolins were often removed from the corm extracts by hydrolysis during processing. As a consequence the potency of the extract was reduced and was no longer as effective in alleviating the symptoms of BPH. It was concluded, therefore, that the sterolins were the most important component of the extract. Subsequent studies showed that a more purified sterolin-rich fraction could be obtained from *Hypoxis* corms after heat inactivation of the phytosterol glycosidases and that these extracts were the most effective in the treatment of BPH (PEGEL and LIEBENBERG, 1973 a).

This resulted in several patent applications to register the procedures for the extraction of sterols and sterolins from *Hypoxis* plant material. These included the South African Patent

Application No. 72/1855 of 5 April 1973 (PEGEL and LIEBENBERG, 1973 a), the Federal Republic of Germany Patent No. 2,312,285 of 20 September 1973 (PEGEL and LIEBENBERG, 1973 b) and the United Kingdom Patent Specification No. 1,417,272 of 10 December 1975 (PEGEL and LIEBENBERG, 1975). Patents were by then also being applied for by other research groups such as the Federal Republic of Germany Patent No. 2,251,695 of 25 April 1974 (REISCH, 1974). Patent applications dealing with claims with respect to *Hypoxis* extracts and the phytosterols and which will not be referred to here, have generally been summarised in two later United States Patents No. 4,160,860 of 10 July 1979 (PEGEL, 1979) and No. 4,198,401 of 15 April 1980 (PEGEL, 1980).

Chronic sterolin deficiency is generally presented symptomatically by arthritis, inflammation and BPH, particularly among the elderly. Although sterolins are a natural constituent of plants, their therapeutic value and importance as an essential food component had not been recognised prior to the clinical trials using *Hypoxis* extracts (PEGEL, 1984). Since a minimum daily dose of 0.01 mg  $\beta$ -sitosteryl  $\beta$ -D-glucoside (the most common natural phytosterolin) is required in a normal nutritional diet, it was concluded that any pharmaceutical products developed to treat BPH should provide a daily sterolin dose of between 0.01-0.10 mg.

The average amount of sterolin, calculated as  $\beta$ -sitosteryl  $\beta$ -D-glucoside, extracted from *H. hemerocallidea* corms is 9 mg / 100 g of spray-dried powder (PEGEL, 1979). Sitosterolins make up approximately 90% of the sterolins extracted from these corms, while the remainder consists of campesterol, stigmasterol and spinasterol (VAN STADEN, 1981). Calculations also showed that 100 ml of aqueous *Hypoxis* extract contains 1.0 mg of dissolved sitosterolin (PEGEL, 1984). As already mentioned the boiled extract is usually administered as a cold tea in 100 ml portions five times daily. PEGEL (1984) therefore concluded that the original *Hypoxis* tea prescribed would have provided more than enough sterolin to successfully treat BPH, hence its usefulness in herbal remedies.

Subsequently, capsules containing dried *Hypoxis* corm powder with a standardised 10 mg of sterol and 0.1 mg of sitosterolin as the active agent (in a ratio of 100:1), were then manufactured and successfully tested. Ultimately capsules based on these ingredients were manufactured and sold in Germany, specifically for the treatment of BPH, under the trade name of Harzol® (PEGEL, 1984). This therapeutic product has been the market leader for the treatment of BPH in Germany since its introduction there in 1974 (VANDERHAEGHE and BOUIC, 1999). It has not been released in either the USA due to

stringent controls or South Africa, however, even though results showed that BPH was improved in the presence of  $\beta$ -sitosterolin (BERGES *et al.*, 1995).

As summarised by VANDERHAEGHE and BOUIC (1999), further research on this product eventually led to the formulation in South Africa of a patented capsule, Moducare™ Sterinol™. Each of these capsules contains sterols and sterolins at twice the concentration of those in Harzol® capsules, i.e. 20 mg sterols and 0.2 mg sterolins (100:1). These phytosterols have been shown to modulate the immune system and by so doing may play an important role in treating diseases associated with a defective immune system. These diseases include BPH, chronic fatigue syndrome, and lupus among others, but there is still no conclusive evidence to support some of these claims. Initial research has shown, however, that they help to not only increase the number of T-lymphocyte cells in the body, but also stimulate the activity of the T-cells (BOUIC *et al.*, 1996). Moducare™ capsules are sold as a therapeutic immune system booster in health shops and pharmacies.

#### **1.4.2.2 Hypoxoside Extracts**

In the meantime R.W. Liebenberg's quest to find a cure for cancer had been continuing quietly. Many of the early reports and patents dwelt on the favourable medicinal properties of the *Hypoxis* corm extracts. This led to further research and additional patent applications in which it was claimed that the high biological activity of the extracts might in fact be due to the presence of other unidentified compounds besides the sterols and sitosterolins (PEGEL, 1979, 1980). In these patent applications the latter author not only described new extraction procedures, but also the preparation of *Hypoxis* capsules, tablets, dragées, ointments and creams. Pharmacological investigations on rats showed that in both the acute and chronic toxicity tests, no toxic phenomena or organ changes were detected. The extracts produced had a beneficial prophylactic and / or curative effect on several disease conditions such as intestinal ulcers, BPH, diseases of the urinary tract, blood and blood-forming organs, and cardiovascular systems such as varicose veins and haemorrhoids. Dermatological diseases included eczema, acne and other forms of dermatitis, while diseases of the skeletal system and muscles which benefited from the extract were inflammation, arthritis, rheumatism and high uric acid levels. Clinical investigations on 1198 patients showed that daily doses of 50-1000 mg of the extract were the most effective in the treatment of the majority of these diseases (PEGEL, 1979, 1980).

The assumption that other unidentified compounds, resulting in the high biological activity measured, were present in the extracts was proven correct when a new diglucoside,

hypoxoside, was isolated from corm extracts of *H. obtusa* Burch (MARINI-BETTOLO *et al.*, 1982). The same compound was independently isolated from several South African *Hypoxis* species namely *H. acuminata*, *H. colchicifolia* (= *H. latifolia*), *H. hemerocallidea* (= *H. rooperi*), *H. nitida*, *H. obtusa* and *H. rigidula*, as well as from *Spiloxene schlechteri* (DREWES *et al.*, 1984). Identified as norlignans, this group of related glycosides has been isolated from only the Hypoxidaceae. Hypoxoside is the major constituent of the genus *Hypoxis* with overall yields of 3.7% recorded from corm extracts of *H. obtusa* (MARINI-BETTOLO *et al.*, 1982) and between 3.5% and 4.5% from extracts of the other species examined (DREWES *et al.*, 1984). In the latter publication a method for the synthesis of the tetramethoxy derivative of hypoxoside was also described. Although this aglucone, trivial name rooperol, is readily obtained by  $\beta$ -glucosidase hydrolysis of hypoxoside, it was not detected in the *in vivo* corm extracts at the time (DREWES *et al.*, 1984). Since then rooperol has been successfully synthesised (POTGIETER *et al.*, 1988).

Again this discovery resulted in several patent applications being lodged in which the preparation of hypoxoside and rooperol was described and their use as anti-cancer agents claimed. Patents pertinent to South Africa are the European Patent Application No. 0,092,226 of 26 October 1983 (DREWES and LIEBENBERG, 1983 a), United Kingdom Patent Application No. 2,120,650 of 7 December 1983 (DREWES and LIEBENBERG, 1983 b), the European Patent Application No. 0,206,765 of 30 December 1986 (WENTELER *et al.*, 1986), and two United States Patents, Nos. 4,644,085 of 17 February 1987 (DREWES and LIEBENBERG, 1987 a) and 4,652,636 of 24 March 1987 (DREWES and LIEBENBERG, 1987 b). The claim that hypoxoside, rooperol and their derivatives are effective in the treatment of inflammation was also made in the PCT International Patent Application No. WO 95/34296 of 21 December 1995 (ALLISON *et al.*, 1995).

These compounds, or norlignans, are considered to be the agents, other than the sterols and sitosterolins, responsible for many of the medicinal properties attributed to *Hypoxis* corm extracts, but this has not yet been unequivocally proven. Hypoxoside is considered today to be one of the most studied phytochemicals obtained from an African plant (ALBRECHT, 1996). A comprehensive biopharmaceutical investigation of hypoxoside has been presented by KOCH and BROSCH-STARZENGRUBER (1991). Additional studies have also indicated that hypoxoside exerts analgesic effects (NICOLETTI *et al.*, 1996) and that methanolic extracts of *H. hemerocallidea* corms show hypoglycaemic activity thus lending credence to the traditional use of the plant in the management of adult Type II *Diabetes mellitus* in South Africa (OJEWOLE, 2000).

Reported as used to treat headaches and inflammation (HUTCHINGS and VAN STADEN, 1994), *H. hemerocallidea* plants were therefore screened for prostaglandin-synthesis inhibitory activity, but were not classified as active as the extracts tested did not reach the minimum criteria set (JÄGER *et al.*, 1996). The authors emphasised, however, that a negative result did not conclusively mean the plant was without anti-inflammatory activity as the active compounds could work at another site in the inflammation process. It is significant, however, that compounds from *Hypoxis* corm extracts are also being tested in controlled clinical trials in South Africa, in conjunction with Moducare™ capsules, to determine their efficacy as daily supplements to stabilize the immune systems of HIV-positive patients (BOUIC *et al.*, 1996).

Hypoxoside is also being examined as both a natural anti-tumour compound (KOCH and BROSCH-STARZENGRUBER, 1991) and a potential non-toxic prodrug for the treatment of inflammation, certain malignancies and HIV-infection (ALBRECHT, 1996). Research has also shown that while hypoxoside is generally inert, rooperol shows high pharmacological activity (ALBRECHT *et al.*, 1995 a, b). Nevertheless questions have been raised with respect to the toxicity of *H. hemerocallidea* plants. Following extensive clinical and research trials conducted on corm extracts of both *H. hemerocallidea* and other *Hypoxis* species at Tygerberg Hospital, Cape Town, ALBRECHT (2000) has been able to show, however, that these corm extracts and tinctures are not toxic. The anti-cancer potential of these molecules is of great interest to medical research, however. To this end hypoxoside was shown to be adequately soluble in aqueous body fluids and that its dissolution rate was sufficiently high to provide fast absorption following oral administration (KOCH and BROSCH-STARZENGRUBER, 1991). Initial tests also indicated that the analogues, dehydroxy- and bis-dehydroxy-rooperol, may be preferentially absorbed by humans during treatment (KRUGER *et al.*, 1994).

As discussed in SMIT *et al.* (1995), high  $\beta$ -glucuronidase and sulphatase activities have been observed in experimental tumours. This led to the idea of designing a therapeutic prodrug, which has yet to be developed for human use, that could be selectively activated by these enzymes at its site of action. Since the aglucone, rooperol, is readily obtained by  $\beta$ -glucosidase hydrolysis of hypoxoside (DREWES *et al.*, 1984) hypoxoside and rooperol, isolated from the corms of both *H. hemerocallidea* and *H. colchicifolia* (= *H. latifolia*), were therefore examined in a series of research studies and phase 1 clinical trials with a view to developing an oral prodrug for cancer therapy.

Initial experiments showed hypoxoside to be cytotoxic for murine and melanoma cell lines *in vitro*, but that this was dependent on the production of the toxic aglucone, rooperol, by the deconjugation of hypoxoside by endogenous, heat labile  $\beta$ -glucosidase in the fetal calf serum (THERON *et al.*, 1994). Furthermore it was found that neither hypoxoside nor rooperol were present in the circulation of experimental animals after oral ingestion, but that the phase II metabolites (glucuronides and sulphates) were (KRUGER *et al.*, 1994). Like hypoxoside these conjugated metabolites are also non-toxic to cells *in vitro* but can be activated when treated with glucuronidase. Because of the specific action of rooperol, ALBRECHT *et al.* (1995 a, b) therefore proposed that hypoxoside could perhaps be useful as an oral, non-toxic, multifunctional prodrug for cancer therapy.

Having established the above, the main aim of current research is therefore to achieve selectivity in cancer chemotherapy through the activation of rooperol metabolites at the site of certain tumours which contain relatively high levels of glucuronidase. The first two clinical phase 1 trials on lung cancer patients demonstrated an absence of hypoxoside toxicity (SMIT *et al.*, 1995). During the determination of dosage levels, in which the patients were given various doses incorporating 1600, 2400 and 3200 mg of a standardised *Hypoxis* plant extract in 200 mg capsules, it was found that neither hypoxoside nor rooperol appeared in circulation. A daily dose of 2400 mg of extract was also sufficient to reach metabolite levels near  $100 \mu\text{g ml}^{-1}$  which had been shown to be tumouricidal (cytotoxic) after enzymatic deconjugation to rooperol (ALBRECHT *et al.*, 1995 a). The important role of rooperol in these applications and the fact that *H. colchicifolia* corm extracts contain a higher proportion of dehydroxy and bis-dehydroxy rooperol than the extracts of *H. hemerocallidea*, also led KRUGER *et al.* (1994) to stress the importance of selecting the correct *Hypoxis* species for possible therapeutic application.

Traditional healers also claim that *Hypoxis* plants are the most effective medicinally when applied or used in a mixture with other medicinal plants or herbs (BRYANT, 1966; MAHLABA, 1999). The mixture is always administered for a specific period of time under the guidance of the traditional healer (SINGH, 2000). It would be interesting to determine the roles of both hypoxoside and rooperol in these applications.

### 1.4.3 HYPOXIS AND THE POPULAR HEALTHCARE INDUSTRY

As a result of these investigations, the increase in HIV positive-infections and the intensive promotional marketing of Moducare™, claims with respect to the incredible medicinal properties of *Hypoxis* corm extracts have been widely publicized in newspapers and

popular magazines (LEE, 1996; LOUW, 1997; YOUNGHUSBAND, 1997; BALETA, 1997; HEARD, 1999) and reported on radio stations (MATSAU, 2000). The South African Broadcasting Corporation has also screened promotional TV programmes, viewed by the author and confirmed by DREWES and HORN (1999), which have attracted the attention of the general public desperate to find relief from incurable diseases such as AIDS and cancer. In spite of the fact that all published research on the development of Moducare™ is based on an internationally patented mixture of sterols and sterolins (BOUIC, 2000), people have developed high expectations from the "miracle" *Hypoxis* plants because of their perceived healing properties. The question that remains to be answered, however, is how this new demand will impact on the popular healthcare industry, the informal plant markets and, most importantly, the natural populations of *Hypoxis* plants.

The healthcare industry in South Africa is divided into several entities. These are the formal system of medicine, the natural health care and homeopathic systems, other cultural systems of healing, and the large informal traditional medicinal system (VAN WYK *et al.*, 1997). It is estimated that in the latter system in South Africa, approximately 80% of black patients consult traditional healers and use herbal remedies for primary health care (GUMEDE, 1989; MANDER, 1997). Turnover in the South African informal medicinal plant or "muthi" trade was estimated to be approximately R270-million per annum in the late 1990s, with more than 700 known plant species actively traded (DOLD and COCKS, 2002).

Traditional healers in South Africa have always collected medicinal plants and barks which they require from wild populations. This method of obtaining plants is also employed on a small scale by the farming community and on a very large scale by plant gatherers for the informal trade in medicinal plants (CUNNINGHAM, 1988 a, 1988 b). The corms of *Hypoxis* plants are also collected in the wild. These are prepared for use by being dried in the sun first and then stored. Aqueous extracts or "tea" can be prepared from these dried corms when required (PEGEL, pers. comm.). Corms are sold either fresh, sun-dried or in powder form at medicinal plant markets.

Since the wide publicity surrounding *Hypoxis* and the many unsubstantiated claims that infusions of the corm can cure AIDS, cancer, arthritis and hypertension, the demand for these plants by healers, their patients and medicinal plant traders has increased markedly. Brisk trade in these plants on the streets of Pretoria and Johannesburg, Gauteng Province, in herbal shops and markets, and in the townships was witnessed by MATSAU (2000) who counted at least four out of every ten hawkers in a single street in central Johannesburg

selling *Hypoxis* corms at a cost of between R3.00 and R7.00 each. The species offered for sale were not given. MATSAU (2000) also commented that some of the sellers interviewed seemed to be more concerned about the immediate success of their businesses and the healing power of *Hypoxis* than about the destructive harvesting of the plants from natural populations and the negative impact this would have on their trade in future.

Similarly the sale of *H. hemerocallidea*, *H. colchicifolia* and *H. rigidula* corms as a cure for HIV and AIDS was personally witnessed at Durban's large Warwick Triangle Medicinal Market, KwaZulu-Natal Province. The corms were not sold individually but in the more traditional way of a small pile consisting of five corms for R10.00. Cheaper than in Gauteng, *Hypoxis* corms were available from nearly every stall visited at the market and were observed to be the most sought after item from the market that day. A market seller also explained in great detail that the shape of the corm determined how and to whom it should be prescribed. The smooth, rounded base of the *H. colchicifolia* corms indicated to her that these could be prescribed to treat women's problems. The protruding clump of fibres and leaf bases at the apex of both *H. hemerocallidea* and *H. rigidula* corms, however, resembled male genitalia and she would therefore sell these to treat men's problems. With the increase in the popularity of and trade in *Hypoxis* corms, a another widespread survey on current usage, demand and harvesting methods of these plants would be extremely valuable.

In a previous survey it was found that geophytic plants were poor field indicators of demand and supply by the informal trade as their corms and roots were totally removed during harvesting. As a result the availability / scarcity of wild plants such as *Hypoxis* was difficult to establish. Instead in the survey, the usage of individual geophytes had first to be ascertained to accurately determine their conservation status and current demand. Therefore in an attempt to obtain an indication of the quantities supplied of the most commonly sold medicinal plants, 54 traders in KwaZulu-Natal were interviewed to determine their approximate annual plant sales (CUNNINGHAM, 1988 a).

With respect to actual sales of *Hypoxis* plants, CUNNINGHAM (1988 a) estimated that in 1988 the annual demand for *H. colchicifolia* corms was 164 x 50 kg maize bags. Although the number of bags sold was given for *H. colchicifolia*, the number of corms (ie 191) per bag was only given for *H. hemerocallidea*. The figure of 191 was therefore used to calculate that approximately 31 324 *H. colchicifolia* corms were sold that year by the 54 traders interviewed. This excludes corms sold to other provinces and outside the trading circle. Based on the 1988 retail price of R10.00 per 50 kg bag, individual *Hypoxis* corms

were sold for approximately R0,06 each. As outlined the cost at the Durban Medicinal Plant Market had increased by the end of 2002 to R2.00 per plant regardless of the species (personal observation) and to between R3.00 and R7.00 in Johannesburg. Increased cost often indicates a decrease in availability and therefore an increase in demand.

Results from an even more recent survey undertaken in the Eastern Cape Province and conducted after the popularization of *Hypoxis*, showed that *H. hemerocallidea* was the most frequently traded medicinal plant species in the areas surveyed. The greatest quantity of plant material sold was also that of *H. hemerocallidea*. Annual sales reached 11 000 kg with a market value of R322 500. With a mean price of approximately R30 per kg, it was the 8<sup>th</sup> most expensive plant sold (DOLD and COCKS, 2002). Using the figure of 4 corms per kg (CUNNINGHAM, 1988 a), it was calculated that the retail price was approximately R8.50 per corm at these markets. Although quantitative data was not given, DOLD and COCKS (2002) concluded that 93% of the plants listed in the survey were harvested unsustainably.

Ever since the production of Harzol<sup>®</sup>, however, sun-dried, powdered *Hypoxis* corms have been sold commercially on a small scale in South Africa under the original trade name, Prostamin<sup>®</sup>, and recently as Hypoxin<sup>®</sup> (PEGEL, pers. comm.). This trade has always been predominantly in a small section of the middle income health care sector but, since the general popularisation of *Hypoxis*, demand for these plants from the general population has increased phenomenally, not only for medication, but to supply business ventures. It was estimated that there are approximately 30 private entrepreneurs operating from the Johannesburg, Pretoria and Nylstroom areas alone (MATSAU, 2000). This has resulted in many self-prepared remedies being produced for the popular market (SINGH, 2000).

These products take the form of crushed dried flakes, capsules, liquids and dried powders and are sold privately, in health shops and at flea-markets. Products are variously labelled as "Green Gold" capsules by Nisha Moodley, a natural herbal remedy; "Cure Coffee" a herbal in powder form by Foxees; "Africa Potato<sup>™</sup>", the first 100% natural and pure form of the *Hypoxis* available commercially as granules or capsules. The latter product is advertised as helping with immune deficiency disorders such as colds and flu, cancer, AIDS, Yuppie flu, arthritis, appetite disorders, tuberculosis and eczema, as well as helping with anxiety and insomnia, all of which are unsubstantiated claims. Other products are produced under more controlled conditions by registered companies such as Phyto-Force, whose premises double up with the South African College of Herbal Medicine and Health. Their products include an *Hypoxis* cream recommended for fungal and bacterial infections

of the skin, and an *Hypoxis* tincture to build up the bodies' immune response, to detoxify the liver and as an anti-candida agent (PHYTO-FORCE DISTRIBUTORS, 1999; SOUTH AFRICAN COLLEGE OF HERBAL MEDICINE AND HEALTH, 1999). At the time of the interview, the statement was made that the concentration of the active ingredients in the tincture could not be guaranteed (TONES, pers. comm.).

It has been shown that extracts of *Hypoxis* deteriorate even when refrigerated due to the poor shelf life of the aqueous solution of hypoxoside (KOCH and BROSCHE-STARZENGRUBER, 1991). Yet an extract, labelled "African Potato Extract", is being offered for sale to the public as a dark brown tea in a 750 ml bottle for approximately R10.00 per bottle. According to DREWES and HORN (1999) the instructions are to keep the extract refrigerated and to take a brandy tot portion three times daily. To test the efficacy of this extract aliquots were examined by thin layer chromatography (TLC) (DREWES and HORN, 1999). Its components were then compared to those of pure hypoxoside from *H. hemerocallidea* and sitosterols from Moducare™. These showed that the raw aqueous extract contained far less phytosterols per dose than Moducare™, at least three other unknown compounds were present, and that degradation had occurred even though the extract had been stored in the refrigerator. It was concluded that these extracts should be taken with caution until a suitable herbal preparation incorporating the best ingredients from *Hypoxis* appears on the market.

A disturbing article appeared in The Sunday Tribune under the heading "*Schoolboy's remarkable 'wonder potato'*" (SUNDAY TRIBUNE, 1999). The article stated that this young schoolboy was making his fortune from the African Potato or *Hypoxis hemerocallidea*. He was producing a tea from these plants and selling this in pharmacies or via mail-order. The claim was made that this 'wonder potato' helped to relieve gout, arthritis, rheumatism, high blood pressure, exhaustion, haemorrhoids and cancer. As a result of his success he had been selected as a finalist in the ABSA / Rapport Top-Entrepreneur competition, of which he was eventually declared the winner. Unfortunately the judges did not take into account that all this product was obtained from wild *Hypoxis* plants collected from natural populations. Harvesting *Hypoxis* plants from the wild is illegal (MATSAU, 2000). Although removed from private land, this fact should perhaps have received a higher priority particularly as *H. hemerocallidea* has been included by SCOTT-SHAW (1999) in his book on rare and threatened plants of KwaZulu-Natal and neighbouring regions. Although not yet threatened it is listed as requiring cultivation in order to meet increasing demand.

Popularisation of *Hypoxis* has inevitably led to the large scale collection of *Hypoxis* corms throughout South Africa to supply not only the traditional medicinal plant trade but also outlets at flea-markets, small business ventures and popular health shops. Fresh corms are also sold directly to the public via health shops and even fruit and vegetable outlets. On enquiring about the source of these plants, the author was informed that corms were supplied by plant gatherers who normally collect and sell plants to vendors at the medicinal plant markets. There are also unconfirmed reports that truck-loads of corms are being removed from farmland in northern KwaZulu-Natal to supply both inland and overseas markets. There have been similar reports for Gauteng Province (MATSAU, 2000). Such extensive, and illegal harvesting of *Hypoxis* plants at an unsustainable rate led MATSAU (2000) to conclude that the genus will become threatened within a very short period of time and that its conservation status will have to be reassessed.

Since their introduction to the popular health market, *H. hemerocallidea* plants have commonly been referred to as the “African Potato” and *iLabatheka* (LOUW, 1997). However neither of these names was used to describe *H. hemerocallidea* in the literature reviewed (Table 1). First heard by the author on a TV programme, botanists are generally in agreement that “African Potato” has been mistakenly used for *Hypoxis* in this instance (POOLEY, 1998; DREWES and HORN, 1999; SINGH, 2000; MATSAU, 2000). *Plectranthus esculentus* has in fact always been known as the African or Wild Potato (POOLEY, 1998). Similarly *iLabatheka* is the vernacular name commonly given to *H. colchicifolia* (= *H. latifolia*) and *Dioscorea dregeana* (= *isidakwa*) (DOKE *et al.*, 1990). It is also used to describe medicine for causing madness or excitement. Extracts of *H. colchicifolia* are in fact used to induce delirium in patients. Although *Hypoxis* plants are commonly referred to by these names today the correct vernacular name for *H. hemerocallidea* is *inkomfe* (POOLEY, 1998).

SMITH (1966) over 30 years ago recognised that for a vernacular name to have permanent value it had to be linked to a botanical name describing one plant only. He therefore recorded the vernacular names of identified plants and linked these to correctly identified herbaria specimens. As shown by the general use of vernacular names listed in Table 1, this does not yet apply to the genus *Hypoxis*. This has resulted in the general public not knowing whether they have obtained the correct species for cultivation or collection for medicinal purposes (VOS, pers. comm.).

From this literature survey it is apparent that although the medicinal constituents and applications of *H. hemerocallidea* have been well researched, there is much confusion with

respect to the correct identification of the various *Hypoxis* species and their medicinal properties. Not yet developed as a crop plant, *Hypoxis* corms are also being removed from natural areas in enormous numbers to meet the new demand. This is putting great pressure on wild populations, a situation which needs to be addressed to prevent *Hypoxis* from becoming another threatened genus in South Africa. To assess the current status of the genus, aspects dealing with the taxonomic and botanical background of the Hypoxidaceae were reviewed and are presented in the following chapter.

## *Chapter 2*

# THE GENUS *HYPOXIS* L.: A LITERATURE REVIEW

## 2.1 INTRODUCTION

The literature reviewed in Chapter 1 clearly demonstrated that many *Hypoxis* species are difficult to identify with certainty. This has been compounded by the fact that several, sometimes incorrect, common and vernacular names are often used to describe more than one species or genus, even from different families. These names usually refer, in the case of *Hypoxis*, to the qualities of individual species such as their star-shaped, yellow flowers, grasslike foliage, yellow mucilaginous corms or so-called poisonous nature. Adding to the confusion is that the family Hypoxidaceae as well as the genus *Hypoxis* are also both frequently referred to as the Star Lily "Family" (LETTY *et al.*, 1962) or the Yellow Star "Family" (GELFAND *et al.*, 1985).

In addition there is even uncertainty as to the origin of the generic name *Hypoxis*. An unusual version is that it is derived from the Greek word *hypoxys* meaning "somewhat acid" (ANON, 1998). Most often cited, however, is that the name originates from the Greek words *hypo* (below) and *oxys* (pointed). The latter has been interpreted in several ways and is said to allude not only to the inferior ovary with its sharply narrowing base (HEIDEMAN, 1979; PAGE, 1984), but also to the sharp points of the "inferior" petals (BRYAN, 1989), or "inferior" perianth segments (BRUCE-MILLER, 1995). In a Greek / Latin dictionary the translation of *hyp-*, *hypo-* is given as "below, under, beneath and lower", and *oxycarpus* as "bearing sharp, pointed fruit". These agree with HEIDEMAN's (1979) interpretation. The confusion still surrounding the classification of *Hypoxis* also supports the earlier observation by THOMPSON (1976) that a comprehensive revision of the family Hypoxidaceae is urgent. Its current systematic status will be discussed briefly.

## 2.2 THE FAMILY HYPOXIDACEAE : SOME SYSTEMATICS

The taxonomic history of the monocotyledonous family Hypoxidaceae R.Br. has been summarised among others by THOMPSON (1972), DARNLEY GIBBS (1974 a, b, c), WOOD (1976), HEIDEMAN (1979), DAHLGREN and CLIFFORD (1982), NORDAL *et al.*

(1985), NICOLETTI *et al.* (1992) and ZIMUDZI (1993). It is apparent from these accounts that both the taxonomic delimitations and phylogenetic relationships of this family have been subject to much debate and alteration since its establishment in 1814 by Robert Brown when he separated the genera *Hypoxis* and *Curculigo* from the Amaryllidaceae and raised them to the rank of family, the "Hypoxideae" (BROWN, 1814. Cited from STAFLEU and COWAN, 1976). Brown's name is conserved and registered as Hypoxidaceae R.Br. (DARNLEY GIBBS, 1974 b). Overall, two main schools of thought with respect to the status of the Hypoxidaceae have emerged since 1814.

Following the first school of thought Lindley, in 1836, retained the name of "Hypoxideae" but reduced the family status to that of tribe. The tribe "Hypoxideae" was again returned to the family Amaryllidaceae (NORDAL *et al.*, 1985). This view was supported by many of the earlier systematists (DAHLGREN and CLIFFORD, 1982) as well as by BAKER (1896) in his key to the "tribe Hypoxideae" in the Flora Capensis, NEL (1914), and HILLIARD and BURTT (1978). It is even listed as such in the INDEX KEWENSIS (1992).

The second school followed the classification system of BROWN (1814) in which the family status of the "Hypoxideae" retained but changed to the Hypoxidaceae. This view became widely accepted after HUTCHINSON (1934) limited the Amaryllidaceae to plants with umbellate inflorescences (DAVIS, 1966; WILLIS, 1966; THOMPSON, 1972, 1976; WOOD, 1976; HEIDEMAN, 1979, 1983; DAHLGREN and CLIFFORD, 1982; DAHLGREN *et al.*, 1985; NORDAL *et al.*, 1985; HILLIARD and BURTT, 1986; HERNDON, 1988; DAVIES and LLOYD, 1990; REID and ARCHER, 1993; ZIMUDZI, 1993, 1994 a; SNIJMAN, 2000). Chemical analyses provided further proof for this separation when alkaloids were reported absent in the Hypoxidaceae, but present in the Amaryllidaceae (DARNLEY GIBBS, 1974 a). It would appear, however, that consensus with respect to the family status has still not been reached as illustrated by the contradictions expressed by HILLIARD and BURTT in 1978 and 1986, and the INDEX KEWENSIS entries for 1989 (DAVIES and LLOYD, 1990) and 1992.

Similarly, the phylogenetic relationships of the family remain unresolved. Briefly relationships between the Hypoxidaceae, Liliaceae, Amaryllidaceae, Orchidaceae and Haemodoraceae, within the orders Liliales, Amaryllideae, Haemodorales or equivalent have been proposed (DARNLEY GIBBS, 1974 b, 1974 c). Recent evidence in which morphological, embryological and chemical characters were plotted to display phylogenetic affinities among the monocotyledons, defined the Hypoxidaceae as a separate family

within the order Asparagales, superorder Liliiflorae (DAHLGREN and CLIFFORD, 1982). The family was included in the Asparagales on the basis of its starchless seeds, phytomelan-encrusted seed coats, and lack of vessels in the stem and leaves. A previously suggested affinity between the Hypoxidaceae and Velloziaceae, based on their mutual possession of epigynous flowers, paracytic stomata and fibrous leaf bases, was also noted. DAHLGREN *et al.* (1985) suggested that these similarities were due rather to convergent evolution, thus justifying separating the families and placing the Velloziaceae in the order Velloziales.

Morphological characters used and other general information on the Hypoxidaceae has been summarised and is readily available in WATSON and DALLWITZ (1992).

### 2.2.1 THE GENERA

A cosmopolitan family, the Hypoxidaceae is represented by several genera worldwide with the exception of Europe and northern Asia (WILLIS, 1966). Its distribution is widespread and ranges from temperate to tropical regions. Although *Hypoxis* L. is accepted as the type genus for the family, agreement with respect to the constituent genera has not been reached and continues to vary (BAKER, 1878; NEL, 1914; THOMPSON, 1972, 1976, 1978; HILLIARD and BURTT, 1978; DAHLGREN and CLIFFORD, 1982; WATSON and DALLWITZ, 1992; SNIJMAN, 2000).

The most recent key to the genera of the tribe "Hypoxideae" was presented by HILLIARD and BURTT (1978) in their series on plants from southern Africa. The eight genera tentatively accepted worldwide in the revision are *Curculigo* Gaertn., *Empodium* Salisb. (= *Forbesia* [Eckl. ex] Nel), *Hypoxis* L., *Molineria* Colla, *Pauridia* Harv., *Rhodohypoxis* Nel., *Saniella* Hilliard and Burtt, and *Spiloxene* Salisb. (= *Ianthe* Salisb.). The Australian genera *Campynema* Labill. and *Campynemanthe* Baill. were excluded on the grounds that they "do not seem to be closely related to the other genera". ZIMUDZI (1993), however, still argued that only *Hypoxis* and *Curculigo* appeared distinct enough to warrant generic status, but provided no details of the other genera. He concluded that a universal generic concept for the Hypoxidaceae does not yet exist and repeated THOMPSON'S (1976) observation that a comprehensive revision of the family is necessary.

It is generally accepted, however, that in southern Africa, the Hypoxidaceae is represented by the genera *Hypoxis*, *Saniella*, *Rhodohypoxis*, *Empodium*, *Pauridia*, and *Spiloxene* of which the last five are endemic to the region (HILLIARD and BURTT, 1978; SNIJMAN,

2000). The first three genera occur mainly in summer rainfall areas while the last three are found in the winter rainfall areas of the Cape Province, South Africa (THOMPSON, 1972; HILLIARD and BURTT, 1978).

## **2.3 THE GENUS *HYPOXIS* L.**

The genus *Hypoxis* L. was established by Linnaeus in 1759 (CRAMER, 1964). The American species, *H. erecta* L. (an illegitimate name based on the type of *Ornithogalum hirsutum* L. = *H. hirsutum* L. Coville), was originally selected as the type specimen for the genus (ZIMUDZI, 1993). Its members are widely distributed throughout the tropical and subtropical regions of the Americas, south-east Asia, Australia and sub-Saharan Africa, with the largest concentration occurring in the eastern Cape, South Africa (BAKER, 1896; HEIDEMAN, 1979; REID and ARCHER, 1993).

Since its establishment, *Hypoxis* has been comprehensively described by several authors as chronicled by ZIMUDZI (1993). The diagnostic characters accepted for the genus are hairy flowers with free perianth segments and no perigone tubes, trilocular inferior ovaries without a beak, subglobose seeds with black or brown testae, six stamens with filaments attached medianly in the sinuses of the anther thecae, and anthers similar on both surfaces (HILLIARD and BURTT, 1978; NORDAL *et al.*, 1985). The primitive type of slow-growing, perennial, vertical rootstock or corm with its yellow pigmentation, is characteristic of both *Hypoxis* and *Curculigo* (HILLIARD and BURTT, 1978).

### **2.3.1 SPECIES OF THE GENUS *HYPOXIS***

Species delimitation within *Hypoxis* has proven difficult not only because of the apparent occurrence of apomixis and hybridization, but also because systematists have given the same morphological characters different taxonomic values (NICOLETTI *et al.*, 1992). Although many species have been described since the establishment of the genus, the total number worldwide is unknown due in part to recent reductions to synonymy (WOOD, 1976) and the formation of large species complexes in regional revisions (NORDAL *et al.*, 1985; ZIMUDZI, 1993). Reports fluctuate from between 50-60 species worldwide (BAKER, 1896), to approximately 80 (BOND and GOLDBLATT, 1984), 88 (PHILLIPS, 1951), 90 (DYER, 1976; DAHLGREN *et al.*, 1985; SINGH, 2000; SNIJMAN, 2000), and 100 (HILLIARD and BURTT, 1987).

The reported number of species in southern Africa varies from 41 (BAKER, 1896; PHILLIPS, 1951), to 43 (REID and ARCHER, 1993), 45 (DYER, 1976; SNIJMAN, 2000) and 50 (HILLIARD and BURTT, 1987). SNIJMAN (2000) noted that southern Africa is a centre of diversity for *Hypoxis*, which is represented in all countries and provinces of this region except Botswana and the Northern Cape Province. As discussed in Chapter 1 with respect to the uses of *Hypoxis* being re-cited over and over again without new, novel additions to the literature, it is apparent that a similar situation exists with respect to the number of species cited. The above figures also appear to have been largely extracted from previous literature and not as a result of original research. This lends support to the often expressed view that *Hypoxis* presents substantial taxonomic problems and requires urgent revision (WOOD, 1976) as does the family Hypoxidaceae.

The two major keys to the genus *Hypoxis* in southern Africa are those of BAKER (1896) and NEL (1914). Again, not only do the diagnostic characters and number of *Hypoxis* species differ in each of these keys, but they are also used by different Herbaria, thus adding to the taxonomic confusion (HEIDEMAN, 1979; NORDAL *et al.*, 1985).

The diagnostic characters for NEL'S (1914) key were obtained purely from herbarium specimens (HEIDEMAN, 1979). Since *Hypoxis* plants, particularly the larger species, shrink considerably during drying resulting in morphological changes, HEIDEMAN (1979) suggested this as a possible contributing factor to the large number of species in Nel's work. Taxonomic studies of East African *Hypoxis* using living plant material also indicated that characters such as leaf width, peduncle length and inflorescence shape used by BAKER (1896), and number of veins per leaf and leaf shape used by NEL (1914) show continuous growth and variation and, therefore, were totally unsuitable for species delimitation (NORDAL *et al.*, 1985). Both the latter authors and HERNDON (1988) found that seed and fruit characters provided a more consistent basis for separating species, but these are often absent from herbarium specimens. In contrast, BRUCE-MILLER (1995) found leaf morphology a useful character as it was relatively consistent within a species, although extremely variable between species of the eastern Cape which were examined.

It is interesting to note here, however, that even though inflorescence morphology varies greatly within an *Hypoxis* species, these characters were successfully used to separate *H. sessilis* Linnaeus and *H. wrightii* (Baker) Brackett, provided inflorescences from plants at similar stages of post-fire development were compared. Comparison of inflorescences from plants at different stages of post-fire recovery, however, suggested that numerous intermediates connected these two *Hypoxis* species (HERNDON, 1988). It was just such a

comparison of dissimilar morphological characteristics of plants at different stages of growth which led to the proposal that all North American *Hypoxis* should be regarded as a single species (BRITT, 1967). Similarly, *H. rooperi* Moore was reduced to a synonym of *H. hemerocallidea* Fisher and Meyer by WOOD (1976), yet the differences in morphology of comparable plants of this species observed by BRUCE-MILLER (1995), led her to suggest that either *H. hemerocallidea* should be split into two varieties or the status of *H. rooperi* should be re-considered. Similar occurrences have also been observed by the author.

These findings emphasise the importance of comparing *Hypoxis* specimens at similar stages of development, either age and / or seasonal, for accurate identification and species delimitation. The use of fresh material would obviously also be advantageous. The author's personal observations of *Hypoxis* plants during the course of this study would support this view. These aspects should be considered in any future revisions, particularly to clarify the large species complexes which are currently being created based on the occurrence of polyploidy, apomixis and the overlapping of morphological characteristics (NORDAL *et al.*, 1985; ZIMUDZI, 1993).

In the latter study it was suggested that seven *Hypoxis* species and one large species complex should be recognised, namely *H. filiformis*, *H. angustifolia*, *H. cuanzensis*, *H. galpinii*, *H. goetzei*, *H. rigidula* and *H. multiceps*, and an *H. villosa* complex (ZIMUDZI, 1993). This was justified by measuring 34 morphological characters from approximately 800 *Hypoxis* specimens and applying multivariate analysis. Although the characters examined were also obtained from a few fresh specimens collected in Zimbabwe the majority were taken from herbarium specimens. The accuracy of this type of analysis must be questioned considering the objections raised with respect to age, shrinkage and use of dried herbarium specimens as outlined above.

It has become apparent from the available literature reviewed here that taxonomic data on both the family Hypoxidaceae and the genus *Hypoxis* L. is inclined to be anecdotal, confused and sometimes even contradictory. The fact that several *Hypoxis* species have medicinal applications also seems to have been ignored in much of the literature dealing with taxonomic issues. Unfortunately it would seem that it is still "impossible to construct a satisfactory simple key to this genus" (COMPTON, 1976) and, until the entire genus is revised, "*Hypoxis* will remain a headache to the systematist" (WILSENACH and WARREN, 1967).

### 2.3.2 MORPHOLOGICAL CHARACTERISTICS OF *HYPOXIS*

Morphological characteristics have generally been used in identification keys to differentiate between the species of *Hypoxis*. The general morphology of *Hypoxis* plants from eastern, central and southern Africa has been thoroughly described by WOOD (1976), HEIDEMAN (1979, 1983), NORDAL *et al.* (1985), ZIMUDZI (1993), SINGH (1999) and SNIJMAN (2000). The more distinguishing characters only will be discussed.

A prominent feature of these perennial herbaceous geophytes is the vertical, fleshy, primitive rootstock or corm with its yellow internal pigmentation which oxidises and turns black rapidly when exposed to air. The corms vary in shape from subglobose to cylindrical. The author's personal observations are that the former is more common among juveniles and smaller species such as *H. filiformis* Baker and *H. angustifolia* Lam., while the latter is more prominent among the larger species such as *H. hemerocallidea* and *H. colchicifolia*, and is a feature of mature corms. Dimensions of the corms of East African *Hypoxis* species have been reported to range from between 10 mm to 80 mm in diameter and 10 mm to 90 mm in length (NORDAL *et al.*, 1985), while those of species found further south range from 5 mm to 110 mm in diameter and 8 mm to 119 mm in length (PAGE, 1984; ZIMUDZI, 1993).

Growth is vertical and continuous throughout the life of the plant. The fleshy, adventitious, contractile roots which emerge from around the corm in distinct rings, die back seasonally leaving rows of prominent scars or holes on the corm. New roots are formed annually in rows immediately above the previous season's roots and / or scars. Unfortunately the root scars cannot be used to determine corm age as all the roots produced in one season do not die simultaneously and some persist into the following growing season (HEIDEMAN, 1979).

The leaves arise directly from the meristematic region of the corm apex in either a tristichous or rosulate arrangement. In some species, for example *H. hemerocallidea*, the leaves are arranged in three precise ranks. In others, cataphylls ensheath the leaf bases to form a short leaf column which extends from the corm to the soil surface. The leaf columns of *H. colchicifolia* and *H. galpinii* Baker are narrow at the base and widen out above ground so that the foliage becomes distinctly funnel-shaped. In contrast, the leaf columns of *H. rigidula* Baker and *H. acuminata* Baker are extended well above ground level to produce a rod-like pseudostem (HEIDEMAN, 1979). The simple leaves vary from linear to lanceolate in shape, few to many, erect to prostrate. They grow continuously throughout

the growing season due to intercalary meristems at their bases. This results in great variation in shape and size depending on the developmental stage of the plant (NORDAL *et al.*, 1985; HERNDON, 1988). *Hypoxis* plants become dormant in winter but the dried leaves remain attached to the corm as there is no abscission area (HEIDEMAN, 1979). Subsequently the new leaf bases are often surrounded by bristles, the remnants of the prominent vascular strands from the previous season's leaves.

A basic character of the genus, with the exception of *H. interjecta* Nel which has totally glabrous leaves, is the presence of hair on all the aerial parts (HEIDEMAN, 1979). The hairs are either double, usually with unequal arms, or in stellate clusters, and vary in length, thickness, density, leaf position and colour which may be white, grey, yellow or red-brown. Hair type is constant within a population, but more than one type is usually found within a species (ZIMUDZI, 1993). The types of leaf indumentum were classified as types A to F using the above characters in a very useful key by NORDAL *et al.* (1985).

Axillary peduncles terminating in an inflorescence are produced early in the growing season while the leaves are still short. The inflorescence types range from simple, through corymbose to racemose. A correlation has been found between the number of flowers and inflorescence type, with the corymbs being few-flowered and the racemes many-flowered (NORDAL *et al.*, 1985). The star-shaped flowers are usually yellow, occasionally white, with an inferior ovary and free perianth segments which persist on the fruiting capsules to maturity. The capsules are either a pyxis having circumscissile dehiscence, or loculicidal with longitudinal dehiscence, but there is no seed dispersal mechanism (HERNDON, 1988).

Each fruit may contain as many as 50 seeds containing copious oily endosperm. Each seed has a distinct funicle and projecting micropyle in close proximity to one another and a hard testa which ranges from black to brown and is usually sculptured. Recent studies have shown the testa to be papillate, rarely smooth. A useful diagnostic feature is that the papillate seeds can be divided into two groups on the basis of the presence or absence of a special folded cuticular coating on the papillae (NORDAL *et al.*, 1985; HERNDON, 1988).

The seed testa are also encrusted with phytomelan which is an inert, opaque, brittle, charcoal-like substance, very rich in carbon (DAHLGREN and CLIFFORD, 1982). Usually overlooked, this feature is of taxonomic importance as the thickness of the crust can vary considerably between families and is very thick in the Hypoxidaceae. It was observed that

the seeds of the more primitive taxa have a thicker phytomelan crust ( $\pm 180 \mu\text{m}$ ) (HUBER, 1969). This also concurs with the opinion that *Hypoxis* corms are of a primitive type.

### 2.3.3 CYTOLOGY AND REPRODUCTION OF *HYPOXIS*

Species delimitation within the genus *Hypoxis* has remained difficult due in large part to the morphological differences observed both between and within species. WILSENACH (1967) speculated, however, that the morphological variation found in *Hypoxis* species is not as a result of the influence of environmental factors since the different types commonly occur together in the same habitat.

An important study on the development of the ovule and seed in the genera *Ianthe* (= *Hypoxis*, now = *Spiloxene*) and *Pauridia* was conducted by DE VOS (1948; 1949) and deserves mention. The aim was to determine whether comparative embryology could be used as a tool to distinguish between the genera examined. Results showed great similarity between these genera confirming their correct placement in the Hypoxidaceae. Developmental differences in embryology were so slight between individuals, however, that the same characters could not be used to differentiate between species.

In 1967 the first important cytological observations on some *Hypoxis* species were published. Cytology as a means to separate species also proved to be very complex as it was found that the chromosome numbers of the larger forms of *Hypoxis* were often greater than 80 and therefore difficult to determine from root tip preparations (WILSENACH and PAPENFUS, 1967). The initial chromosome numbers determined using this method varied between  $2n = 16$  to 96, but the basic chromosome number could not be conclusively determined (WILSENACH, 1967).

Pollen was therefore analysed to determine the haploid chromosome numbers instead. These were found to be low ( $n = 7$ ) for *H. filiformis*, a small species, and high ( $n = 96$ ) for *H. hemerocallidea* (= *H. rooperii*), a large species. From this data, WILSENACH and WARREN (1967) concluded that polyploidy played an important role in the speciation of *Hypoxis* and proposed that the smaller species with low chromosome numbers reproduce sexually while those with high numbers are apomictic. This was investigated further by studying embryo-sac development in these two species. *H. filiformis* was selected because it has a low chromosome number ( $n=7$ ) and its populations show very little morphological variation. In contrast *H. hemerocallidea* is a large species with a high, but variable, chromosome number ( $n = 86$  to 96) and whose populations show morphological variation.

Although both degeneration of the megaspore mother cell and twin embryo-sacs were observed, apomixis could not be proven.

A subsequent cytological analysis of East African species of *Hypoxis* has confirmed the occurrence of low chromosome numbers in the smaller and more homogenous species ( $2n = 14$  for *H. angustifolia* Lam.) and high numbers for the larger species or *H. obtusa* Burch. complex (40 to 200) (NORDAL *et al.*, 1985). A basic chromosome number of seven was proposed. Seeds were produced in the absence of pollen in one case only, however, thus indicating that apomixis might occur. These results led ZIMUDZI (1994) to speculate that the complex cytology and reproduction of the genus has a bearing on the species problem, but could not prove this conclusively. The suggestion by HERNDON (1988) that morphological characters, such as inflorescences, should only be compared if they are from plants at similar stages of development might be of value in solving this problem if taken into consideration when designing cytological studies in future.

#### 2.3.4 CHEMICAL CONSTITUENTS OF *HYPOXIS*

Chemical constituents of plants have played an increasingly important role in taxonomy. The Hypoxidaceae were given family status on the basis that they contain no saponins and are the only plants from which hypoxoside has been isolated. An examination of the phenolic constituents of monocotyledonous plants also revealed that the Hypoxidaceae have a primitive pattern of phenolic constituents (BATE-SMITH, 1968). The chemical constituents of the Hypoxidaceae, with emphasis on the corms of *H. hemerocallidea* (= *H. rooperi*), are summarised in HÄNSEL *et al.* (1993).

Most chemical analyses of the Hypoxidaceae have in fact been carried out on *Hypoxis* plants. Phenolic constituents recorded as present in *Hypoxis* are quercetin, caffeic acid and p-coumaric acid (BATE-SMITH, 1968). Both chelodonic acid (DAHLGREN and CLIFFORD, 1982) and flavonols (WATSON and DALLWITZ, 1998) also occur in *Hypoxis*, but alkaloids, steroidal saponins (DAHLGREN *et al.*, 1985), proanthocyanidins and ellagic acid (WATSON and DALLWITZ, 1998) have been recorded as lacking. Other compounds isolated include zeatin and zeatin glycoside (VAN STADEN, 1981), the carbohydrates, starch, glucose, sucrose and fructose (BEWS and VANDERPLANK, 1930) and the fatty acids, palmitic, septadecanoic, sepdecatrienoic, octadecenoic, octadecadienoic and stearic acids (VAN STADEN and BAYLEY, 1988). Of greatest economic importance to date has been the extraction of sterols and sitosterolins from the corms of *H. hemerocallidea* and the

subsequent formulation of capsules prescribed to treat BPH and latterly to modulate the human immune system as discussed in Chapter 1.

An interesting seasonal change in cytokinin-like activity was also measured in the leaves, roots and corms of *H. hemerocallidea* (PAGE and VAN STADEN, 1982). Results showed that the cytokinin compounds not only accumulated in the leaves during leaf maturation and decreased with leaf senescence, but also increased in the corms prior to the resumption of visible growth after winter dormancy and then decreased as growth commenced. It was suggested that the cytokinins were utilized rapidly or metabolized during growth.

Hypoxoside, an unusual bis-(3,4-dihydroxyphenyl)pent-1-en-4-yne glycoside, is the most studied compound produced by *Hypoxis*. It was isolated initially from the corms of *H. obtusa* (MARINI-BETTOLO *et al.*, 1982) and *H. hemerocallidea* (= *H. rooperi*) (DREWES *et al.*, 1984) and its structure determined. Hypoxoside is also present in the corms of *H. acuminata*, *H. colchicifolia* (= *H. latifolia*), *H. nitida*, *H. rigidula* and *Spiloxene schlecteri* (DREWES *et al.*, 1984). Since the discovery of hypoxoside several studies have been carried out to isolate and identify compounds in different species of *Hypoxis* which might be of interest to the pharmaceutical industry. None has been used as a taxonomic character to differentiate between two or more species, however, as far as can be established from the literature reviewed.

Amongst these chemicals are the related compounds, nyasoside (MARINI-BETTOLO *et al.*, 1985) and nyasicoside (GALEFFI *et al.*, 1987) isolated from corms of *H. nyasica* Baker collected in Malawi. More recently two new monoglucosides, mononyasine A and mononyasine B, as well as hypoxoside, were obtained from corms of *H. nyasica* (MESSANA *et al.*, 1989). Also reported is acuminoside, a new terpenoid glycoside and its aglucone, geraniol, from *H. acuminata* (BREDENKAMP *et al.*, 1989). A naturally occurring derivative of hypoxoside, 1-(3",4"-dihydroxyphenyl)-5-(4'-hydroxyphenyl)pent-1-en-4-yne, was also isolated from *H. hemerocallidea* (DREWES *et al.*, 1989) and interjectin, a derivative of nyasicoside, from *H. interjecta* and *H. multiceps* (MARINI-BETTOLO *et al.*, 1991). Further phytochemical analysis of *H. obtusa* resulted in the isolation of the known compounds, acuminoside, hypoxoside and nyasoside plus a new phenolic glycoside named obtusaside (MSONTHI *et al.*, 1990).

Hypoxoside has proved difficult to produce synthetically. *In vitro* cultures were therefore examined as a means to obtain this compound in bulk. Only the malformed root-type

cultures were shown to contain hypoxoside, but at too low a concentration to justify *in vitro* culture as a method to for its large scale production (PAGE and VAN STADEN, 1987).

Because of the perceived medicinal importance of hypoxoside and the continued interest shown in its synthesis, attempts were made to determine its biosynthetic pathway. Using *in vitro* root producing callus of *H. hemerocallidea*, cinnamic acid and phenylalanine were shown to be precursors thus indicating hypoxoside to be a shikimate pathway derivative (BAYLEY and VAN STADEN, 1988). In subsequent studies both these (BAYLEY and VAN STADEN, 1990 b) and various other radio-labelled compounds (BAYLEY and VAN STADEN, 1991) were incorporated into hypoxoside in intact *H. hemerocallidea* plants. Extracts of the corms, roots and leaves showed that the corms had the highest concentration of hypoxoside, then the roots and finally the leaves. BAYLEY and VAN STADEN (1990 a) concluded that although the corm appeared to be the major site for hypoxoside biosynthesis, the biosynthetic potential of the leaves and roots should not be dismissed. In a later study, when [<sup>14</sup>C]hypoxoside was applied to the leaves or corms of *H. hemerocallidea* plants most of the radioactivity was later detected in the corms (VAN STADEN and UPFOLD, 1994). It was concluded therefore that hypoxoside is rapidly hydrolysed in the leaves from where the end products are exported to the corm. Only a small amount, however, is exported in return from the corm to the leaves.

The intense foaming action observed to occur among plants used in traditional medicine in Malawi resulted in their being examined for molluscicidal activity, including *H. hemerocallidea*. A mortality rate of only 33.3% was recorded among molluscs exposed to the *Hypoxis* corm extracts, however, and was too low for the extract to be effectively used as a molluscicide (AMUSAN *et al.*, 1995). Similar results were obtained using *H. nyasica* corm extracts (KAMWENDO *et al.*, 1985). No novel compounds responsible for the observed foaming action were identified and saponins were reported as absent from the *Hypoxis* species examined.

All the important chemical compounds discussed have been extracted from the corms of the various *Hypoxis* species analysed to date. Whole plants have therefore been destructively harvested to obtain corms for extraction impacting negatively not only on the supply of plants, but in obtaining reproducible corm extracts with the same concentration of compounds as used in previous investigations. At this point the observation by TYLER (1986) should perhaps be repeated which is that an important constraint encountered by the pharmaceutical industry is the increased difficulty to obtain sufficient, correctly identified medicinal plants from dwindling natural populations for the intensive evaluation

required to develop plant-derived drugs. This has been further compounded in southern Africa where plant products are used daily and wild plants have remained the main source of supply to the traditional and other medicinal plant trades. Existing plant populations are not an infinite resource, however, and the introduction of these medicinal plants as viable commercial crops needs to be investigated.

### **2.3.5 CULTIVATION OF *HYPOXIS***

Traditional healers in South Africa have always collected *Hypoxis* plants from the wild for medicinal purposes, as have the farming community and informal medicinal plant traders. Popularisation of these plants has led to even greater demand from the healthcare and pharmaceutical industries and the middle income group. Not yet cultivated commercially, *Hypoxis* corms have simply been removed from natural areas to meet demand.

It would appear, however, that *Hypoxis* seeds are generally difficult to germinate and require long periods of post-ripening (WOOD, 1976; HEIDEMAN, 1979). Problems associated with seed propagation of *H. hemerocallidea* were investigated (HAMMERTON, 1985). Results indicated that the seeds were difficult to germinate due to both a coat-imposed and embryo dormancy (HAMMERTON and VAN STADEN, 1988) and that seed germinability was related to seed density and varied with the site and date of harvest (HAMMERTON *et al.*, 1989). Reasons for the failure of 60% of the experimental seeds to germinate could not be proven, however, and should be investigated further (HAMMERTON, 1985). In contrast the genus, *Rhodohypoxis*, grows easily from seed (KILLICK, 1976).

All the seed trials mentioned were conducted in the colder areas of Pietermaritzburg or Johannesburg. The cold conditions experienced in winter may have affected both the flowering and seed production of these plants as similar problems with respect to seed germination were not experienced in later trials conducted in the warmer climate of Durban. Horticultural procedures relating to seed propagation of *H. hemerocallidea* are outlined in GILLMER and SYMMONDS (1999) and HAWKER *et al.* (1999).

It was originally argued, however, that with the high incidence of polyploidy shown by the large *Hypoxis* species, seed propagation may not be recommended particularly if large numbers of superior chemotypes are required to be cloned for the pharmaceutical industry. *In vitro* propagation was therefore proposed for this application. Procedures for the *in vitro* culture of *H. hemerocallidea* were therefore investigated (PAGE, 1984) and protocols for

the multiplication of this species from corm (PAGE and VAN STADEN, 1984) and flower bud explants (PAGE and VAN STADEN, 1986) developed. These procedures were then modified for the commercialisation and mass production of *H. hemerocallidea*, *H. acuminata*, *H. rigidula* and *H. obtusa* via a continuous micropropagation system (APPLETON and VAN STADEN, 1995 a). *H. obtusa* has also been successfully produced *in vitro* but *H. nyasica* and *H. angustifolia* failed to regenerate under the same conditions (VINESI *et al.*, 1990). Subsequently, however, *H. angustifolia* was successfully propagated in tissue culture (APPLETON and VAN STADEN, 1995 b). *Rhodohypoxis baurii*, a small member of the Hypoxidaceae has also been cultivated *in vitro* (UPFOLD *et al.*, 1992).

Results of trials to determine the best growing media to wean *in vitro* cultures of *H. hemerocallidea* and *H. nitida* have been reported (DIVES, 1991). Data on large scale field cultivation of *Hypoxis* species has not been published. A small field trial was undertaken in and near Pietermaritzburg to establish the effect of soil type and fertilisation on growth and establishment of *H. hemerocallidea* plants produced *in vitro*. The effect of different herbicides was also investigated in the same trial (McALISTER and VAN STADEN, 1995). These aspects have been summarised in HAWKER *et al.* (1999).

## 2.4 OBJECTIVES

In a project undertaken to develop a conservation policy for the sustainable use of and trade in indigenous / medicinal plants, the escalation in plant trade was attributed to population growth, rapid urbanisation, unemployment and inadequate medical facilities. In turn the increase in plant trade has resulted in indiscriminate gathering of wild plants by interested parties. Such widespread harvesting combined with the development of virgin land has caused irreparable damage to the environment, habitat and plant numbers (CUNNINGHAM, 1988 a, 1988 b). This and other evidence highlighted the urgent need to review the issues of the exploitation and conservation of indigenous plant resources in Africa (CUNNINGHAM *et al.*, 1992; ANATI *et al.*, 1994).

As *Hypoxis* plants are herbaceous geophytes and therefore destructively harvested, no evidence of their former presence in heavily exploited natural localities is left. Situations like this and the lack of published data on the demography of indigenous plants led CUNNINGHAM (1988 a) to conclude that an accurate assessment could not be made of either the sustainability and effects of harvesting on natural populations of geophytes, or whether demand was exceeding supply. Because *Hypoxis* plants do not reproduce vegetatively and possess dormant seeds which are difficult to germinate (HAMMERTON

and VAN STADEN, 1988), their natural replacement could be outstripped by demand from the escalating herbal trade industry. To conserve existing natural populations and avoid an imbalance occurring between supply and demand, methods for their propagation should be developed. Despite all the attention, as reviewed, *H. hemerocallidea* is still not cultivated commercially. Supplies of *H. hemerocallidea* must be ensured, however, as this plant could prove to be the single most important commercial species this decade to not only the pharmaceutical industry (VANDERHAEGHE and BOUIC, 1999), but also the traditional healing profession. With time and ongoing research many of the other *Hypoxis* species may in fact also fall into this category.

Continued interest in the genus, *Hypoxis*, therefore stems from its:

1. sustained use worldwide in traditional medicine;
2. recognised pharmaceutical value to western medicine;
3. anticipated use as a multifunctional oral prodrug for cancer;
4. potential economic importance; and
5. threatened natural habitat.

From the literature reviewed it became apparent, however, that the two species of *Hypoxis* which are most frequently bought and utilised on a regular basis in traditional medicine are *H. hemerocallidea* and *H. colchicifolia*. Most investigations have been carried out on *H. hemerocallidea*, but very little has been published on *H. colchicifolia*. The latter species has been identified, however, as an important potential prodrug for the treatment of cancer (KRUGER *et al.*, 1994), but has unfortunately also been declared as becoming difficult to find in the wild in KwaZulu-Natal (NAIDOO, 1998).

The aim of this study is, therefore, to investigate and produce, for both the pharmaceutical industry and the traditional medicinal trade, assured sources of supply of *H. colchicifolia* Baker, a valuable medicinal plant identified for the important role it can play in the development of an anti-cancer prodrug. The successful introduction of these wild *Hypoxis* plants as viable commercial crop plants will, however, depend on the production of large numbers of plants initially. For this to succeed various methods by means of which plants can be propagated on a large scale to supply the various industries, must be investigated and developed. A popular technique by means of which clones of high yielding medicinal plants can be produced is micropropagation.

### *Chapter 3*

## ***HYPOXIS COLCHICIFOLIA* BAKER: A REVIEW AND POPULATION STUDIES**

### **3.1 INTRODUCTION**

The preceding literature review has shown that although 11 species of *Hypoxis* have been cited as used for traditional purposes in southern Africa, only two, *H. hemerocallidea* and *H. colchicifolia*, are commonly used in South Africa. A third species, *H. rigidula*, is used to a much lesser extent. The first two are also the only species of *Hypoxis* mentioned in surveys of the most popular medicinal plants sold by herbal traders either directly from markets in the Province of KwaZulu-Natal (CUNNINGHAM, 1988 a, 1988 b) or via mail-order from herbal shops in Gauteng (the Witwatersrand) (WILLIAMS, 1992). Recently both species have been recorded as still regularly collected and used by traditional healers in KwaZulu-Natal for medicinal purposes (NAIDOO, 1998). In the Eastern Cape Province, *H. hemerocallidea* was identified as the most frequently traded medicinal plant species at the markets surveyed. Only one other *Hypoxis* species, provisionally identified as *H. filifolia*, was mentioned and rated 56 out of the 60 most traded plant species (DOLD and COCKS, 2002). In other countries in sub-Saharan Africa *H. obtusa* appears to be the most widely utilized *Hypoxis* species. All are highly valued and sought after for their medicinal properties.

Current preference for these species is not clear, other than the large-scale popularization of *H. hemerocallidea*, but could arguably be due to either their superior medicinal efficacy, large corm size, abundance, conspicuousness on the ground, or increased scarcity of the other *Hypoxis* species with traditional uses. These reasons are difficult to quantify, however, as pertinent published biological and / or ecological information on each species is lacking. In addition plant gatherers still collect *Hypoxis* from the wild. Plants are not cultivated as they do not regenerate vegetatively and their seeds appear to be dormant (WOOD, 1976; HAMMERTON and VAN STADEN, 1988). Since *Hypoxis* plants are destructively harvested to obtain the underground corm, the part generally used for medicinal purposes (Table 1), little or no evidence of their previous distribution or

abundance within exploited natural populations is left. It is therefore difficult to assess the effect previous harvesting has had on the natural populations of *Hypoxis*.

At a conference on botanical biodiversity (biological diversity) in southern Africa, the point was also made that although important reviews on the conservation and utilization of plant biodiversity in Africa had been undertaken, they were very general and contained little information or detail on botanical resources themselves (HUNTLEY, 1994). Similarly, in an overview of research in tropical areas (HEDBERG, 1987) and at a landmark workshop held in Bangkok, it was emphasized that much published research on the economic value and sustainable harvesting of non-timber products from tropical forests by rural peoples used untested assumptions based on indirect, anecdotal evidence about the usage and sustainable harvesting of wild populations (GODOY and BAWA, 1993). It is therefore evident that even in the well studied area of tropical forests, quantitative analyses of the effect of harvesting on natural populations are lacking and that without these appropriate conservation and management plans cannot easily be designed (HALL and BAWA, 1993).

By exploring the "Traditional Botanical Knowledge" of local people using qualitative interviewing and observational methods, participants in other ethnobotanical studies have observed that the knowledge traditional societies have of the distribution of wild resources is sophisticated. It would appear that they have, on the whole, developed patterns or methods of resource use which are sustainable in both the short and long term. It was therefore suggested that these traditional methods should be investigated as tools to manage wild resources (COTTON, 1996). A second opinion, however, is that although traditional healers may harvest wild species on a sustainable basis, traditional land ownership systems are based on common property rights which are fragile systems easily unbalanced by population increase and agriculture. Tribal activities can therefore result in land degradation and plant extinctions (GIVEN and HARRIS, 1994). Conversely indiscriminate resource use can also impact negatively on traditional methods. Therefore, before traditional methods can be effectively implemented, qualitative data on traditional methodology should be verified quantitatively. This would require a multi-disciplinary approach incorporating a broad range of data, both traditional and biological, to successfully develop biological resource management programmes (COTTON, 1996).

Although judicious harvesting of medicinal plants is practised by traditional healers in southern Africa (MAVI, 1994), it is also well known that wild plant populations are undergoing irreparable damage due to the combined effect of rapid urbanisation, development of open spaces, agriculture and indiscriminate gathering to supply the

expanding herbal trade industry (CUNNINGHAM, 1988 b; 2001). Concern has therefore been expressed that the excessive demands placed on plant species and ecosystems could lead to a situation in which many species, about which little is known, reach depletion. The lack of botanical information to support the wise management of plant species in the region has been strongly emphasised (ANATI *et al.*, 1994). To maintain a stable, sustainable plant resource it is, therefore, vital to determine its conservation status and develop harvesting strategies based on relevant and accurate biological data on plant survival, reproduction, growth rates, population densities and utilization.

This scenario is also applicable in South Africa where the trade in indigenous medicinal plants remains buoyant and plants with high value are increasingly placed under threat of extinction, particularly at a local level (SCOTT-SHAW, 1999). The conservation status of only two *Hypoxis* species, *H. hemerocallidea* and *H. ludwigii*, has, however, been recorded. Both fall into the lower risk (least concern) category of rare and threatened plants at a provincial level. It was found, however, that the conservation status of a plant species can change when only threatened plants used for traditional purposes are considered. This is because the large spatial scales used to draw up provincial categories do not accurately account for species at threat within a smaller scale such as a rural area of several thousand square kilometres. Surprisingly local extinctions of heavily utilized species were found to be common. *H. hemerocallidea* is among the list of medicinally important plants shown to be locally extinct within small areas of KwaZulu-Natal (SCOTT-SHAW, 1999).

This is supported by NAIDOO (1998) who reported that healers interviewed in areas of KwaZulu-Natal were finding it increasingly difficult to source certain prized medicinal plants from traditional collecting sites in the wild near Izingolweni and Ulundi in KwaZulu-Natal. Both *H. hemerocallidea* and *H. colchicifolia* were included among these plants. Demographic studies showed these species were unable to withstand the pressure of collection. This led to the conclusion that *H. hemerocallidea* and *H. colchicifolia* cannot be harvested on a sustainable basis and therefore require careful management and active protection to ensure population survival (NAIDOO, 1998). If the redefinition of the conservation status of threatened species at a local level is followed (SCOTT-SHAW, 1999), this could be interpreted to indicate that *H. colchicifolia* should now also be placed in the conservation category of being locally extinct in certain traditional collecting sites in KwaZulu-Natal.

To accurately determine the conservation status of individual *Hypoxis* species, however, their usage must be known. The availability and / or scarcity of *Hypoxis* plants in the wild is

difficult to establish as geophytic plants are poor field indicators of demand and supply because their corms and roots are totally removed during harvesting. Data with respect to the usage, market value and individual costs of *H. hemerocallidea* and *H. colchicifolia* corms is presented in Chapter 1. This showed, from surveys conducted at the large markets in KwaZulu-Natal and the Eastern Cape Province, that approximately 31 000 and 44 000 corms of *H. hemerocallidea* were sold annually at the time that the respective areas were surveyed. Collectively this totalled in excess of 75 000 corms from selected areas in just these two provinces alone and excludes corms sold at peripheral markets, exported to other provinces and collected by healers for private use in their practices. In addition approximately 31 300 *H. colchicifolia* corms were sold in KwaZulu-Natal.

The long term effect of the annual removal of so many corms must have a detrimental effect on wild populations particularly, as NAIDOO (1998) observed, that large corms were removed in preference to smaller corms and that this adversely affected seedling recruitment. Rate of re-establishment is also difficult to estimate because, although large *Hypoxis* plants such as *H. colchicifolia* are thought to live to 25 years or more, the exact age of large plants at the time of harvest is unknown and no field data on corm growth, seed establishment or hypoxoside yields are available to confirm this. Natural replacement of *Hypoxis* plants could therefore be outstripped by demand from the escalating herbal trade industry. CUNNINGHAM (1988 a, 2001) concluded that quantitative data on the effects of over exploitation of medicinal plants needs to be assimilated. Of particular importance is data on the biomass and population dynamics of individual geophytes found in grasslands, as these species have always been "lumped together as forbs", including *Hypoxis*.

With respect to the medicinally important species of *Hypoxis*, research has centred around *H. hemerocallidea* and *H. obtusa* and the extraction and identification of their active compounds or norlignans, specifically hypoxoside and rooperol (Chapter 1). Little accurate data has been published on the growth rates, reproduction, population densities, natural occurrence or harvesting pressures of the *Hypoxis* species listed in Table 1. Because *H. colchicifolia* occurs only in KwaZulu-Natal (WOOD, 1976) and appears to be declining in number (NAIDOO, 1998), it is essential to research and collate all aspects of, and published information available on, this important medicinal plant. This in order to more fully meet the requirements identified as necessary to formulate a management or harvesting strategy to conserve and maintain *H. colchicifolia* as a stable, sustainable resource.

## 3.2 *HYPOXIS COLCHICIFOLIA*: A LITERATURE REVIEW

### 3.2.1 SYSTEMATICS, DISTRIBUTION AND MORPHOLOGICAL CHARACTERISTICS

In the European spring of 1854, Sir William J. Hooker, director of the Royal Botanic Gardens, Kew, took delivery of a consignment of plants in a “wardian case” from Major Robert Garden of the 45<sup>th</sup> Sherwood Foresters, a regiment stationed at Pietermaritzburg, South Africa. The wardian case, a glazed glass container used to transport live botanical specimens between the colonies and Kew, contained plants collected in Natal (= KwaZulu-Natal). Among this collection was an *Hypoxis* plant collected by Dr William Stanger, amateur botanist and secretary of the Horticultural Society of Durban (McCRACKEN and McCRACKEN, 1990). It was named *H. latifolia* Hook., and illustrated by Walter Hood Fitch in *Curtis’s Botanical Magazine* 80:T4817 (1854) (HILLIARD and BURTT, 1986). This seems to be the first record of *H. colchicifolia*, however, as, following a suggestion by WOOD (1976), *H. latifolia* Hook. and *H. oligotricha* Baker were recently reduced to *H. colchicifolia* Baker (HILLIARD and BURTT, 1986). The basis for this reduction was that there is apparently no preserved type specimen of an *Hypoxis* plant collected by Major Garden prior to 1854, cultivated at Kew and subsequently described as *H. latifolia* Wight in 1853.

*H. colchicifolia* is distinct within the genus and easily identified in the field by virtue of its large form and leaf size (Plate 1). It is a very robust species with no close ally in the genus, *H. galpinii* being the closest morphologically except for its hairier inflorescence and narrower leaves. The most recent description of *H. colchicifolia* is in WOOD (1976). Distinguishing characters are the large, round, perennial corm, 40 mm to 70 mm in diameter, large contractile roots, and strongly ribbed, firm, overlapping leaves which are glabrous except for a few hairs on the margins. The outer leaves are shorter than the central ones (Plate 1). The leaves are described as broad, 25 mm and 110 mm at the widest point, and varying between 110 mm and 600 mm in length. *Hypoxis* plants are acaulescent with their leaves arising directly from the corm, often in three ranks. The leaves of *H. colchicifolia* are also three ranked, but this is not obvious as they overlap to form a funnel (WOOD, 1976). The racemose inflorescence, fruit and seed of *H. colchicifolia* are all illustrated in WOOD (1976).

Knowledge of the growth patterns of *Hypoxis* plants is very limited. Their leaves grow continuously throughout the growing season and die back during winter dormancy.



**Plate 1.** *Hypoxis colchicifolia* is distinct within the genus. Plants are easily identified by virtue of (A) their bright yellow flowers produced concurrently with the leaves after the dormant winter period, and (B) the large oval shaped corms of mature plants whose leaves reach a height of 700 mm as illustrated. (C) Corms of younger plants are globose with long contractile roots at the base compared to the short contractile roots near the crown of the elongated mature corm in (B) (Bar = 1800 mm).

Although described as deciduous (WOOD, 1976), JEPPE (1975) noted that during dormancy the leaves of *H. oligotricha* (= *H. colchicifolia*) become stiff and dry and remain attached to the plant throughout winter. The latter author also recorded November and December as the flowering period, while WOOD (1976) recorded August to February. Corm growth and development has not been studied in depth because of the difficulties encountered with seed germination (WOOD, 1976; HEIDEMAN, 1979). The corms of several *Hypoxis* species from Zimbabwe are, however, illustrated in ZIMUDZI (1993).

The known distribution of *H. colchicifolia*, as illustrated in WOOD (1976), is described as being concentrated wholly in KwaZulu-Natal south of the Tugela River from the Estcourt district to the Mtamvuma River in the south. Two specimens collected outside this area are thought to be doubtful identifications since the plant specimen, Burt-Davy 13457 (Vryheid), has hairs on the leaves (WOOD, 1976), and no other similar plants have been found in the same area as Moss 13679 (Milner Park, Johannesburg) (HILLIARD and BURTT, 1986). Several new records have been included on the distribution map in POOLEY (1998).

Although *H. colchicifolia* has been reported to grow naturally in poor and sandy soils in undisturbed or disturbed grassland (WOOD, 1976; POOLEY, 1998), there are no published records of its original abundance. BEWS, however, reported in 1921 that "*H. latifolia*" is common in the lowveld areas of the Midlands, Natal (= KwaZulu-Natal). Interestingly GIBSON (1975) later described *H. oligotricha* (= *H. colchicifolia*) as a "rare and very handsome plant" indicating a possible decline in its abundance between 1921 and 1975.

### **3.2.2 SOME TRADITIONAL USES OF *H. COLCHICIFOLIA***

References to the traditional uses and medicinal properties of *Hypoxis* species, including those of *H. colchicifolia*, could not be found in any of the taxonomic literature reviewed other than in the recent field guide by POOLEY (1998). In most of the other literature reviewed (Chapter 1), however, although referred to, records of the traditional uses of *H. colchicifolia* were generally found to be repetitive and anecdotal. The claim that *H. colchicifolia* plants are toxic due to the presence of haemanthine also appears to be unsubstantiated. Otherwise the well known traditional uses of *H. colchicifolia* are summarised in Table 1 (Chapter 1) and will not be discussed again here.

In a recent herbal, however, some medicinal applications of *H. colchicifolia* not generally discussed were specifically mentioned (PUJOL, 1990, 1993). Its corms are apparently

frequently used by the Zulu and Xhosa peoples in a mixture of magical plants and bark called *Intelezi*, which is renowned for its psychoactive powers. Magically, *H. colchicifolia* is apparently also so effective that an emotionally upset person can be calmed by simply carrying a piece of a corm in their pocket. Medicinally *H. colchicifolia* "tea" is commonly used to calm the heart, stop nausea and vomiting, and induce good feelings, an appetite and sleep. In the earlier edition of PUJOL (1990), however, the illustration of *H. colchicifolia* was incorrectly named *Liliaceae Lancifolium* Spp. Wild. This has subsequently been changed in PUJOL (1993) to *H. oligotricha*, but not yet correctly named *H. colchicifolia*.

### 3.2.3 SMOKE EXTRACTS FROM LEAVES OF *H. COLCHICIFOLIA*

Fire is a widespread practice used in traditional resource management (COTTON, 1996). Under natural conditions fire has a direct stimulatory effect on seed germination within certain plant populations. Plant-derived smoke also provides an important cue for seed germination (BROWN and VAN STADEN, 1997). Several attempts have been made to identify the active molecules responsible for this. In one such study aqueous smoke extracts derived from the leaves of several plants, including *H. colchicifolia*, were examined to determine to what extent germination stimulants are produced by different types of plants and plant products (JÄGER *et al.*, 1996). Aqueous smoke extracts prepared from the leaves of *H. colchicifolia* were shown to stimulate the germination of light-sensitive lettuce seeds particularly at dilutions of 1:10 and 1:100. Active compounds separated out from the smoke extract by thin layer chromatography at R<sub>f</sub> 0.7 to 0.8, significantly stimulated seed germination, but were not identified. The response of *H. colchicifolia* seeds themselves to the smoke extracts obtained from its leaves was not, however, investigated.

### 3.2.4 ACTIVE COMPOUNDS

Hypoxoside and its aglucone, trivial name rooperol, have also been isolated from the corm extracts of *H. colchicifolia* plants, but the total yields of these compounds were not specifically given (DREWES *et al.*, 1984). Rooperol, readily obtained by  $\beta$ -glucosidase hydrolysis of hypoxoside, is of special interest to medical research because of its anti-cancer potential. The important role of rooperol in anti-cancer applications and the fact that *H. colchicifolia* corm extracts contain a higher proportion of dehydroxy and bis-dehydroxy rooperol than those of *H. hemerocallidea*, therefore led KRUGER *et al.* (1994) to stress the importance of selecting the correct *Hypoxis* species for possible therapeutic application. The pharmaceutical industry has experienced limited success in the development of new plant-derived drugs, however, often because difficulties encountered to obtain sufficient

plants from dwindling wild populations for investigation (TYLER, 1986). In view of its potential value, it is therefore vital to ensure a continued supply of *H. colchicifolia* plants for the future.

### 3.3 SUMMARY

Although habitat rather than single species conservation is more desirable, pertinent quantitative data on individual plant species with high usage values, such as *H. colchicifolia*, must be acquired to implement and manage conservation programmes successfully. Notwithstanding, it is also accepted that the cultivation and development of alternative supplies of scarce and popular medicinal plants requires high priority (CUNNINGHAM, 2001). From the literature reviewed it is also now apparent that little is known about the abundance, distribution, re-establishment, population structure, growth patterns, seed germination, hypoxoside / rooperol yields, age, harvesting patterns or cultivation of *H. colchicifolia* plants. Although a recent study showed that *H. colchicifolia* is not harvested on a sustainable basis in the areas surveyed in KwaZulu-Natal (NAIDOO, 1998), it is clear that additional quantitative data on the natural populations of *H. colchicifolia* must be collected to determine the availability, density and viability of this species to justify its cultivation.

### 3.4 OBJECTIVES

The initial objectives of this section of the present study are therefore to provide:

- an historical overview of data on the abundance of wild *H. colchicifolia* plants;
- some comparable field observations and numerical data on the effect of urban environmental practices on the density and survival of both *H. colchicifolia* and *H. hemerocallidea* populations;
- numerical data on the long term effects of the grassland management practices of fire and mowing on field populations of *H. colchicifolia* and *H. hemerocallidea*; and
- data on the composition of a population of *H. colchicifolia* plants, with emphasis on corm size, mass and hypoxoside yield.

## 3.5 EXPERIMENTAL

It is proposed to achieve these objectives by:

- reviewing available literature and examining historical items held by Herbaria, Natural Science Museums and Botanical Gardens to obtain new records on the abundance / density of *H. colchicifolia*;
- collecting field data on *H. colchicifolia* and *H. hemerocallidea* populations in both near-pristine (undisturbed) and disturbed habitats selected within the environs of suburban Pietermaritzburg, KwaZulu-Natal, a region where several *Hypoxis* species occur naturally, to obtain data on the extent of unexploited and exploited populations. This data will be used to determine the effect of urban practices on the density, frequency and long-term sustainability of these plants in their natural environment;
- conducting a survey of *H. colchicifolia* and *H. hemerocallidea* plants remaining in experimental plots used in a study to determine the long term effects of mowing and burning on the condition of Tall Grassveld (= grassland) at a site near Pietermaritzburg. Data will be used to determine the effects of some grassland management practices on the density and survival of these geophytes; and
- identifying and collecting a population of *H. colchicifolia* plants destined for destruction by urban development. Data will be collected on the composition, morphological characteristics and corms of these plants. There is also no information available on the optimum corm size or age at which to harvest *H. colchicifolia* corms to maximise hypoxoside yields. Hypoxoside will therefore be extracted and analysed for a range of corms representative of the population in order to ascertain whether there is a relationship between corm size, weight and hypoxoside yield.

## 3.6 MATERIALS AND METHODS

### 3.6.1 HISTORICAL RECORDS OF *H. COLCHICIFOLIA*

#### 3.6.1.1 Literature Review

Published literature was reviewed.

### 3.6.1.2 Other Historical Records

A search was conducted for new records on the abundance of *H. colchicifolia* at the:

- Bews Herbarium, University of Natal, Pietermaritzburg.
- Natal Museum, Pietermaritzburg.
- Natural Science Museum, Durban.
- Pietermaritzburg Botanic Gardens.
- Durban Botanic Gardens, Parks Department, eThekweni Municipality.

### 3.6.2 A SURVEY OF *H. COLCHICIFOLIA* AND *H. HEMEROCALLIDEA* PLANTS IN A NEAR-PRISTINE AND DISTURBED NATURAL AREA

#### 3.6.2.1 Locality and Description of Sampling Sites

In South Africa pristine vegetation types are nature reserves and conservation areas while other managed areas are "near-pristine" (LOW and REBELO, 1996). Undeveloped natural open spaces in suburban Pietermaritzburg were scoured for localities with both "near-pristine" and disturbed vegetation in which plants of *H. colchicifolia* and *H. hemerocallidea* occurred. The adjacent sampling sites selected were situated in a protected greenbelt area bordering the Blackburrow Spruit on Cleland Road, Hayfields and managed by the Pietermaritzburg Municipal Parks and Recreation Department.

**Site 1. Near-pristine, control site of least disturbance.** The site was typical of Tall Grassland or Grassveld, the dominant veld type around Pietermaritzburg (TAINTON *et al.*, 1978; ACOCKS, 1988), with several *Acacia* trees, tall grasses, and open spaces. Dominant woody species were *Acacia sieberiana*, *A. karroo* and *A. nilotica* while the most abundant grasses were *Themeda triandra* and *Hyparrhenia* species (Plate 2 A and B). The site formed part of the greenbelt lying adjacent to the spruit (river) and was bounded by Cleland Road in the north and the fence along the N3 Highway in the south. The field sloped down from the western boundary towards the Blackburrow Spruit on the eastern side. It had been minimally managed and very rarely burnt. The indigenous plants were protected and permits to collect seeds or plant material from the field were not issued. *H. colchicifolia* and *H. hemerocallidea* (Plate 3B) were dominant features of the landscape and taken, for the purposes of this study, to be as closely representative of a pristine or near-pristine population as found in natural vegetation today (Plate

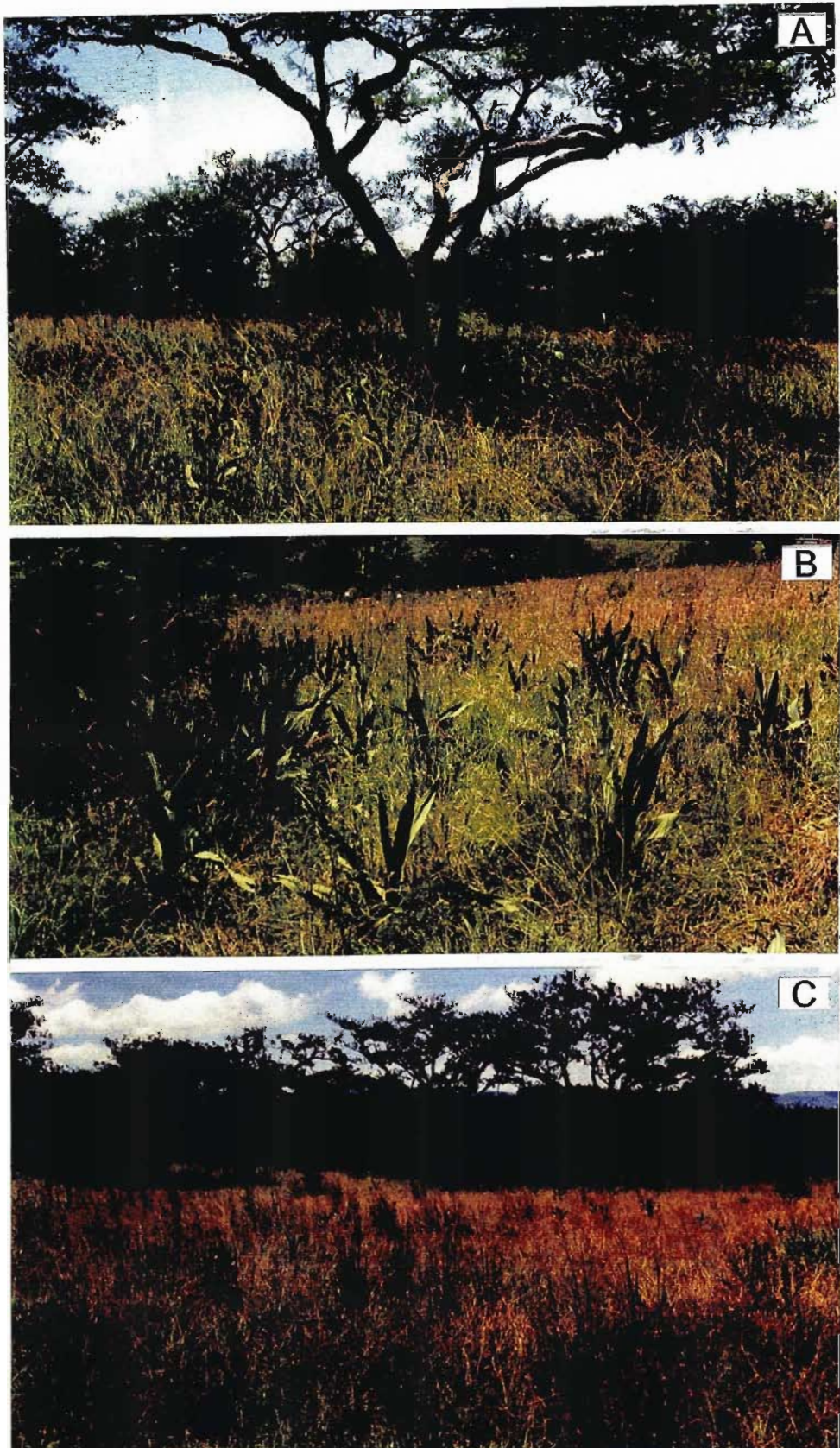
2 A and B). Site 1 was therefore selected as the control site representing an undisturbed or near-pristine environment.

**Site 2. A disturbed, partially managed site.** Site 2 was in an open grassland area with predominantly tall grasses, mostly *Themeda triandra* and *Hyparrhenia* species, a few scattered stunted *Acacia* trees, *Aloe* species, *H. hemerocallidea* and *H. colchicifolia* and many small indigenous perennials, annuals and geophytes (Plate 2 C). It was positioned directly adjacent to the western boundary of Site 1, which formed its eastern boundary, and immediately behind the Hayfields Veterinary Clinic on its northern border. The fence along the N3 Highway formed the southern boundary, while a second treed area demarcated the western boundary. The field was semi-managed by *ad hoc* burning and mowing due to its proximity to a residential area. Grasses were therefore short and tufted for most of the year except in spring when they were left undisturbed. The tall grasses were then harvested annually by hand for thatching. Disturbed patches and small holes throughout this site indicated that plants were also harvested from this area.

### 3.6.2.2 Sampling Procedures

The standard recommendation to obtain quantitative information on plant communities is to use a quadrat or plot of standard size (WRATTEN and FRY, 1980; COX, 1985). The choice of quadrat size depends on the size and density of the plants being sampled. To be valid there should be approximately ten plants per unit (Prof. D. YEATON, pers comm.). Quadrat shape is determined by the topography and the ease with which the quadrat can be laid out. Circular plots can be easily demarcated using a central pole and a freely rotating radius line which is usually a cloth tape measure.

Initial random samples taken at the study site showed that there were fewer than 10 plants 10 m<sup>2</sup> units. For the purposes of this study circular plots with an area of 100 m<sup>2</sup> were therefore used. Ten sampling locations per site were chosen at random in order to adequately record the number of plants present. In Site 1 a tree was selected as the central pivot for each plot to include *Hypoxis* plants growing both under the trees and in the open spaces (recommendation by Prof. D. YEATON). In Site 2 where trees were sparse, 5 plots were under trees and 5 in the open. All *H. colchicifolia* and *H. hemerocallidea* plants within each plot were counted. Plants found on the edge of the plot were included in the sample if their corms were more than halfway inside the plot boundary, but those more than halfway outside the boundary were excluded. From this data the density or the



**Plate 2.** Study sites 1 and 2 in the greenbelt area adjacent to the Blackburrow Spruit and Cleland Road. (A) and (B) Control Site 1 with trees and dense stands of *H. colchicifolia* in close association with the low-growing *H. hemerocallidea* plants. (C) Partially managed Site 2, adjacent to Site 1 (trees in background), in which *H. colchicifolia* and *H. hemerocallidea* plants are less frequent.

number of individuals per unit area, and the frequency or the fraction of sample plots containing the species, could be calculated for each species and comparisons made. These sites were surveyed at the end of May just prior to the onset of dormancy and when large, leafy plants were abundant.

### **3.6.3 A SURVEY TO DETERMINE THE LONG TERM EFFECTS OF MOWING AND BURNING ON THE DENSITY AND SUSTAINABILITY OF *H. COLCHICIFOLIA* AND *H. HEMEROCALLIDEA* POPULATIONS IN TALL GRASSLAND**

#### **3.6.3.1 Locality and Description of Sampling Site**

The trial site, used in a study to determine the long term effects of mowing and burning on the condition of Tall Grassveld, was situated on the Grassland Science Plateau at the Ukulinga Research Station, University of Natal, Pietermaritzburg, about 9 km south west of Pietermaritzburg at an altitude of approximately 900 m above sea level. This was a gently undulating area on shallow soil underlain by shale (DILLON, 1980). Deep soil and a moisture gradient were found in the depression leading to the dam between Block II and Block III (C. MORRIS, pers. comm.) (Figure 1).

This experimental site was selected for investigation because of the predominance of *H. colchicifolia* and *H. hemerocallidea* plants in some of the sub-plots and obvious absence in others. The presumption was made that this effect could be due to the different treatments used in the ongoing grassland management trial. It was therefore argued that a survey of the remaining *Hypoxis* plants could provide useful numerical data on the long term effects of the grassland management practices of fire and mowing on the density and survival of field populations of these geophytes.

#### **3.6.3.2 Treatments**

Treatments have continued uninterrupted since the inception of the trial at the beginning of the 1950 / 1951 season (TAINTON *et al.*, 1978; DILLON, 1980). The plot design consisted of 4 summer utilization (whole plot) and 11 aftermath removal (sub-plot) treatments applied in 6-year cycles. Each trial block therefore consisted of a total of 44 treatments or sub-plots. These were replicated 3 times (Figure 1). Each sub-plot was 13.5 m x 18 m (243 m<sup>2</sup>) in area. The treatments were as follows:

Summer Utilization Treatments (mown for hay) (whole plot).

- A. Control with no mowing (utilization) in summer
- B. One hay cut (mow) early in summer (November or December)
- C. One hay cut late in summer (February or March)
- D. Two hay cuts coinciding with B and C

Aftermath Removal Treatments (burn or mow) (sub-plot).

- 1. Control with no mowing or burning as an aftermath treatment
- 2. Annual burn, first week in August
- 3. Annual burn, after first spring rain of 12.5 mm in 24 hours
- 4. Biennial burn, first week in August
- 5. Biennial burn, after first spring rain of 12.5 mm in 24 hours
- 6. Biennial burn in autumn (summer utilization treatments not applied)
- 7. Triennial burn, first week in August
- 8. Triennial burn, after first spring rain of 12.5 mm in 24 hours
- 9. Triennial burn in autumn (summer utilization treatments not applied)
- 10. Annual mow, first week in August
- 11. Annual mow, after first spring rain of 12.5 mm in 24 hours

### **3.6.3.3 Sampling Procedures**

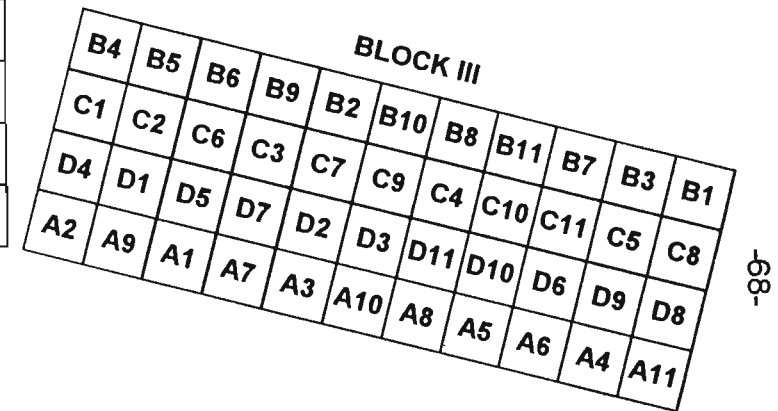
To maintain uniformity between this survey and the survey of the sites on Cleland Road, it was necessary to determine plant density within 100 m<sup>2</sup> quadrats at all the sites. However, only two 100 m<sup>2</sup> quadrats could fit into the small sub-plots (243 m<sup>2</sup>) of the trial site at Ukulinga. As determined in an exploratory survey of the sites, the use of smaller quadrats did not adequately record the presence or absence of the *Hypoxis* species in each site. It was recommended therefore that in these small sub-plots the entire population of plants within each sub-plot should be counted and the density 100 m<sup>2</sup> calculated (Prof D. YEATON, pers. comm.). DILLON (1980) had also concluded that the plot sizes of this trial should have been larger for more accurate, consistent results and adequate statistical analysis. Therefore the total numbers of *H. colchicifolia* and *H. hemerocallidea* plants remaining in each sub-plot were counted and plant density 100 m<sup>2</sup> calculated.

Treatments A to D and 1 to 11 as outlined in Section 3.6.3.2 have been applied regularly to the plots and sub-plots in 6-year cycles. Due to time constraints imposed on this study

# GRASSVELD BURNING AND MOWING TRIAL UKULINGA RESEARCH STATION

## PLOT DESIGN

BLOCK I											BLOCK II										
B11	B6	B9	B8	B2	B10	B1	B3	B4	B7	B5	D2	D7	D6	D4	D10	D5	D11	D8	D1	D9	D3
A1	A8	A11	A2	A6	A3	A9	A4	A7	A5	A10	A9	A8	A2	A11	A6	A7	A5	A4	A3	A10	A1
C8	C2	C11	C9	C6	C4	C10	C3	C5	C1	C7	C4	C5	C10	C2	C11	C8	C6	C1	C9	C3	C7
D1	D8	D10	D2	D4	D11	D7	D5	D3	D6	D9	B8	B5	B11	B7	B10	B6	B3	B1	B2	B4	B9



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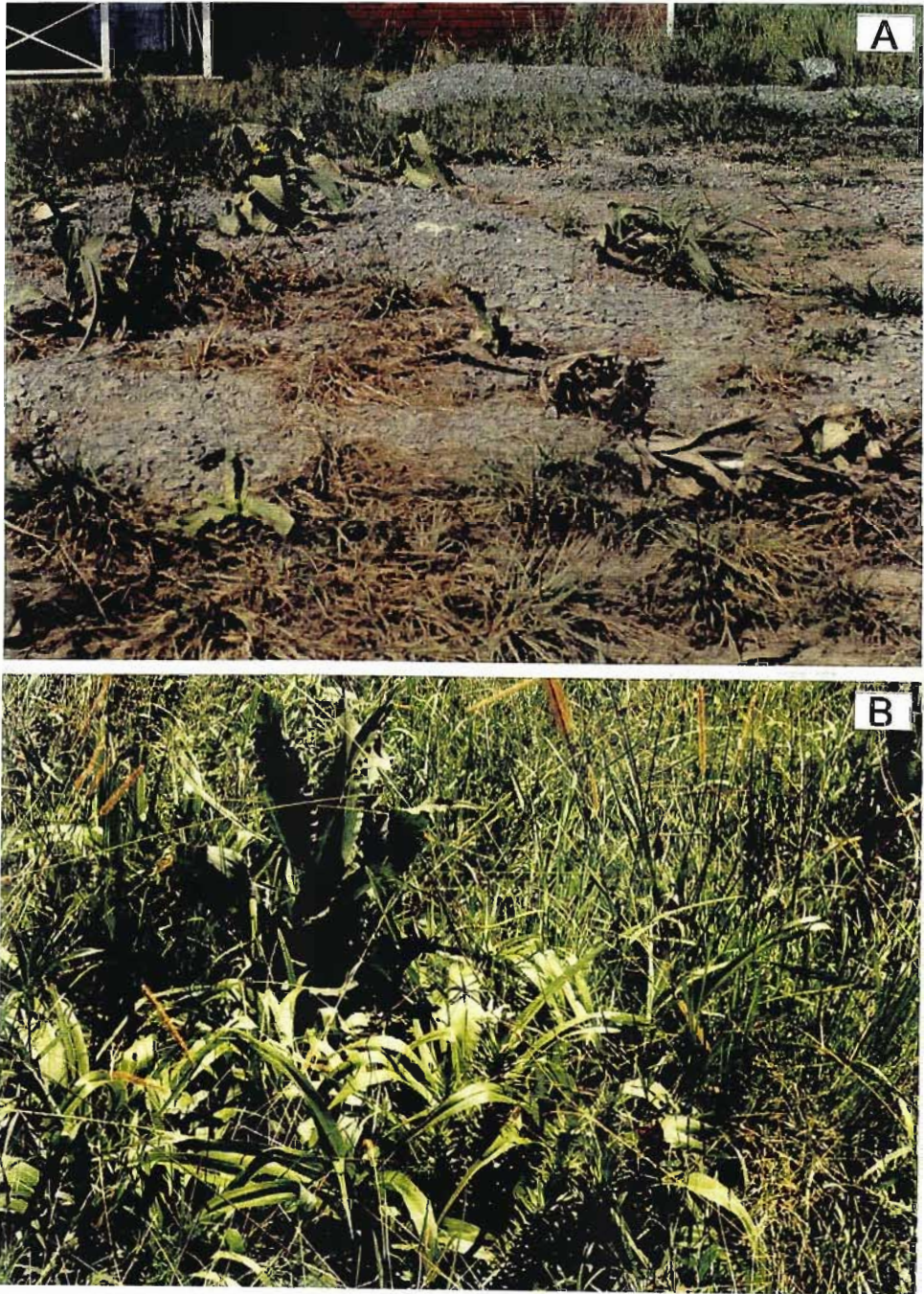
### KEY

SUMMER UTILIZATION TREATMENTS - (MOWING) - A, B, C, D

AFTERMATH REMOVAL TREATMENTS - (BURNING OR MOWING IN SUB-PLOTS) - 1 TO 11

**Figure 1.** Plot design of trial to determine the long term effects of burning and mowing on Tall Grassveld on the plateau at the Ukulinga Research Station, University of Natal, Pietermaritzburg. There were four utilization treatments (A,B,C,D) and eleven removal treatments (1 - 11) resulting in 44 treatments per block. This was replicated three times (blocks I, II, III). Deep soil was found in the depression leading to the dam between blocks II and III. There was also a moisture gradient in this area.

the 3-year or halfway point was considered to be the best time for sampling. The remaining plants of both *H. colchicifolia* and *H. hemerocallidea* were therefore counted at the end of May, halfway through the eighth 6-year cycle. The triennial burning in autumn had been delayed that year, however, and as a result the treatment 9 sub-plots had not been burned at the time of sampling.



**Plate 3.** (A) The mechanical destruction of populations of *Hypoxis colchicifolia* plants during the construction of Sunny Hills Victorian Housing Development. Plant of *H. hemerocallidea* in upper right hand corner. (B) *H. hemerocallidea* plants, in foreground, grew in close association with *H. colchicifolia* plants, in background, in all the surveyed sites.

### **3.6.4 COMPOSITION, LEAF AND CORM CHARACTERISTICS, AND HYPOXOSIDE YIELDS OF A POPULATION OF *H. COLCHICIFOLIA* PLANTS**

#### **3.6.4.1 Locality and Description of Sampling Site**

A property in Dunsby Ave., Lincoln Meade, Pietermaritzburg and on which the Sunny Hills Victorian Housing Development was under construction, was selected as a suitable sampling site. Not only were all the *H. colchicifolia* plants growing there being destroyed (Plate 3), but the suburb was adjacent to Hayfields with vegetation and topography similar to that of the greenbelt locality on Cleland Road (refer section 3.6.2). Permission to collect plants was obtained from the developer, Mr G.Reid, Image Construction, Hayfields.

#### **3.6.4.2 Sampling Procedures**

An undisturbed population of *H. colchicifolia* was identified on the property and all the plants collected. Measurements were taken of the leaves, roots and corms. Hypoxoside content was determined for a representative range of corms. The remaining plants were planted in the Department Botanical Gardens, University of Natal, Pietermaritzburg.

#### **3.6.4.3 Determination of Hypoxoside Yields**

The method used for hypoxoside extraction and analysis was modified from those of PAGE (1984) and BAYLEY (1989) as described by McALISTER and VAN STADEN, (1995).

## **3.7 RESULTS AND DISCUSSION**

### **3.7.1 HISTORICAL RECORDS OF *H. COLCHICIFOLIA***

#### **3.7.1.1 Literature Review**

Refer to Chapters 1, 2 and 3 for a review of published literature. No quantitative data on the original abundance or density of *H. colchicifolia* populations in pristine habitats was found in the published literature. With the increased urgency to conserve important medicinal plants during the 1980s, however, studies began to focus more on obtaining data on the supply of, and demand for, heavily utilized medicinal plants by conducting surveys on plant usage by herbal traders and the informal medicinal plant markets. Studies were

also directed towards establishing whether wild plants are harvested on a sustainable basis. Some important historical records with respect to the abundance of *H. colchicifolia* plants are summarized chronologically as follows:

- 1853 There was no published data recording the abundance of *H. colchicifolia* plants at the time that the type specimen was collected.
- 1921 "*H. latifolia*" (= *H. colchicifolia*) was recorded as being common in the lowveld areas of the Midlands, Natal (= KwaZulu-Natal) (BEWS, 1921).
- 1975 *H. oligotricha* (= *H. colchicifolia*) was described as a "rare and very handsome plant" (GIBSON, 1975), indicating a possible decline in its abundance between 1921 and 1975.
- 1988 Calculations indicated that approximately 31 324 corms of *H. colchicifolia* and *H. hemerocallidea*, were sold annually in KwaZulu-Natal by 54 informal traders alone (CUNNINGHAM, 1988 a).
- 1998 Availability of *H. colchicifolia* plants was recorded as being uncommon, collecting sites as more distant, and the quantity of wild plants collected by traditional healers from a single site as being 2 to 3 corms per visit. In comparison the availability of *H. hemerocallidea* plants was recorded as common, but not abundant with 3 to 5 corms collected from a site per visit. Furthermore demographic studies showed these species were unable to withstand the pressure of collection for medicinal purposes as the available *H. colchicifolia* and *H. hemerocallidea* plants within the study sites were depleted within seven months from the commencement date of the study (NAIDOO, 1998).

### 3.7.1.2 Other Historical Records

No new records with respect to the abundance of *H. colchicifolia* plants were found at either the Natural Science Museum or the Durban Botanic Gardens, eThekweni Municipality. The Herbaria collections also gave no indication of abundance. Mature plants were observed growing in natural areas within the boundaries of the Pietermaritzburg Botanic Gardens, however, but again there were no historical records on its previous abundance or availability.

The Natal Museum, Pietermaritzburg, surprisingly provided some interesting historical information. This was only visual, however, without supporting quantitative data. The dried funnel shaped leaves of *H. colchicifolia* plants were prominently displayed in the dioramas

of all the Large Mammal Display Cabinets housing the old mammal collections (Plate 4). They had been collected from the grasslands surrounding Pietermaritzburg and painted green to produce “natural looking” vegetation. As per M. MOLONEY and P. CRAUSE (pers. comm.) the components of the displays had been maintained unchanged since the animals were placed in the cabinets during the post second world war era and into the early 1950s. The last work was done on these collections in 1954 and the cabinets were last revamped in 1958 by C. Holiday (B. STUCKENBERG, pers. comm.).

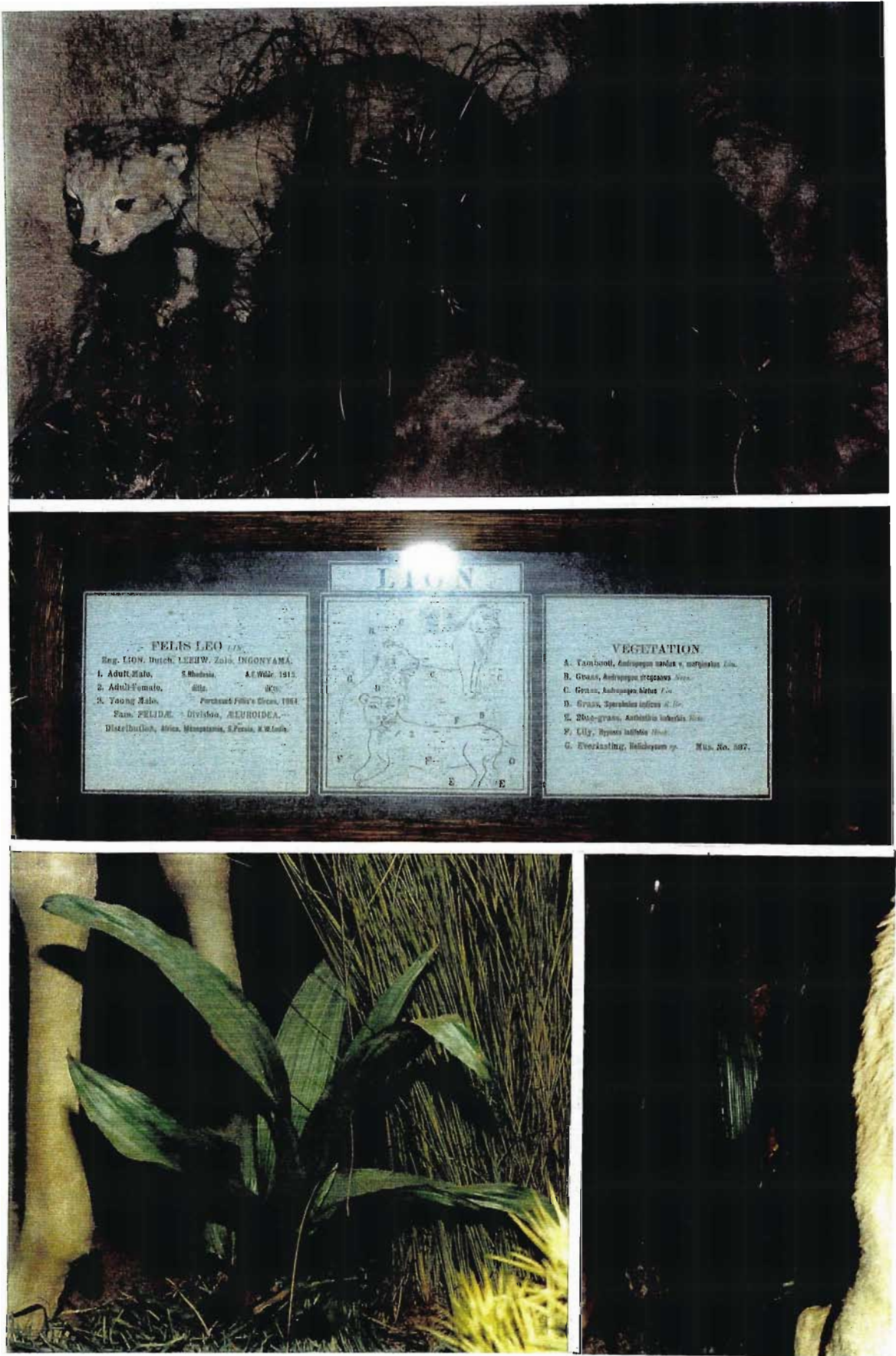
Good examples of the earliest displays are “Coke’s Hartebeest Display Cabinet” - Museum No 535, and “The Lion Cabinet” - Museum No 597. The vegetation listed on these original display labels and displayed in the cabinets was selected to best illustrate the grassland vegetation of the animals’ habitat at that time. Only one *Hypoxis* species, namely *H. latifolia* Hook (= *H. colchicifolia*), was selected as representative of the grasslands and prominently displayed (Plate 4). This species must therefore have been a dominant feature of the grasslands in the 1940s and 1950s supporting BEWS observation made in 1921.

Although historical records on plant abundance at these localities do not exist, increased numbers of *H. colchicifolia* plants have been observed between 1996 and 2002 on occasional visits to the Game Valley Lodge and Tala Game Reserves in Cramond and Eston respectively. These plants are becoming more and more conspicuous in conserved grassland areas and particularly where alien vegetation has been removed. It would appear therefore that in the KwaZulu-Natal midlands, *H. colchicifolia* plants can become re-established in areas previously used for grazing and agriculture. Several other *Hypoxis* species were also seen at the Game Valley Lodge Reserve.

### **3.7.2 RESULTS: SURVEY OF *H. COLCHICIFOLIA* AND *H. HEMEROCALLIDEA* PLANTS IN NEAR-PRISTINE AND DISTURBED NATURAL AREAS**

#### **3.7.2.1 General**

Mature *H. colchicifolia* plants are the largest of all the *Hypoxis* species and were far more conspicuous than the *H. hemerocallidea* plants in both Sites 1 and 2. Both species tended to be clustered in groups or populations, particularly under trees. These plants were present in far greater numbers than observed at Game Valley Lodge or Tala Game Reserve. Mature and juvenile *H. colchicifolia* plants were easily identified as the juvenile plants had narrow, curled leaves and no inflorescences in contrast to the very broad, heavily ribbed leaves characteristic of the tall, flowering mature plants. Mature and juvenile



**Plate 4.** Painted specimens of *H. colchicifolia* (= *H. latifolia*) are a dominant feature in the dioramas of the grassland habitats of large mammals displayed in the Large Mammal Display Cabinets housing the old mammal collections of the late 1940s and early 1950s (Natal Museum, Pietermaritzburg).

**Table 2.** The total number, density 100 m<sup>2</sup> and frequency of *H. colchicifolia* and *H. hemerocallidea* plants in the control Site 1, a near-pristine area with minimal disturbance, and the disturbed Site 2, a partially managed area, situated within the protected greenbelt, Cleland Road, Hayfields.

	<i>H. colchicifolia</i>			<i>H. hemerocallidea</i>
	Mature	Juvenile	Mature + juvenile	Mature + juvenile
<b>SITE 1. Near-pristine, control site of least disturbance</b>				
Total no. plants in 1000 m <sup>2</sup>	511	26 (4.8%)	537	304
Density 100 m <sup>2</sup>	51.1	2.6	53.7	30.4
Standard deviation	± 29.89	± 1.43	± 30.80	± 23.82
Frequency	1	1	1	1
<b>SITE 2. A disturbed, partially managed site</b>				
Total no. plants in 1000 m <sup>2</sup>	103	30 (22.6%)	133	83
Density 100 m <sup>2</sup>	10.3	3	13.3	8.3
Standard deviation	± 8.58	± 4.64	± 11.80	± 8.74
Frequency	1	0.5	1	0.9
% of number of plants in Site 1	20.2%	115.4%	24.8%	27.3%

plants could therefore be counted separately in each quadrat. In contrast mature and juvenile *H. hemerocallidea* plants could not be separated as all the plants had inflorescences and the total number of plants in each 100 m<sup>2</sup> quadrat were therefore counted. The survey results are presented in Table 2 and Figure 2.

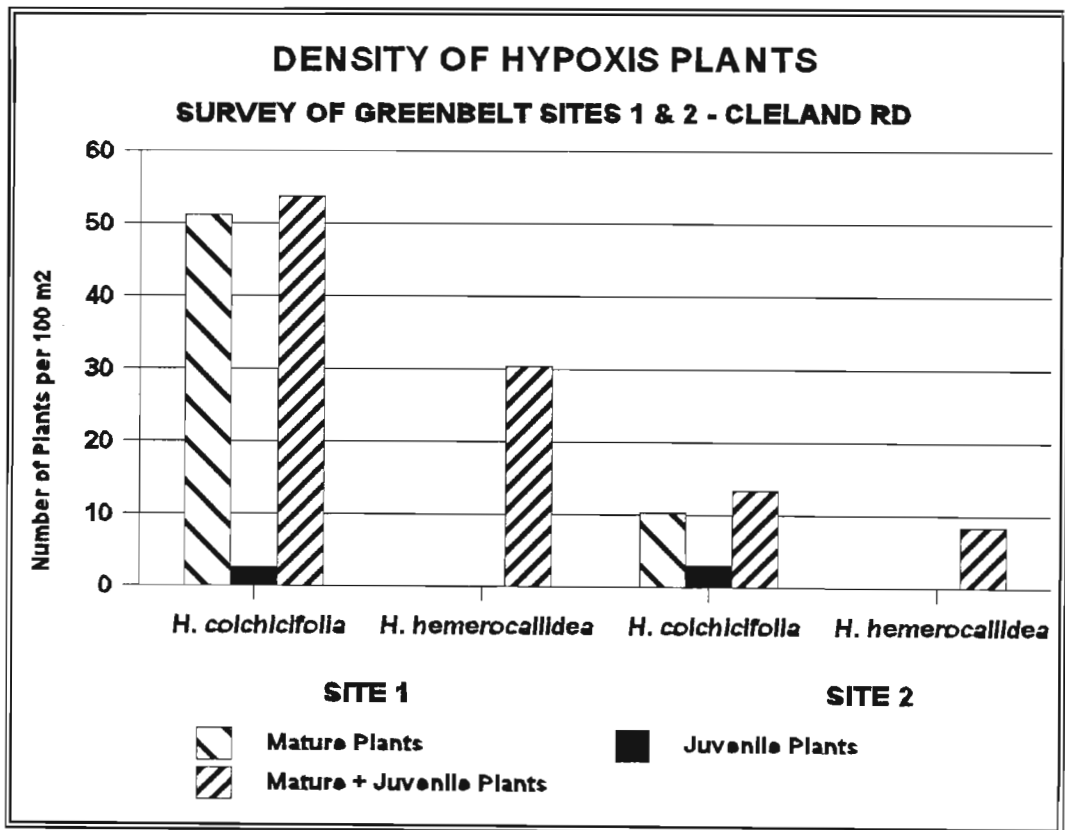
### 3.7.2.2 Site 1. Near-pristine, control site of least disturbance

Plant density in the near-pristine, control Site 1 was approximately 54 plants 100 m<sup>2</sup> for *H. colchicifolia* and 30 plants for *H. hemerocallidea*. A large deviation of 30.8 and 23.8 was recorded for each species respectively, indicating an uneven distribution of plants over the 1000 m<sup>2</sup> surveyed. A frequency of 1 calculated for each species clearly shows, however, that plants of both species were present in all the quadrats surveyed. These results support the observation that both *H. colchicifolia* and *H. hemerocallidea* occur in populations or groups. With a frequency of 1 and a density of 2.6 plants 100 m<sup>2</sup>, juvenile *H. colchicifolia* plants were also shown to occur throughout the near-pristine site indicating

successful seedling recruitment which was low as only 4.8% of the total number of *H. colchicifolia* plants were juveniles.

### 3.7.2.3 Site 2. A disturbed, partially managed site

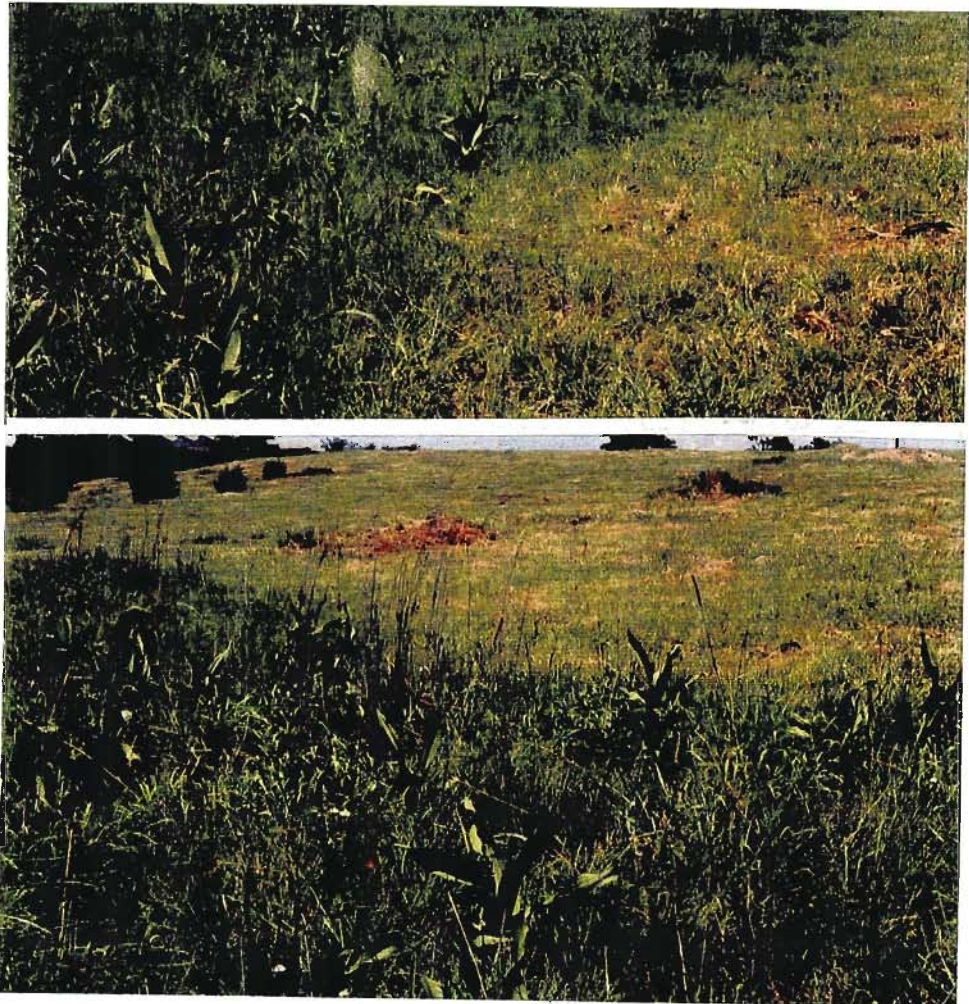
In comparison to the near-pristine area, the density of *H. colchicifolia* in the disturbed, partially managed Site 2 was only 13 plants 100 m<sup>2</sup> which was 75.2% less than the 54 plants 100 m<sup>2</sup> in Site 1. The density of *H. hemerocallidea* was 8 plants 100 m<sup>2</sup> or 72.7% less than the 30 plants 100 m<sup>2</sup> in Site 1. Plant frequency remained constant at 1 for *H. colchicifolia* and only slightly less at 0.9 for *H. hemerocallidea*, however, indicating that although actual plant numbers had been greatly reduced, both species were still present throughout the partially managed site.



**Figure 2.** The density 100 m<sup>2</sup> of *H. colchicifolia* and *H. hemerocallidea* plants in the near-pristine, control site of least disturbance (Site 1) and the disturbed, partially managed site (Site 2) situated in a protected greenbelt area on Cleland Road, Hayfields, Pietermaritzburg.

In contrast, the density of juvenile *H. colchicifolia* plants was similar in both sites at 3 plants 100 m<sup>-2</sup> indicating no change in seedling recruitment. The frequency recorded for these juvenile plants in the partially managed site was only 0.5, however, which was half that recorded for juveniles in Site 1, indicating a 50% decrease in the distribution of seedlings in the disturbed site. This could be due to the decrease in number of mature plants and therefore a decline in population size.

While collecting the above data, subjective observations at the time showed that there were few if any *Hypoxis* specimens in the frequently mown roadside area along the N3 Highway on the other side of the fence and adjacent to the study Sites. Damage to these plants and the destruction of *H. colchicifolia* and *H. hemerocallidea* populations by the implementation of roadside maintenance practices was very evident as illustrated in Plate 5. These plant populations were also being destroyed by residential encroachment.



**Plate 5.** Subjective observations showed that there were few if any *Hypoxis* plants on the frequently mown roadside area adjacent to the study sites on Cleland Road. This is in complete contrast to the numbers of plants visible in the adjacent near-pristine control Site 1. Plant populations are also being destroyed by residential encroachment in the background.

The above data and observations clearly show that mature plants and individual populations of both *Hypoxis* species examined had been destroyed in the partially managed site in the greenbelt area compared to the adjacent near-pristine site. It can therefore be concluded that naturally occurring populations of *H. colchicifolia* and *H. hemerocallidea* plants are adversely affected by the urban management practices of intermittent burning, mowing and hand cutting of grass for thatching.

### **3.7.3 RESULTS: SURVEY TO DETERMINE THE LONG TERM EFFECTS OF MOWING AND BURNING ON THE DENSITY, FREQUENCY AND SUSTAINABILITY OF *H. COLCHICIFOLIA* AND *H. HEMEROCALLIDEA* POPULATIONS IN TALL GRASSLAND**

#### **3.7.3.1 General Observations**

Plants of both *Hypoxis* species were conspicuous and easily recognized within the experimental sites even though they were entering the winter dormancy period. Their dormancy patterns were different, however, as the leaves of *H. hemerocallidea* plants had died back completely, but those of *H. colchicifolia* had dried *in situ* as they senesced. They remained firmly attached to the underground corm confirming this observation by JEPPE (1975). Further investigation showed that copious numbers of *H. colchicifolia* seeds had collected in the leaf axils. Later during winter small animals, possibly Oribi, rodents or rabbits observed at the site, had gnawed through the leaf bases and the dried leaves had then been blown about, scattering the seed. There was no evidence to suggest that animals had grazed on green *Hypoxis* plants or their corms.

Other observations made at the time of the survey showed that the topography of the trial site was unevenly distributed across the experimental blocks and several large bare, stony patches of ground with little grass cover had appeared at the top of the site. There were few *H. colchicifolia* plants on these patches but *H. hemerocallidea* plants were prolific. This is in keeping with the finding that *H. hemerocallidea* plants prefer poor soils with little nutrients (McALISTER and VAN STADEN, 1995). Apparently, variations in soil type, moisture content, plant density and rabbit burrows had occurred since the onset of the trial (C. MORRIS, pers. comm.). The effect of these changes and more than 45 years of grassland management on plant diversity within the trial plots has, however, not been investigated. Although baseline data on the two *Hypoxis* species at the onset of the trial was not compiled, further personal observations showed large numbers of *H. colchicifolia* plants growing under trees and fences in the undisturbed areas around the trial site. Their

prevalence in the surrounding area would indicate their presence throughout the site at the start of the grassland trial.

With respect to the overall vegetation, exotic trees, namely *Melia azedarach* (Syringa), *Jacaranda mimosifolia* (Jacaranda) and *Acacia mearnsii* (Black Wattle), had become established in the control sub-plots 1 of treatment A only. Vegetation growth beneath these exotics was poor and the stands of *H. colchicifolia* and *H. hemerocallidea* plants were not as extensive as those under the indigenous trees on Site 1, Cleland Road. Grasses in the control sub-plots were tall but clumped with open spaces between them.

Grasses on the sub-plots of the aftermath removal treatments 6 (biennial burn in autumn) and 9 (triennial burn in autumn) were also tall and clumped because they had not been mown (C. MORRIS, pers. comm.). Summer utilization treatments were not applied to the autumn burn plots because at least two years herbage accumulation was required for this veld to be effectively burnt in autumn (TAINTON *et al.*, 1978). Autumn burns had also not yet taken place at the time of sampling. Plants of both *Hypoxis* species were present on these plots. Data published on the effect of burning and mowing on the vegetation of the grassland plots was found to be very variable and therefore not analysed statistically (TAINTON *et al.*, 1978). In a later study DILLON (1980) concluded that the sub-plots were too small for adequate fires and therefore accurate statistical analysis. For this reason and because long term monitoring of demographic data is essential to accurately determine population variables, the current study should be considered as an exploratory examination.

### **3.7.3.2 Density and frequency of *H. colchicifolia* and *H. hemerocallidea***

Data collected is presented as mean density or number of plants 100 m<sup>2</sup>, and plant frequency or the fraction of the sample plots containing the plant species (Table 3). Treatment trends clearly indicate that each species had responded differently to the mowing and burning regimes. A clear pattern emerged for the total number of plants 100 m<sup>2</sup> recorded for *H. colchicifolia* (Figure 3) but not for *H. hemerocallidea* (Figure 4).

With the exception of treatment 6, the mean density recorded for all the *H. colchicifolia* plants was greater in all the summer utilization treatment (SUT) A sub-plots which are not mown in summer, when compared to the mean density recorded in the sub-plots of the summer utilization treatments B, C and D, all of which have been mown annually in summer since the onset of the trial (Table 3, Figure 3). It must be repeated that the

aftermath removal treatments 6 and 9, the bi- and triennial autumn burn sub-plots respectively, cannot be included in this comparison as they were not mown in summer according to the schedules of the whole plot treatments A, B, C and D (refer also Figure 1). A frequency of one was also recorded for the mature *H. colchicifolia* plants in 10 of the 11 aftermath treatments in the summer utilization treatment A, but not in B, C or D with the exception of treatments 6 and 9 (Table 3). Overall this would indicate that summer mowing has a detrimental effect on mature *H. colchicifolia* plants. In comparison the treatment trends obtained for *H. hemerocallidea* were very variable, providing no clear indication of the effect mowing and burning might have had on plant density or frequency.

If the response of *H. colchicifolia* plants to individual aftermath removal treatments (ART) is examined, however, other treatment trends become apparent. The sub-plots of ART 1 form the control in which there were no mowing or burning aftermath treatments in either SUTs A, B, C or D, and should be examined first. Plant density was high in the overall control treatment A 1 which was neither burnt nor mown, and negligible in B1, C1 and D1 which were all mown in summer but did not receive aftermath treatments (Figure 3). A frequency of 1 was recorded for A1, but less than 1 for B1, C1 and D1. This clearly indicated, and supported the initial overall conclusion, that grassland mowing in summer is detrimental to both the density and frequency of *H. colchicifolia* plants. Again the effect of the treatments on *H. hemerocallidea* plants in ART 1 was inconclusive.

In the aftermath removal treatments 2, 3, 4, 5, 7, 8, 10 and 11, the sub-plots of A, B, C and D were all subjected to either annual (2, 3), biennial (4, 5), or triennial (7, 8) burning or mowing (10, 11) in spring. Again the A sub-plots were not mown in summer, but the sub-plots of B, C and D were also all mown in summer. In all these treatments the density of *H. colchicifolia* plants was less affected by the aftermath removal treatments in A than by the combination treatments in B, C or D. This trend was even more marked in A7 and A 8 which were burnt only every three years in spring. Plant density in the control treatment A1 was, however, still greater than those recorded for A2, 3, 4, 5, 7, 10 and 11 (Figure 3). These results indicated, therefore, that the density of *H. colchicifolia* plants was also detrimentally affected by the aftermath removal treatments of burning and mowing in spring particularly when these were applied in combination with the summer utilization treatment of mowing.

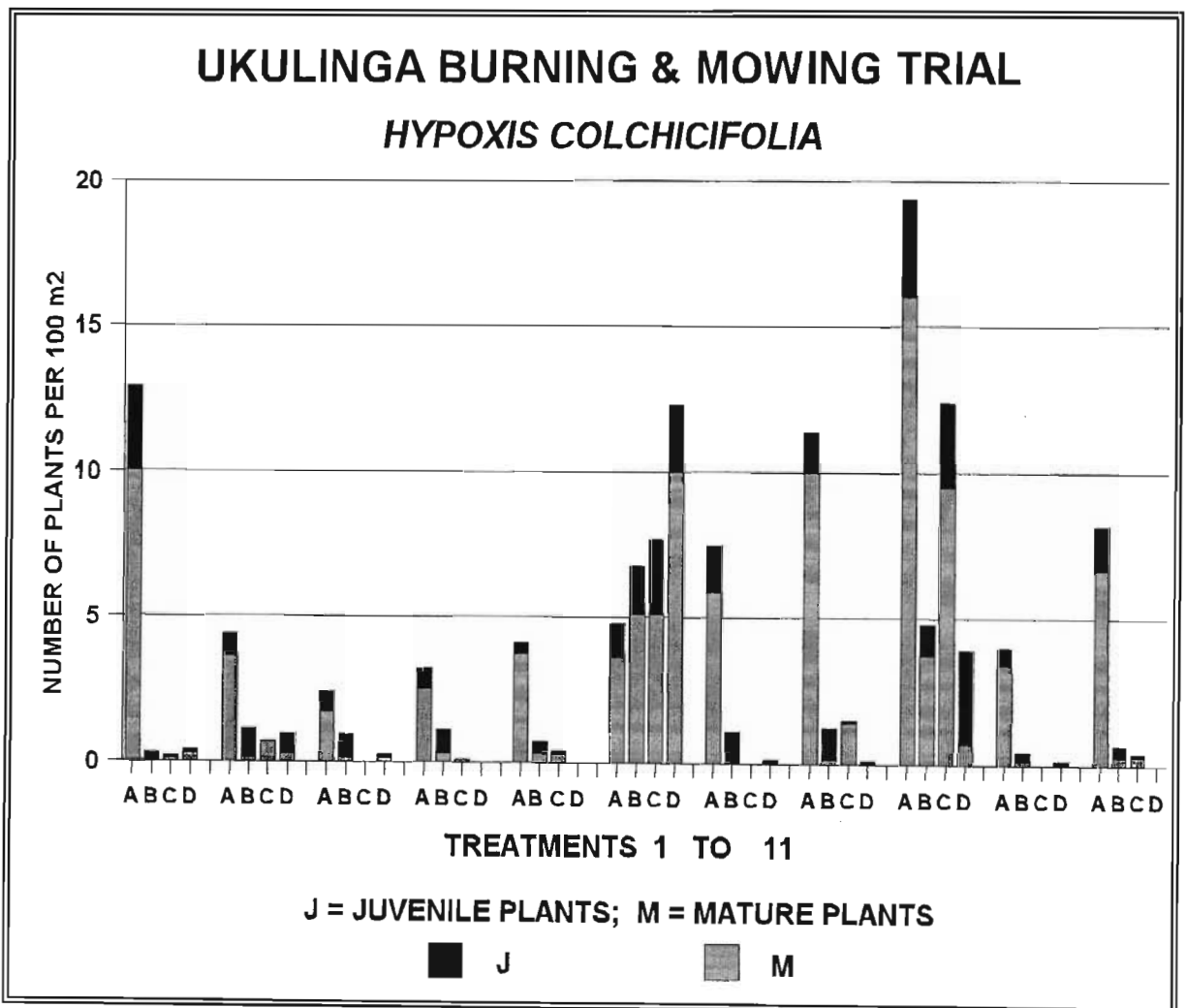
If summer utilization mowing treatments are not applied then the grassland management practices least detrimental to the density of *H. colchicifolia* plants were the aftermath removal treatments of biennial (ART 6) and triennial (ART 9) burning in autumn, and to a

**Table 3.** Mean density 100 m<sup>2</sup> of *H. colchicifolia* and *H. hemerocallidea* plants remaining in the 44 sub-plots of the field trial to determine the long term effects of mowing and burning on Tall Grassland. M = Mature plant, J = Juvenile plant, D = Mean Density 100 m<sup>2</sup>, F = Frequency, SUT = Summer Utilization Treatment (A, B, C, D), ART = Aftermath Removal Treatment (1 to 11). Refer to Section 3.6.3.2 for key to treatments.

Treatments 6 and 9: Summer utilization treatments are not applied to autumn burn plots.

ART	SUT A (control, no mowing)						SUT B (one cut in Nov or Dec)					
	<i>H. colchicifolia</i>				<i>H. hem</i>		<i>H. colchicifolia</i>				<i>H. hem</i>	
	M		J		M + J		M		J		M + J	
	D	F	D	F	D	F	D	F	D	F	D	F
1	10	1	2.9	1	20.3	0.67	0	0	0.3	0.33	0	0
2	3.6	0.7	0.8	0.67	9.7	0.67	0.14	0.3	1	1	4	0.67
3	1.7	1	0.7	1	17.8	0.67	0.14	0.3	0.8	0.67	0.14	0.33
4	2.5	1	0.7	1	5.6	0.67	0.3	0.3	0.8	1	2.5	0.67
5	3.7	1	0.4	0.67	4.9	1	0.3	0.3	0.4	0.67	1.9	0.67
6	3.6	1	1.2	0.67	2.3	1	5.1	1	1.7	1	2.7	0.33
7	5.9	1	1.6	1	4.5	1	0	0	1.1	0.67	1	0.67
8	10	1	1.4	1	1.4	0.67	0.14	0.3	1.1	0.67	12.2	1
9	16	1	3.4	1	1.9	0.67	3.7	1	1.1	0.67	14.3	1
10	3.4	1	0.6	1	3.4	1	0.14	0.3	0.3	0.33	6.2	0.33
11	6.7	1	1.5	1	9.7	1	0.27	0.7	0.4	0.33	14	0.67
ART	SUT C (one cut in Feb or March)						SUT D (two cuts at B and C)					
1	0.1	0.3	0.1	0.33	0.6	0.67	0.27	0.7	0.14	0.33	12.2	0.33
2	0.7	0.7	0	0	1	0.33	0.27	0.3	0.7	1	0.14	0.33
3	0	0	0	0	0.14	0.33	0.14	0.3	0.14	0.33	2.9	0.67
4	0.1	0.3	0	0	13.3	0.67	0	0	0	0	1.6	0.33
5	0.3	0.7	0.1	0.33	5.5	0.67	0	0	0	0	10.8	0.67
6	5.1	1	2.6	0.67	3.3	1	10	1	2.3	1	1.9	0.67
7	0	0	0	0	0.4	0.67	0	0	0.14	0.33	1.9	0.33
8	1.4	0.3	0.1	0.33	12.6	0.67	0	0	0.13	0.33	17.4	0.33
9	9.5	1	2.9	0.67	11.7	1	0.7	0.7	3.2	1	9.5	1
10	0	0	0	0	6.6	0.33	0	0	0.14	0.33	17.2	0.67
11	0.3	0.7	0.1	0.33	5	0.33	0	0	0	0	10.6	0.33

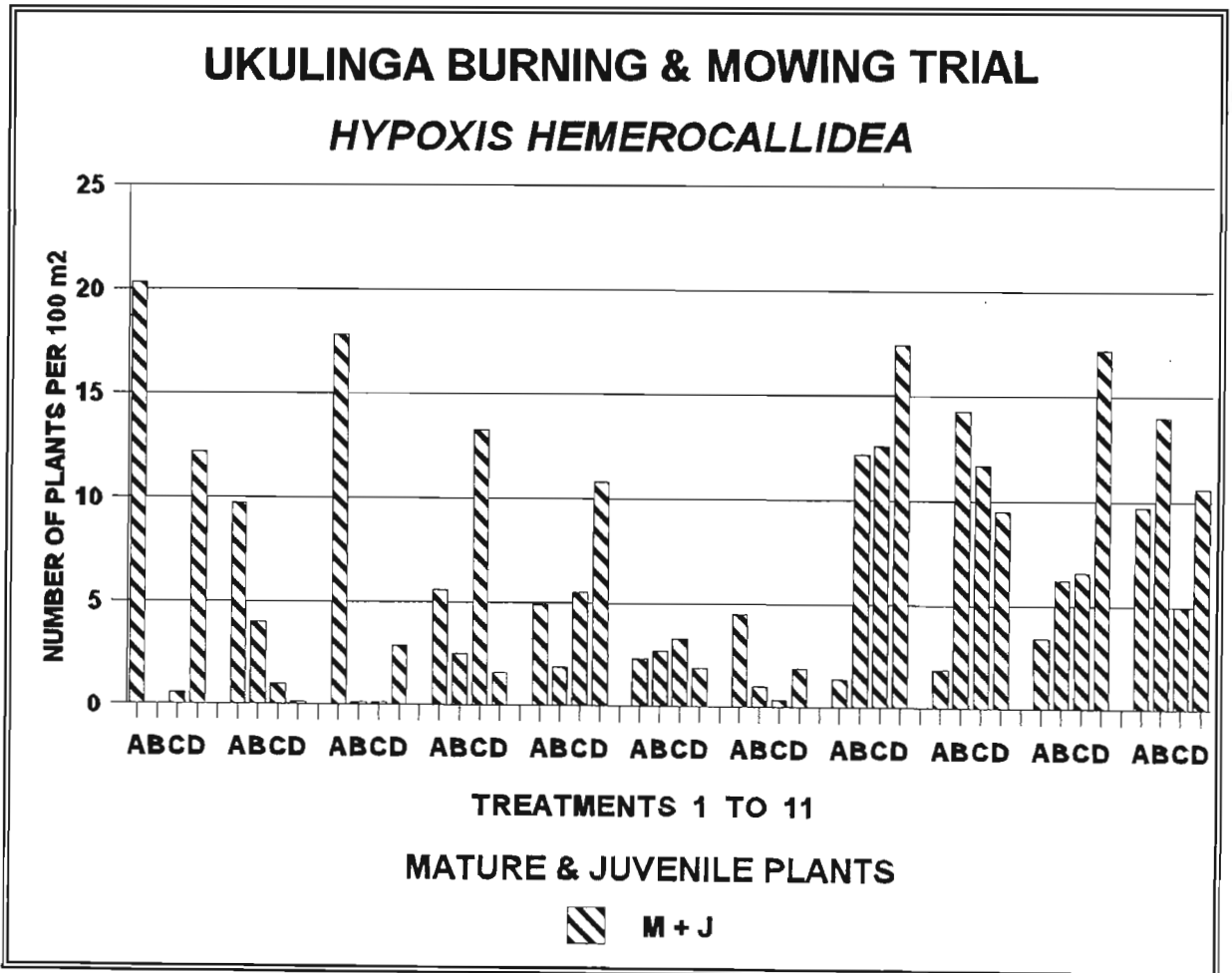
lesser extent, triennial burning in spring (A7 and A8). All these treatments provided the plants with sufficient time between applications to become established and perhaps produce seed. Earlier studies have shown that autumn grassland burns (ART 6 and 9) are cool because the plant material burnt is still green whereas winter and spring burns are hot as the plant material is dry. It was suggested therefore that this temperature difference could have a profound effect on the regrowth of geophytes after fires (DILLON, 1980). Data presented here suggests, however, that both the timing and frequency of the treatments might also be crucial to geophyte survival, and that the practice of cutting grassland in summer destroys not only the regrowth, but the *H. colchicifolia* plants themselves.



**Figure 3.** Mean number or density 100 m<sup>2</sup> of mature and juvenile *H. colchicifolia* plants remaining in each of the 44 sub-plots of the field trial to determine the long term effects of mowing and burning on Tall Grassland. Refer to Materials and Methods, Section 3.6.3.2 for key to the summer utilization (A, B, C, D) and aftermath removal treatments (1 to 11).

The density of the juvenile *H. colchicifolia* plants recorded in the sub-plots suggests that seedling recruitment continued despite treatments. However, the frequency calculated indicated that these juvenile plants were not evenly distributed throughout the site. Exceptions were the sub-plots of the summer utilization treatment A, the control A1, and the triennial burn treatments in autumn (ART 6 and 9). It was observed, however, that seeds were distributed in winter when the dried leaf heads were blown throughout the trial site. DILLON (1980) also came to the conclusion that the sub-plots were too small. As a result seeds may have been carried to adjacent sub-plots where they germinated.

Conclusive trends were not obtained for *H. hemerocallidea* plants. It is suggested that this might be because *H. hemerocallidea* plants have a completely different geophytic growth pattern to *H. colchicifolia* plants, but there is no data to support this viewpoint.



**Figure 4.** Mean number or density 100 m<sup>2</sup> of *H. hemerocallidea* plants remaining in each of the 44 sub-plots of the field trial to determine the long term effects of mowing and burning on Tall Grassland. Refer to Materials and Methods, Section 3.6.3.2 for key to the summer utilization (A, B, C, D) and aftermath removal treatments (1 to 11).

**Table 4.** Mean density of *H. colchicifolia* and *H. hemerocallidea* plants 100 m<sup>2</sup> as determined for a near-pristine site, a semi-disturbed, partially managed site and selected sites subjected to grassland management practices of mowing and burning shown to be least detrimental to plant survival.

Sites	Mean Plant Density 100 m <sup>-2</sup>			
	<i>H. colchicifolia</i>			<i>H. hemerocallidea</i>
	Mature	Juvenile	Mature + juvenile	Mature + juvenile
Near-pristine, least disturbance (Cleland)	51.1	2.6	53.7	30.4
Semi-disturbed, partially managed	10.3	3	13.3	8.3
Control not mown or burnt A 1(Ukulinga)	10	2.9	12.9	20.3
Biennial autumn burn, not mown 6 A - D	6	2	8	2.8
Triennial autumn burn, not mown 9 A - D	7.5	2.7	10.2	9.4

### 3.7.3.3 Comparison between the mean densities 100 m<sup>2</sup> of *H. colchicifolia* and *H. hemerocallidea* plants from all the localities surveyed in this study

A comparison between the densities 100 m<sup>2</sup> of *H. colchicifolia* and *H. hemerocallidea* plants determined for each of the localities surveyed might be useful to determine the plant density that could arguably be expected for these two highly utilized *Hypoxis* species in near-pristine vegetation, and to what extent urban and grassland management practices have been detrimental to their abundance. Mean densities are summarised in Table 4.

Results show that *H. colchicifolia* is abundant at a density of ±54 plants 100 m<sup>2</sup> in a near-pristine locality with minimal disturbance. However, plant density was significantly reduced by approximately 75% by the urban management practices of intermittent mowing and burning. Harvesting of plants may also have contributed to the decline in abundance. If it is assumed that the density of *H. colchicifolia* plants in a pristine locality is the same as in the near-pristine site, then over a period of 45 years the density of the plants in the control plot of the trial to determine the long-term effect of mowing and burning on tall grassland, was also significantly reduced by nearly 76%. Although the control plot was neither mown nor burnt, plant abundance was adversely affected by the surrounding disturbance. Similarly on the plots with the least destructive practices of bi- and triennial burning in autumn, the plant density was reduced by nearly 85% and 80% respectively (Table 4). The

other destructive grassland management practices of mowing and burning resulted in the *H. colchicifolia* populations being significantly reduced by nearly 100% (Table 3).

Surprisingly the density of the popular *H. hemerocallidea* plants in the near-pristine locality was less than that of *H. colchicifolia*. Partial urban management practices also had the effect of significantly reducing the density of *H. hemerocallidea* from  $\pm 30$  plants  $100 \text{ m}^2$  to  $\pm 8$ . Although the treatment trends for *H. hemerocallidea* showed no absolute pattern in the long-term grassland trial, the practices of mowing and burning also had the effect of reducing plant density considerably.

### **3.7.4 RESULTS: COMPOSITION, LEAF AND CORM CHARACTERISTICS AND HYPOXOSIDE YIELDS OF A POPULATION OF *H. COLCHICIFOLIA* PLANTS**

#### **3.7.4.1 Population Composition, Leaf and Corm Characteristics**

The population of *H. colchicifolia* plants examined consisted of 61 plants which ranged in size from small, non-flowering to large, flowering specimens of unknown age. Criteria to determine the age of *Hypoxis* plants have not been established. HEIDEMAN (1979) suggested counting the annual rings of contractile roots and old root holes on the corms. Used in an attempt to determine the ages of the *H. colchicifolia* plants comprising the population, this suggestion proved inconclusive, however, as the number of rows of roots produced by the plant per annum is not known. The holes near the base of many corms were also obscured by callus. However, between 10 and 12, and 37 and 39 rows of roots and root holes were counted on corms 23 and 61 (the largest) respectively. It was estimated that if two rows of roots emerge per annum (excluding basal roots) then these plants would be  $\pm 5$  and 19 years old.

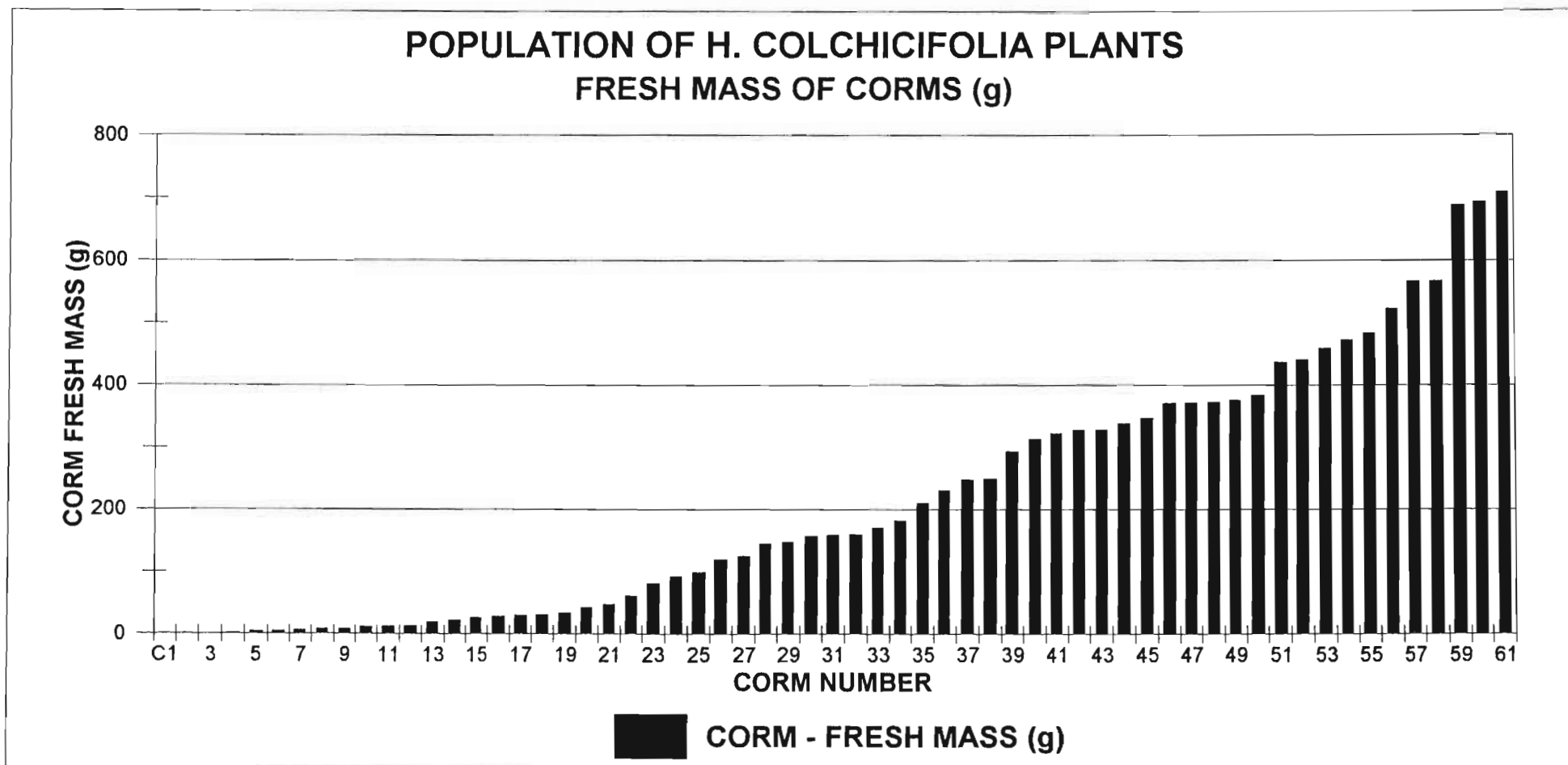
The corms appeared to be fairly representative of the population, however, increasing gradually in size and weight from the smallest corm, 7mm in diameter, 20 mm long and with a fresh mass of 1.1 g, to the largest which was 100 mm in diameter, 143 mm long and weighed 708.6 g (Table 5). By plotting the fresh mass (grams) of all the corms comprising the population, a stepwise change in mass, incorporating two to four corms per step, is apparent (Figure 5). If these steps represent the number of seedlings that survived each year, and two to three seedlings were recruited annually as shown to occur in  $100 \text{ m}^2$  of near-pristine vegetation (refer Section 3.7.2.2), then the largest corm in the population could be at least 20 years old. This figure is surprisingly similar to the estimated 19 years obtained by counting two rows of roots or root holes per annum.

**Table 5.** The range of leaf and corm measurements obtained for each group (A to F) of *H. colchicifolia* plants comprising the population harvested at the Sunny Hills Victorian Housing Development site.

CHARACTERS	RANGE OF MEASUREMENTS					
	PLANT GROUPS A to F and Plant Numbers					
	A No 1 to 8	B 9 to 19	C 20 to 27	D 28 to 34	E 35 to 45	F 46 to 61
Inflorescence	x	x	x	✓	✓✓✓	✓✓✓✓
Leaf width (mm)	3 - 21	21 - 50	62 - 81	54 - 81	64 - 118	70 - 137
Leaf length (mm)	260 - 465	343 - 650	530 - 780	680 - 840	615 - 835	612 - 960
Corm diameter (mm)	7 - 17	20 - 40	43 - 59	53 - 63	62 - 75	74 - 106
Corm length (mm)	15 - 46	34 - 75	53 - 85	74 - 103	93 - 122	87 - 150
Fresh Corm Mass (g)	1.1 - 7.9	7.9 - 33.9	42.3 - 124.7	144.4 - 182.4	210 - 437	371 - 708.6

A wide range of measurements was obtained for the leaves and corms (Table 5). To present this data meaningfully, the plants were divided into groups. Roots were used as the criterion to group the smallest plants. In group A the roots emerged from the base of each corm (nos. 1 to 8). In group B each corm had two rows of contractile roots near the circumference and the basal roots had died (nos. 9 to 19). In group C all the corms had three or four distinct rows of roots below which small holes from dead roots were evident (nos. 20 to 27). After the third set of roots the number of rows of new and old roots could no longer be distinguished or used to group the plants. Inflorescence presence was used as the criterion instead. In the fourth group (D) each plant had a single inflorescence (nos. 28 to 34). This increased to two or three in group E (nos. 35 to 45), while in group F each plant had several inflorescences (nos. 46 to 61).

The presence of an inflorescence was used to visually distinguish between juvenile and mature *H. colchicifolia* plants in the field sites on Cleland Road and at Ukulinga (Sections 3.7.2 and 3.7.3). Using this criterion the plants in groups A, B and C would therefore represent the juvenile complement of the population while groups D, E and F represent the mature plants. It was also estimated that plant no. 23 (group C) could be approximately five years old as outlined above. It would appear therefore that *H. colchicifolia* plants do not reach reproductive maturity for several years.



**Figure 5.** The fresh mass (g) (■) of the corms of the 61 *Hypoxis colchicifolia* plants comprising a single population at the Sunny Hills Victorian Housing Development site.

Measurements showed that the leaves vary in width from 3 mm to 137 mm (mean 63 mm) and in length from 260 mm to 960 mm (mean 644 mm). In WOOD'S (1976) description, however, leaf width is given as 25 mm to 110 mm at the widest point and leaf length as 110 mm to 600 mm. An explanation for these differences is that *Hypoxis* plants have a geophytic growth pattern in which the leaves grow continuously throughout the growing season. The *H. colchicifolia* population was harvested in March towards the end of the growing season when the leaves were at their longest, and well after flowering. Herbaria specimens on the other hand are usually collected early in the season while in flower and with new growth. Juvenile *Hypoxis* plants may therefore also have been excluded from Herbarium collections. This is also reflected in the corm diameter measurements which ranged from 7 mm to 107 mm and not 40 mm to 70 mm as given by WOOD (1976) and corm lengths of between 15 mm and 150 mm. Although the ages of the *H. colchicifolia* plants comprising a population could not be accurately ascertained, the data presented should prove useful when plants are in cultivation to establish the optimum stage of development at which plants can be harvested to obtain maximum hypoxoside yield.

#### **3.7.4.2 Hypoxoside Yield**

Total hypoxoside content per corm dry mass (TH CDM<sup>-1</sup>) (mg), and the hypoxoside content per one gram dry mass (H g<sup>-1</sup> CDM) (mg) were determined for the corms of 18 *H. colchicifolia* plants (Table 6). Although the H g<sup>-1</sup> CDM increased with an increase in corm mass, this fluctuated between corms indicating that hypoxoside production is not consistent. These fluctuations were, however, not reflected in the TH CDM<sup>-1</sup> which increased linearly with an increase in corm mass. The hypoxoside content determined ranged from 0.168 mg for the smallest corm, 0.64 mg for corm number 3, which was the first corm examined to have yellow pigmentation, to 2640.76 mg for the largest corm (number 61). Although the hypoxoside content of corm number 46 (CFM 371 g) was less at 2152 mg, the maximum hypoxoside yield of 1.57% was obtained for this corm, after which yield decreased to 0.74% for corm number 61 (CFM 708.58 g). It would appear from these results that maximum hypoxoside yields are obtained from *H. colchicifolia* plants with a corm fresh mass of approximately 350 g to 400 g.

### **3.8 DISCUSSION AND CONCLUSION**

The search conducted of published literature and other historical records of the Hypoxidaceae confirmed that there is no quantitative data on the original abundance,

**Table 6.** Corm fresh mass (CFM) (g), corm dried mass (CDM) (g), total hypoxoside content per corm dry mass (TH CDM<sup>-1</sup>) (mg), and hypoxoside content per one gram corm dry mass (H g<sup>-1</sup> CDM) (mg) determined for a range of 18 corms selected to represent the population of *H. colchicifolia* plants.

GROUP	PLANT / CORM NUMBER	CFM (g)	CDM (g)	TH CDM <sup>-1</sup> (mg)	H g <sup>-1</sup> CDM (mg)
A	1	1.1	0.37	0.168	0.45
	2	1.43	0.51	0.216	0.424
	3	1.5	0.62	0.64	1.032
	4	2.42	0.97	5.316	5.48
	5	4.53	1.91	5.424	2.84
B	9	7.9	3.5	7	2
	10	10.98	3.3	12.54	3.8
	13	19.08	8.32	84.531	10.16
	17	29.54	12.52	124.699	9.96
C	20	42.32	17.4	160.08	9.2
	22	60.71	27.3	205.296	7.52
	23	81.47	31.61	237.707	7.52
D	28	144.41	59.25	803.43	13.56
E	37	247.67	89.06	926.224	10.4
F	46	371	137.25	2152.08	15.68
	53	420	181.5	2450.25	13.5
	56	523	191.27	2597.447	13.58
	61	708.58	358.8	2640.76	7.36

frequency or distribution of *H. colchicifolia* plants in pristine habitats. *H. colchicifolia* plants were a dominant feature of grassland vegetation up to the early 1950s as recorded by BEWS (1921) and displayed in the dioramas at the Natal Museum, however, but were reported as rare by 1975 and are today not readily seen in grasslands other than nature reserves, greenbelts and protected areas. The species is also not listed in LOW and REBELO (1996) as an important component of grasslands in KwaZulu-Natal because geophytes are not discussed individually and are still lumped together as "forbs".

In this study, however, *H. colchicifolia* plants were shown to be abundant in a near-pristine locality, judged on present standards, with a density of 51.1 mature plants 100 m<sup>2</sup> and a

total density of 53.7 plants 100 m<sup>2</sup>, including juveniles. They occur in groups or populations consisting of a range of individuals from seedlings to 20-year-old and older plants. Plant density was also shown to have been vastly reduced in partially managed, disturbed areas to approximately 10.3 mature plants 100 m<sup>2</sup> and 13.3 plants 100 m<sup>2</sup> if juveniles were included. In comparison *H. hemerocallidea* was not as abundant with a total density of 30.4 plants 100 m<sup>2</sup> in the near-pristine habitat and 8.3 plants 100 m<sup>2</sup> in the disturbed site.

In the long term grassland field trial, however, the populations of *H. colchicifolia* plants on the experimental sub-plots had been severely damaged by fire and mowing. The densities of the plants on the control sub-plots and in the near-pristine site were also not comparable with the former being very similar to the density recorded in the partially managed, disturbed site adjacent to the near-pristine site. Although neither burnt nor mowed during the trial period, it would appear that the vegetation on the control sub-plots had been negatively affected by the management practices on adjacent sub-plots resulting in a reduction of the *Hypoxis* populations and increase in alien species. This might be indicative of the general state of grasslands in KwaZulu-Natal at present. It is suggested therefore that the data presented here provides sufficient quantitative evidence that *H. colchicifolia* was originally an abundant and dominant component of pristine or near-pristine grassland vegetation in areas of KwaZulu-Natal, but that this is no longer the situation.

The results can also be used to demonstrate the effect harvesting must have had on natural *Hypoxis* populations. Both species are heavily utilized for medicinal applications and in 1988 in excess of 31 300 corms of each were sold via 54 outlets in Durban only (CUNNINGHAM, 1988 a). Therefore, if mature *H. colchicifolia* plants only were removed by collectors at a rate of 51 plants 100 m<sup>2</sup> then approximately 6.14 hectares of near-pristine grassland would have been denuded of mature *H. colchicifolia* plants to supply these outlets. In contrast approximately 10.4 hectares of near-pristine grassland would be required to obtain the same number of *H. hemerocallidea* plants. If the same number of corms had been removed annually over the next 15 years, then the area of denuded grassland would have escalated to 92.1 and 156 hectares for each species respectively.

Although these estimated areas are small, they were calculated using quantities of plants from heavily populated near-pristine areas and the number of corms sold per annum by 54 traders only. This excluded the large number of corms sold by other traders and traditional healers. Neither were pre-sale losses due to spoilage nor increased demand taken into consideration. The assumption was also made that the plants were not harvested on a sustainable basis since herbsellers admitted to collecting *H. hemerocallidea* plants

throughout the year from Durban's Silverglen Nature Reserve (NOMTSHONGWANA, 1995). These plants were removed even when large corms were scarce and the small corms were discarded instead of being replanted. Natural areas also only make up  $\pm 5\%$  of the Durban Metropolitan area today and it is estimated that only 1% of these are in a condition that closely resembles their original state (HINDSON *et al.*, 1996). Silverglen Nature Reserve would fall into this category yet its natural resources are being over-exploited by the very people who rely on it for their livelihood.

This scenario is applicable throughout South Africa and it can be assumed that in 1988 very few natural areas had near-pristine vegetation. If plant density determined in this study for disturbed areas is used to calculate harvesting rate then 30.4 hectares would have been denuded to obtain 31 300 mature *H. colchicifolia* plants and 37.7 hectares for *H. hemerocallidea*. Over 15 years approximately 456 and 566 hectares of disturbed grassland would have been denuded of these species respectively to supply only 54 traders. Compared to the near-pristine areas, an additional 80% and 73% of disturbed grassland would have been denuded of these species in the same time span. Furthermore, these values were calculated without taking annual increases in demand into consideration and far greater areas have probably been stripped of these species in reality. This is very significant as *H. colchicifolia* plants occur only in KwaZulu-Natal south of the Tugela River. It can therefore be concluded that continued destructive harvesting of these plants at the calculated rate impacts negatively on natural grassland vegetation and that in the long term, the current populations of *H. colchicifolia* will be inadequate to supply future demand.

Information and quantitative data gathered in this study show conclusively that populations of both of the *Hypoxis* species examined are adversely affected by human activity such as partial management of open spaces, manual grass cutting, urban development and the grassland management practices of burning and mowing. These practices collectively resulted in a 75% reduction in plant density on average. Data also showed that both species responded quite differently to the practices of fire and mowing, with *H. hemerocallidea* plant density, although adversely affected, showing no clear response. Results conclusively showed, however, that mature *H. colchicifolia* plants are unable to survive in areas subjected to annual mowing and regular burning, but are able to survive cool bi- and triennial burns at the end of their growing season.

These findings, however, do not agree with the statements that *Hypoxis* corms have a high tolerance to fire and that fire promotes the growth of new leaves and flowering in the genus (SINGH, 1999). This generalization was based on observations that *H. hemerocallidea*

plants produced leaves and flowers shortly after a burn between March and August, irrespective of the month of burning, and that plants which had bloomed earlier in the year had flowered again. No quantitative data was given to support these statements. In the present study, however, the densities of both *H. hemerocallidea* and *H. colchicifolia* plants decreased dramatically when subjected to burning, particularly to burns in August. A higher tolerance to cool bi- and triennial burns in autumn was shown by *H. colchicifolia* plants. In KwaZulu-Natal autumn is from late March through to early June. It would appear therefore that the observations by SINGH (1999) were perhaps made after an autumn burn, indicating that *H. hemerocallidea* plants in fact also respond more favourably to cool fires.

Similarly, the statement that “fire acts as a strong flowering stimulus for *H. sessilis* and *H. wrightii* in southern Florida” (HERNDON, 1988) also appears to contradict the results of this study. On closer examination, however, the prescribed burning practices adhered to in the areas examined were typically low-intensity fires conducted every 5 to 7 years in May or June at the end of the dry season. Although northern hemisphere species which flowered in June to July, these results showed that infrequent low-intensity fires prior to flowering did not destroy the plant populations and therefore support the findings in the current study and those by SINGH (1999). These studies also emphasize the importance of collecting precise data for individual *Hypoxis* species in order to optimize the timing of controlled burning to ensure plant survival.

It has also been implied that soil-stored seeds of *Hypoxis* have fire-stimulated germination and that seed dormancy appears to be broken by smoke (SINGH, 1999). Fire cues, physical or chemical, that could be responsible for seed germination of individual *Hypoxis* species are still unknown. If fire stimulation were true abundant seedlings could have been expected in the sub-plots of the long term trial at Ukulinga which had been subjected to burning, and the disturbed area surveyed, yet plants were more abundant in the unburnt near-pristine areas of least disturbance.

A reason that *H. colchicifolia* plants cannot survive mowing, annual and hot spring fires, but can survive cool bi- and triennial burns in autumn could be due to their late flowering characteristics. Inflorescence data recorded for the plants from the harvested population indicated that juvenile plants only reached flowering maturity after an estimated 3 to 5 years. In contrast *H. hemerocallidea* plants flower frequently (SINGH, 1999). This suggestion is supported by the observation that *Hypoxis hirsuta* plants bloom late in May and therefore grow best in areas where grazing is light or which are mown annually in mid-July after the flowering period (ANON, 1998).

NAIDOO (1998) also recorded the availability of *H. colchicifolia* plants as uncommon and the quantity collected by traditional healers as 2-3 corms per visit. In a harvesting study all viable *H. colchicifolia* plants were removed from the collection site within 7 months of the commencement of the study at a rate of one per visit. In this study annual seedling recruitment was established at about 3 per population of *H. colchicifolia* plants. Annual removal rate would therefore far exceed the replacement rate of 3 seedlings per annum, lending support to NAIDOO's (1998) conclusion that *H. colchicifolia* plants cannot be harvested on a sustainable basis. Results from this study also showed that maximum hypoxoside yields are obtained from *H. colchicifolia* plants from a wild population with a corm fresh mass of approximately 350 g to 400 g. It is therefore suggested that to harvest plants on a sustainable basis then plants of this size only should be used to maximise hypoxoside yields. It has been clearly demonstrated therefore that both traditional resource use and land management practices impact negatively on the sustainability of *H. colchicifolia* populations and that this natural resource requires careful management and active protection to ensure population survival.

It has also been assumed that *Hypoxis* is a typical component of open grasslands and only a few species are able to tolerate shaded conditions found in forest margins (SINGH, 1999). In the present study, however, both *H. hemerocallidea* and *H. colchicifolia* plants grew in abundance in the shade under trees and amongst grasses in the near-pristine habitat surveyed, albeit grassland. These contradictions add support to the statement that details of individual botanical resources are lacking and are an important requirement for biodiversity conservation to succeed.

More than fifty years ago Gerstner suggested cultivation as a means to save rare medicinal plant species as emphasised by CUNNINGHAM (1990), but this has not been implemented on a large scale in southern Africa. Alternative measures and management programmes, such as sustainable harvesting and sustainable use, and other conservation strategies have generally not been successful (VAN STADEN, 1999). This led to the suggestion that improvement of the self-sufficiency of collectors, traders and healers by introducing the concept of cultivation on a small scale would have the effect of reducing the pressure on individual plant populations. The next aim of this study is therefore to investigate cultivation as a means to ensure not only a continued supply of *H. colchicifolia* plants for the informal medicinal trade and the pharmaceutical industry but the future survival of this important medicinal plant in the wild.

## *Chapter 4*

# **IN VITRO REGENERATION OF *HYPOXIS* *COLCHICIFOLIA* PLANTLETS**

## **4.1 INTRODUCTION**

The need to cultivate *H. colchicifolia* to ensure its survival has been established quantitatively using both published and new field data (Chapter 3). Methods to propagate this species have, however, not been investigated. It is also apparent from the literature reviewed that *Hypoxis* plants are generally not cultivated due to the perception that they are naturally abundant, a viewpoint shown to no longer be valid in traditionally high usage areas in KwaZulu-Natal (NAIDOO, 1998; SCOTT-SHAW, 1999). Sellers interviewed in Gauteng, South Africa, were also more concerned about their profit and the healing power of *Hypoxis* (MATSAU, 2000) than the fact that the unrelenting harvesting of wild plants was destroying the very populations they relied on for business. The popularisation of *Hypoxis* plants for the treatment of various ailments has also resulted in *H. hemerocallidea* becoming the most frequently traded medicinal plant in the Eastern Cape Province (DOLD and COCKS, 2002).

Natural *Hypoxis* populations, therefore, continue to be over exploited by plant collectors, traders, herbalists, traditional healers and the general public as a means to supply the formal, informal and popular medicinal plant industries and, more recently, the plant nursery trade. Contrary to popular belief, however, traditional healers and herbalists involved with a rehabilitation mine project had no objections to indigenous plants being propagated for their own medicinal practices (KROON, 1999). In fact they actually recommended which medicinal plants they felt should be translocated and propagated for these purposes. Interestingly their major conservation priority was to rescue and cultivate geophytic plants with large rootstocks which take many years to reach maturity.

Like many geophytes, *Hypoxis* plants rarely multiply vegetatively as their corms possess no axillary meristems and have only a single central meristem as described for *H. hemerocallidea*, the most extensively studied South African species (WOOD, 1976; PAGE, 1984). Natural reproduction is, therefore, from seed. It has been established, however, that

natural seedling recruitment is insufficient to sustain wild populations of either *H. hemerocallidea* or *H. colchicifolia* (NOMTSHONGWANA, 1995; NAIDOO, 1998) at current harvest rates or if subjected to various grassland management practices (Chapter 3).

With no viable alternatives sexual propagation would appear to be the most obvious method to propagate *Hypoxis* plants. Several studies have, however, shown germination of *Hypoxis* seed to be very variable. Although *H. hemerocallidea* seedlings have been obtained (GILLMER and SYMMONDS, 1999), other seed batches have been difficult to germinate (NOMTSHONGWANA, 1995). It was tentatively concluded that these failures were due to the requirement of a long post-ripening period (WOOD, 1976; HEIDEMAN, 1979) and to an embryo and coat-imposed dormancy (HAMMERTON, 1985). The viability of *H. hemerocallidea* seed also varies within a season between harvest sites and dates (HAMMERTON *et al.*, 1989) and seasonally from year to year (NOMTSHONGWANA, 1995). The latter author attributed the variation to the different germination media used, but this was not proven. PAGE (1984) emphasised genetic variability, meiotic abnormalities, the occurrence of high somatic, and variable pollen grain chromosome numbers in the Hypoxidaceae as major disadvantages against propagation by seed.

Translocation of rescued plants has also been suggested as a measure to both save and propagate various species of *Hypoxis*. However, although *H. acuminata*, *H. angustifolia* var. *buchananii*, *H. filiformis*, *H. iridifolia* and *H. hemerocallidea* plants were successfully translocated from a condemned area to new sites, the seed collected from the *H. acuminata* plants failed to germinate (KROON, 1999). Future propagation from seed would be essential for translocation to succeed. It was also suggested that these *Hypoxis* species would make excellent horticultural subjects in an herbaceous border, but methods to propagate the plants, other than by translocation, were not provided (KROON, 1999). More recently while investigating the source of plants used in a landscaped area, about 500 nursery grown *H. hemerocallidea* plants were seen for the first time for sale at an indigenous plant nursery in Gauteng (KNOLL, 2001). Plants would have to be propagated on a far larger scale than this, however, in order to satisfy current demand.

Apparently several *Hypoxis* species from the USA, southern Africa and Australia were introduced to Britain as garden subjects from as early as 1752 (BRYAN, 1989). The records pertaining to *H. colchicifolia* are confusing, however, as they show that a summer flowering plant, *H. latifolia* (= *H. oligotricha*), and which is now = *H. colchicifolia*, was introduced in 1854 while an autumn flowering plant, only 30 cm in height and with the invalid name of *H. colchicifolia*, was introduced in 1884. Sowing or broadcasting seed in

spring (British) was the only propagation method given for all the *Hypoxis* species listed. Other horticultural criteria outlined included planting corms in rockeries to a depth of 5 cm to 10 cm and at a distance of 15 cm to 20 cm. There was no specific evidence that *H. colchicifolia* plants, or any of the other species mentioned, were ever successfully propagated in Britain.

In the USA, *H. wrightii* and *H. sessilis* plants were observed under natural conditions to occasionally reproduce vegetatively by the formation of new ramets (HERNDON, 1988). Small clusters of slow growing seedlings were also seen. Mature plants were observed to survive for many years in the wild. HERNDON (1988) therefore concluded that to successfully grow *Hypoxis* plants, propagation and cultivation methods that could sustain plant production and survival for decades would have to be developed. This would also apply to the cultivation of medicinally important species of South African *Hypoxis* since it is estimated that their corms take at least five to seven years to become medicinally viable (BAYLEY, 1989; MATSAU, 2000) and that mature plants can survive for 20 years or longer in the wild (Chapter 3).

The results of a cultivation study conducted in Pietermaritzburg, South Africa and in which the effect of fertilizers, herbicides and soil type on plant growth and hypoxoside production of *H. hemerocallidea* plants was examined (McALISTER and VAN STADEN, 1995), support HERNDON's (1988) suggestion. It was shown that fertilizer application induced plant growth at the expense of hypoxoside production. Furthermore the greatest plant growth, survival and hypoxoside production was recorded not only for those plants grown in their natural clay / shale soils, but for the herbicide trial control plants which received no herbicides and remained undisturbed in unweeded plots for the duration of the trial. McALISTER and VAN STADEN (1995) therefore recommended that in order to survive under cultivation for any length of time, *H. hemerocallidea* plants should be grown in areas with clay / shale soils and remain undisturbed without the application of herbicides. They also suggested that cultivated plants should receive high levels of nitrogen, phosphorous and potassium initially to encourage corm growth, but that once established fertilizer treatment should be discontinued to encourage hypoxoside production and accumulation in the corms over a prolonged period of time.

All the *H. hemerocallidea* plants used in the cultivation study by McALISTER and VAN STADEN (1995) were produced *in vitro* as per PAGE and VAN STADEN (1984) and VAN STADEN and BAYLEY (1988). Although on average only 35 plantlets were obtained from a mature *H. hemerocallidea* corm utilizing this method (PAGE and VAN STADEN, 1984),

the establishment of mature plants in cultivation clearly demonstrated that *H. hemerocallidea* plants can be successfully propagated *in vitro* for agricultural purposes. It stands to reason therefore that *in vitro* culture should be examined with a view to developing a method suitable for the mass propagation of *H. colchicifolia* plants.

## 4.2 IN VITRO PROPAGATION

### 4.2.1 HISTORY AND COMMERCIALISATION OF IN VITRO PLANT CULTURE

The comment by PIERIK (1987) that “the volume of literature concerning *in vitro* vegetative propagation has become so large it is beyond the scope of this book to discuss it extensively” is even more pertinent today. Publications review and report on not only the history, general principles, methodology, applications and commercialisation of *in vitro* or tissue culture, but also recent research and development in this field. Among the many subject texts referred to in this study were those by DE FOSSARD, 1976; STREET, 1977; KRIKORIAN, 1982; GEORGE and SHERRINGTON, 1984; DODDS and ROBERTS, 1985; ZIMMERMAN *et al.*, 1986; PIERIK, 1987; BHOJWANI, 1990; LINDSEY, 1991; GEORGE, 1993; PRAKASH and PIERIK, 1993; RAZDAN, 1993; VASIL and THORPE, 1994; HERMAN, 1997; and KYTE and KLEYN, 2001. An extensive bibliography on *in vitro* culture prior to 1979 was compiled by PIERIK (1979).

Historically interest in the culture of plant cells, tissues and organs began between 1838 and 1839 with the concept put forward by Schwann and Schleiden that individual living cells of a multicellular organism should be capable of independent development. This gave rise in the early 1900s to the totipotency theory in which it was stated that a totipotent cell is one able to regenerate into a complete organism if exposed to the correct external conditions. Then in 1902 Haberland made the first attempt to cultivate isolated plant cells *in vitro* on an artificial medium, followed in 1904 by Hannig who developed a technique to culture embryos. It was only after the discovery of plant growth hormones, however, and the realisation in 1957 that the formation of plant organs can be regulated in culture by altering the cytokinin : auxin ratio in the growth medium that research into and development of methods to propagate plants *in vitro* escalated.

Since then *in vitro* or tissue culture technology and its application has received immense attention. It is presently widely used in the fields of genetic engineering, molecular biology, production of secondary products, agriculture, forestry and horticulture. The extensive and

growing use of this technology in the regeneration of cloned plants and as an aid to conventional plant improvement, also clearly indicated from the outset that it could be applied in the field of plant cultivation to great advantage. As a result *in vitro* plant propagation, or micropropagation as it is commonly called, developed into a global commercial industry with a total production in 1989 of 513 million plants (PIERIK, 1993). By the year 2000, approximately 250 million plants were being produced annually by 75 commercial tissue culture laboratories in the USA alone (KYTE and KLEYN, 2001).

The orchid industry was the first to implement micropropagation on a large scale during the 1950s (KYTE and KLEYN, 2001), and was responsible in 1985 for more than 55% of the total number of commercially produced *in vitro* plants (ZIMMERMAN *et al.*, 1986). Even though orchid culture has been extremely successful and is a good example of the application of *in vitro* techniques for the continuous clonal propagation of large numbers of quality orchid plants from elite selections, *in vitro* plant regeneration has always been more difficult to induce in monocotyledons than in dicotyledons (EVANS *et al.*, 1983).

Literature on the *in vitro* propagation of monocotyledons published prior to 1983 was reviewed by PAGE (1984) to determine methods suitable for the *in vitro* culture of *H. hemerocallidea*. Although 93 genera had reportedly been placed into aseptic culture, the reasons for the poor response of monocotyledons to tissue culture remained unclear. PAGE (1984) concluded, however, that callus cultures of monocotyledons could be induced to undergo organogenesis by adjusting the cytokinin : auxin ratio. It was also emphasised that the source and physiological age of monocotyledonous explants were important factors which influenced not only organ formation but also the regeneration potential of the explant and therefore the resultant *in vitro* culture. PAGE (1984) also concluded that little factual information with respect to *in vitro* root induction in cultures of monocotyledons had been published.

Subsequently, SAGAWA (1991) made the pertinent observation that, although orchids are generally thought to be easy to propagate *in vitro*, a whole range of responses, including failures, to tissue culture had been obtained for individual genera, species and even genotypes over the years. This was attributed to the family Orchidaceae being extremely heterozygous with over 450 genera and 15,000 species. The author, however, also came to the conclusion that, because this diversity of response had been so poorly documented in the literature, a false impression that orchids can be easily propagated *in vitro* had been created.

Similarly reports of reproducible plant regeneration obtained from tissue cultures of the Gramineae prior to 1980 were few. In most of these early cultures plant regeneration was sporadic, of a short term nature and achieved via shoot morphogenesis (VASIL, 1985). It has also generally been assumed that model *in vitro* systems developed for one plant can be used for another (VASIL, 1987). The cereals and grasses proved this assumption to be incorrect, however, and were very resistant to *in vitro* culture as reported for the Orchidaceae. Lately, however, the improved techniques used to produce embryogenic suspension cultures have proven invaluable in the establishment of *in vitro* cultures of all the important species of cereals and grasses (VASIL and VASIL, 1991).

Great advancements have since been made with the application of *in vitro* systems in plant biotechnology, synthetic seed technology, cryo-preservation, and standard and automated micropropagation systems (HERMAN, 1997). Practically, however, protocols established for particular plants are not always binding. Some procedures may be difficult to repeat due to numerous variables as reported for the Orchidaceae and other monocotyledons and culture media are subject to change. Tissue culture techniques therefore require constant attention and improvement, micropropagation systems for commercial mass propagation must be devised and new plants, which may face extinction and have never been propagated *in vitro*, need to be researched. With such attention being given to plant tissue culture and the improved plant regeneration currently being achieved as a result, *in vitro* culture is often the only practical way to produce the large numbers of plants required for commercial enterprise, including the genus *Hypoxis*.

#### **4.2.2 IN VITRO CULTURE OF THE HYPOXIDACEAE**

Following an upsurge in interest in the medicinal properties of hypoxoside for pharmaceutical purposes during the 1970s and increased need for *H. hemerocallidea* plants, *in vitro* culture was examined as a means to rapidly multiply this species (PAGE, 1984). At the time *in vitro* methods to regenerate plants with underground corms with a single meristem as found among the genus *Hypoxis* had not yet been established. The approach adopted in developing an *in vitro* technique was therefore to induce additional meristems either directly, or indirectly via callus formation, on explants taken from *H. hemerocallidea* plants.

Direct regeneration of *H. hemerocallidea* plants was initiated *in vitro* via meristemoids induced on corm explants. Theoretically, a single explant like this should produce an infinite number of plants *in vitro*. On average, however, only 39 *H. hemerocallidea* plants

were produced from each mature corm using this method (PAGE and VAN STADEN, 1984). Because only 90% of these plants survived acclimatization, only 35 plants were ultimately obtained from each corm via direct *in vitro* regeneration. This was not unusual as a number of bulbous crops have a relatively low rate of multiplication *in vitro* and the plants produced take two to three years to flower (KYTE and KLEYN, 2001). Another distinct disadvantage to the use of *H. hemerocallidea* corm explants was that the mother plant was totally destroyed in the process. Inflorescence peduncles, leaves and unopened flower buds were therefore examined as alternate explant sources. While the former proved unsuccessful (PAGE, 1984), the flower buds were found to be a productive explant source for *H. hemerocallidea* (PAGE and VAN STADEN, 1986).

Plantlets regenerated from explants of *H. hemerocallidea* flower buds, however, were produced indirectly from callus induced on young unopened flower buds, in contrast to direct regeneration from corm explants. It was also estimated that a possible 80 994 *H. hemerocallidea* plants could be regenerated from 100 flower buds in a year using this technique (PAGE and VAN STADEN, 1986). Although a more efficient method than direct regeneration from corms, the disadvantages were that flower buds were only available for three months of the year, plant regeneration was indirect via callus, and only 75% of the plants survived acclimatization. The initial procedures suggested for the *in vitro* production of hypoxoside using these cultures have been reviewed by VAN STADEN and BAYLEY (1988).

Using the protocols as described by PAGE (1984) and PAGE and VAN STADEN (1984) (Table 7), *H. obtusa* was also successfully produced *in vitro* from corm explants, but *H. angustifolia* and *H. nysica* failed to regenerate under the same conditions (VINESI *et al.*, 1990). In contrast to the findings of PAGE (1984) callus was not induced by adding auxins to the *H. obtusa* culture media thus further confirming that the requirements for the *in vitro* propagation of monocotyledonous plants can differ even between species.

A varied response to tissue culture was also shown by three colour varieties of *Rhodohypoxis baurii*, a small member of the Hypoxidaceae endemic to South Africa (UPFOLD *et al.*, 1992). In contrast to *Hypoxis*, multiple shoots developed initially on the corm explants of each variety when placed on an inoculation medium free of plant growth regulators. Thereafter, however, only the red variety continued to multiply significantly. Even the addition of growth regulators did not enhance the regeneration rate of the pink and white varieties, further highlighting differences encountered between *in vitro* cultures of monocotyledons.

Plantlet regeneration and bulbil formation *in vitro* from leaf and underground stem explants have also been reported for *Curculigo orchioides* Gaertn. (Hypoxidaceae) an endangered medicinal plant from India (SURI *et al.*, 1999). Plantlets were obtained through direct regeneration from leaf and stem explants without an intervening callus phase, and via bulbils derived from callus. In contrast to *Hypoxis*, leaf explants were found to be the most suitable material from which to establish *in vitro* cultures of *C. orchioides*, not because the most shoots were produced, but because contamination free cultures were obtained thus eliminating a major problem encountered when using underground organs. The authors also remarked that *C. orchioides* plants responded quite differently in culture to bulbous crops of the Amaryllidaceae and Liliaceae families.

With the spread of HIV / AIDS, worldwide interest has escalated in both the development of natural products as immune system boosters and the superior healing properties of *Hypoxis* corm extracts. Wild populations of medicinally important *Hypoxis* species were therefore again targeted during the 1990s to supply the informal medicinal plant traders and health shops. To alleviate the renewed and unrelenting pressure placed on wild plant populations through harvesting, it became increasingly urgent to not only conserve but also cultivate *Hypoxis*. As a result an attempt was made to develop a standardized micropropagation procedure suitable for the continuous regeneration of all *Hypoxis* species (APPLETON and VAN STADEN, 1995 a). In view of the varied responses to *in vitro* culture recorded among the Hypoxidaceae (Table 7), the original protocol outlined for the direct regeneration of *H. hemerocallidea* plants from meristemoids induced on corm explants (PAGE, 1984; PAGE and VAN STADEN, 1984) was re-examined.

In contrast to the results of PAGE and VAN STADEN (1984), greatest growth response was obtained from decontaminated corm explants inoculated onto an MS initiation medium supplemented with BA and NAA at a concentration of 1 mg l<sup>-1</sup> each (APPLETON and VAN STADEN, 1995 a). If the culture was maintained on this medium, however, large malformed roots were produced at the expense of adventitious shoots. Further tests showed that multiple shoots without malformed roots could be obtained continuously either directly from compact meristematic tissue or indirectly from undifferentiated callus if the plant culture was transferred to an auxin free MS medium with 1 mg l<sup>-1</sup> BA. Fine roots were also produced on shoots at this stage, but, with only poorly developed corms, few of these rooted shoots survived acclimatization. To induce corm formation *in vitro* prior to rooting, clusters of shoots were therefore transferred from the maintenance medium to an MS medium without plant growth regulators (PGRs). Rapid corm formation occurred followed by spontaneous rooting. These rooted plantlets did not separate easily during transplanting

**Table 7.** Protocols developed for the *in vitro* regeneration of some Hypoxidaceae.

PLANT NAME	EXPL	MEDIUM	SHT/RT	R PLTS	REFERENCES
<i>Hypoxis hemerocallidea</i> Fisch. and Mey.	1. Corm	<u>Init M.</u> MS, 1 mg l <sup>-1</sup> BA: 1 g l <sup>-1</sup> Cas H <u>Rt M.</u> MSO	Shts Rts	✗ ✓ 90%	1. PAGE (1984), PAGE & VAN STADEN (1984). 2. PAGE (1984), PAGE & VAN STADEN (1986). 3. PAGE (1984) 4. PAGE (1984)
	2. Flower buds	<u>Init M.</u> MS, 5 mg l <sup>-1</sup> BA: 1 mg l <sup>-1</sup> NAA <u>Rt M.</u> MSO	C, shts, rts Rts	✗ ✓ 75%	
	3. Leaf	As above for 1 and 2	None	✗	
	4. Infl pd	As above for 1 and 2	None	✗	
<i>H. obtusa</i> Burch	Corm	<u>Init M.</u> MS, 1 mg l <sup>-1</sup> BA <u>Rt M.</u> MSO (¼ strength)	Shts, rts Rts	✓ ✓	VINESI <i>et al.</i> (1990).
<i>H. nyasica</i> Bak.	Corm	As above for <i>H. obtusa</i>	None	✗	VINESI <i>et al.</i> (1990).
<i>H. angustifolia</i> Lam.	Corm	As above for <i>H. obtusa</i>	None	✗	VINESI <i>et al.</i> (1990).
<i>H. angustifolia</i> Lam. var <i>angustifolia</i>	Corm	<u>Init M.</u> MSO <u>Main M.</u> MS, 2 mg l <sup>-1</sup> BA <u>Rt M.</u> MSO	Shts, rts Shts Rts	✓ 45% ✓ 100%	APPLETON and VAN STADEN (1995 b).
	Callus	<u>Main M.</u> MS, 0.5 mg l <sup>-1</sup> BA <u>Rt M.</u> MSO	C, shts Rts	✓ 90%	
<i>H. hemerocallidea</i> Fisch. and Mey	Corm	Cont Reg achieved on: <u>Init M.</u> MS, 1 mg l <sup>-1</sup> BA, 1mg l <sup>-1</sup> NAA	C, shts, mrts	✗	APPLETON and VAN STADEN (1995 a).
		<u>Main M.</u> MS, 1 mg l <sup>-1</sup> BA	Shts	✗	
		<u>Rt M.</u> MSO	Rts	✓ 90%	
<i>H. acuminata</i> Baker	Corm	Cont Reg achieved as for <i>H. hemerocallidea</i>	As above	✓ 70%	APPLETON and VAN STADEN (1995 a).
<i>H. obtusa</i> Burch	Corm	Cont Reg achieved as for <i>H. hemerocallidea</i>	As above	✓ 90%	APPLETON and VAN STADEN (1995 a).
<i>H. rigidula</i> Bak.	Corm	Cont Reg achieved as for <i>H. hemerocallidea</i>	As above	✓ 70%	APPLETON and VAN STADEN (1995 a).
<i>Rhodohypoxis baurii</i> (Bak.) Nel	Corm	<u>Init M.</u> MSO <u>Rt M.</u> MSO	Shts, rts Shts, rts	✓ ✓ 90%	UPFOLD <i>et al.</i> (1992).
<i>Curculigo orchioides</i> Gaertn.	1. Stem	<u>Init M.</u> B5, 4.4 µM BA	Shts	✗	SURI <i>et al.</i> (1999).
	2. Leaf	<u>Main M.</u> B5, 35.2 µM BA	C, bulbil	✗	
		<u>Rt M.</u> B5 0	Rts	✓ 90%	

Expl = explant; Sht = shoot; C = callus; Rt = root; Mrt = malformed roots; R Plts = regenerated plants; 90% = % survival; Init M = initiation medium; Rt M = rooting medium; Main M = maintenance medium; B5 = Gamborg basal medium; PGR = plant growth regulators; MS = Murashige and Skoog basal medium; BA = 6-benzylaminopurine; NAA = 1-naphthalene acetic acid; MSO = MS medium - PGRs; B5O = Gamborg medium - PGRs; Cas H = casein hydrolysate; Infl pd = inflorescence peduncle; None = no response; Cont Reg = continuous regeneration.

however, resulting in poor establishment and plants with multiple corms. Since multiple corms are commercially undesirable, an additional stage was introduced into the procedure whereby the corms were separated and rooted individually on MS rooting medium without PGRs. In addition the corms of individually rooted *H. hemerocallidea* plantlets were also used, while still *in vitro*, as an aseptic source of secondary explants to re-inoculate the culture. After transfer to a potting medium of compost : sand (2 : 1; v : v), 90% of the rooted *H. hemerocallidea* plants were successfully acclimatized.

The original aseptic procedure used to propagate *H. hemerocallidea* plants was therefore modified by the introduction of new media and distinct steps until a stable *in vitro* culture could be established from a single *H. hemerocallidea* corm. An efficient micropropagation system from which *Hypoxis* plantlets could be continuously regenerated was thus obtained (APPLETON and VAN STADEN, 1995 a). Using this stepwise procedure the culture was scaled up to produce large numbers of plants in contrast to the 35 plants per corm originally obtained by PAGE and VAN STADEN (1984). Not only had the efficiency of the *in vitro* procedure improved as a result, but it was no longer necessary to destructively harvest more than one mother plant to initiate a very productive culture.

The stepwise micropropagation procedure developed was then used to establish *in vitro* cultures of *H. acuminata*, *H. obtusa* and *H. rigidula* (APPLETON and VAN STADEN, 1995 a). Productivity of the *H. angustifolia* var. *angustifolia* culture was also dramatically improved on introducing the sequential steps into the *in vitro* protocol (APPLETON and VAN STADEN, 1995 b). The response of each *Hypoxis* species to *in vitro* culture was not the same, however, particularly with respect to growth regulation requirements, callus production, shoot multiplication, contamination and browning.

It is well known that plants with high concentrations of phenolic compounds, also found among some *Hypoxis* species (BAYLEY, 1989), are not easy to culture because injury to the tissues during explant preparation stimulates the production of polyphenolases. These enzymes then oxidise the phenolic substances present in the tissue to form dark, growth-inhibiting compounds often toxic to the explant (DODDS and ROBERTS, 1985). *In vitro* these compounds also leach out into the basal medium causing it to turn brown to black in colour. Bleeding or browning, the terms used to describe this phenomenon, is generally detrimental to the culture. It retards growth and multiplication, finally causing the culture material to become black and necrotic. Agar source and media with too high a salt or chloride additive are also thought to be common causes of browning.

Suppression of the oxidation process has been suggested as a way to control browning. This is not easy to achieve *in vitro*, however, as the oxidation reaction of phenols takes place on immediate contact with oxygen. The control compounds used *in vitro* are also normally incorporated into the basal medium and may therefore not be in sufficiently close contact with the plant tissue and phenolics to have an effect on the oxidation process. Control techniques used include presoaking explants in antioxidant solutions before inoculation, adding antioxidants to the medium, transferring the culture to fresh medium immediately discolouration is observed, and limiting exposure to light (DODDS and ROBERTS, 1985; PIERIK, 1987). Activated charcoal is also sometimes added to the medium to adsorb toxic phenolics and their products (KYTE and KLEYN, 2001).

While continuous plantlet regeneration was obtained for *H. obtusa*, *H. hemerocallidea*, *H. acuminata* and *H. rigidula* after introducing the stepwise micropropagation procedure, only a 6% growth response was recorded from the primary corm explants of *H. colchicifolia* due to the negative and toxic effects of browning and internal pathogens (APPLETON and VAN STADEN, 1995 a). Attempts to propagate *H. colchicifolia* plants thereafter were only partially successful. The protocols developed were therefore more fully investigated in this study to specifically formulate a micropropagation system for the mass propagation of *H. colchicifolia* plants.

## **4.3 MATERIALS AND METHODS**

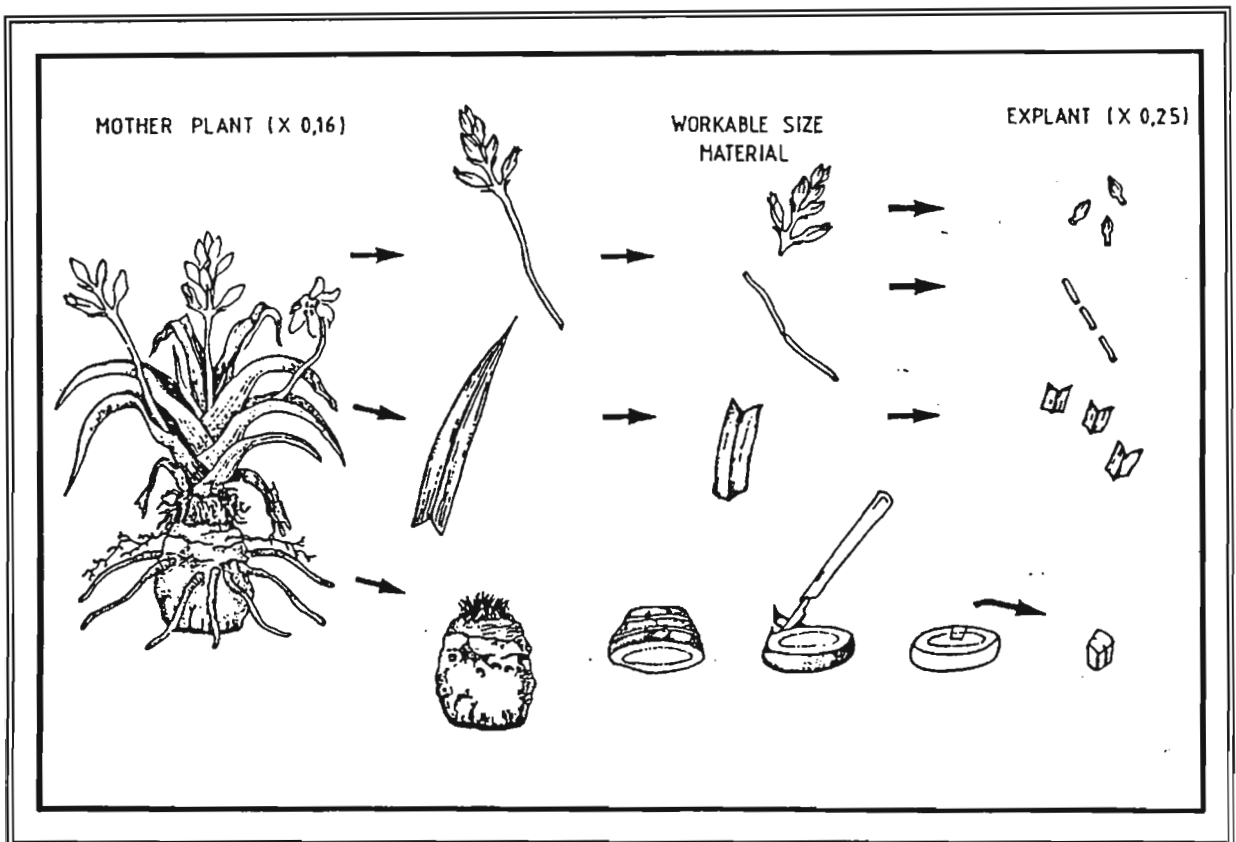
### **4.3.1 PLANT MATERIAL**

Ideally only disease-free plants should be tissue cultured. This is, however, not always feasible if wild plants, which are available only from their natural environment, need to be established *in vitro*. Mature *H. colchicifolia* plants, collected from tracts of grassland scheduled for suburban housing development in Hayfields, Pietermaritzburg, were therefore cleaned and maintained in pots under 10% shade for six months until required for explant material to initiate an *in vitro* culture. The best explant source was determined by aseptically inoculating, onto culture media, segments of corms, leaves, inflorescence peduncles and flower buds removed from these plants and trimmed as illustrated for *H. hemerocallidea* in Figure 6 (from PAGE, 1984). Corm explants were harvested throughout the year while other explants were collected after the dormant winter period. Young, emerging, meristematic leaves approximately four cm in length, were collected in spring (October), mature leaves, inflorescence peduncles and flower buds in summer (December)

and seeds in late summer (March). Viable and non-viable seeds were separated in water. The viable seeds or "sinkers" were retained and the non-viable "floaters" removed as described for *H. hemerocallidea* (HAMMERTON, 1985).

#### 4.3.2 DECONTAMINATION PROCEDURES

There are no standard, set methods to surface decontaminate explants for *in vitro* culture since the concentration of the disinfectants and duration of the treatments used depends on the cleanliness and hardness of the explant material. The basic decontamination procedures to obtain aseptic *H. hemerocallidea* explants used by PAGE (1984) and PAGE and VAN STADEN (1984, 1986) were therefore initially followed to decontaminate all the *H. colchicifolia* explant material. An ethanol dip was routinely used to promote penetration



**Figure 6.** Diagrammatic outline showing the procedure followed by PAGE (1984) to obtain flower bud, inflorescence peduncle, leaf and corm explants of a suitable size from a mature *H. hemerocallidea* plant for *in vitro* inoculation. This procedure was followed during the preparation of explants from the *H. colchicifolia* plants used in this study. (Illustration from PAGE, 1984).

of the major disinfectants tested, ie. mercuric chloride and sodium hypochloride. The concentration of both the ethanol dip and disinfectants, as well as the explant immersion times, were monitored for every decontamination procedure done and modified according to the contamination present and tolerance levels of the explants used. All explants were inoculated onto MS basal medium without PGRs to determine whether the disinfectants and procedures used were adequate and to eliminate those infected with endogenous pathogens. The following decontamination procedures were the most successful for the explants investigated and were followed, with minor adjustments, throughout this study.

#### **4.3.2.1 Leaves, young and mature**

- 1) Leaf sections, 40 mm long, were washed under running tap water for 15 minutes.
- 2) Immersed in a 0.2% benomyl (Benlate™) solution (w:v) + 2 drops Tween 20 (100 ml<sup>-1</sup>) for 15 minutes.
- 3) Rinsed in sterile distilled water twice for 5 minutes each.
- 4) Dipped in 80% ethanol for 30 seconds.
- 5) Surface decontaminated in a 1% sodium hypochlorite (JIK) solution (v:v) plus 2 drops Tween 20 (100 ml<sup>-1</sup>) under aseptic conditions in a Laminar Flow Cabinet for 20 minutes.
- 6) Rinsed in 3 changes of sterile distilled water for 10 minutes each.
- 7) Leaf sections were trimmed, cut into 10 mm<sup>2</sup> explants and inoculated.

#### **4.3.2.2 Flower Buds and Inflorescence Peduncles**

- 1) Unopened flower buds approximately 10 mm in length or inflorescence peduncles 30 mm in length were washed under running tap water for 30 minutes.
- 2) Immersed in a 0.2% benomyl solution (w:v) plus 2 drops Tween 20 (100 ml<sup>-1</sup>) for 5 minutes.
- 3) Rinsed in sterile distilled water twice for 5 minutes each.
- 4) Immersed in 70% ethanol for 2 minutes.
- 5) Surface decontaminated in a 0.1% mercuric chloride (HgCl<sub>2</sub>) solution (w:v) plus 2 drops Tween 20 (100 ml<sup>-1</sup>) for 2 minutes under aseptic conditions.
- 6) Rinsed in 3 changes of sterile distilled water for 10 minutes each.
- 7) Buds placed on sterile filter paper to absorb excess water during trimming.
- 8) Flower buds were cut in half transversely just below the perianth segments to remove the anthers and pollen cleanly prior to inoculation. Peduncles were cut into 10 mm long explants for inoculation.

#### **4.3.2.3 Seeds**

- 1) Viable seeds were washed under running tap water for 30 minutes.
- 2) Immersed in a 0.2% benomyl solution (w:v) plus 2 drops Tween 20 (100 ml<sup>-1</sup>) for 5 minutes.
- 3) Rinsed in sterile distilled water twice for 5 minutes each.
- 4) Immersed in 85% ethanol for 1 minute.
- 5) Surface decontaminated in a 3.5% sodium hypochlorite solution (v:v) plus 2 drops Tween 20 (100 ml<sup>-1</sup>) for 10 minutes under aseptic conditions.
- 6) Rinsed in 3 changes of sterile distilled water for 5 to 10 minutes each.
- 7) Decontaminated seeds were inoculated singly onto basal medium.

#### **4.3.2.4 Corms**

- 1) Corm slices, 10 mm thick, were washed under running tap water for 3 hours.
- 2) Immersed in 0.2% benomyl solution (w:v) plus 2 drops Tween 20 (100 ml<sup>-1</sup>) for 30 minutes.
- 3) Rinsed in 2 changes of sterile distilled water for 5 minutes each.
- 4) Immersed in 95% ethanol for 4 minutes.
- 5) Immersed again in a 0.2% benomyl solution (w:v) (using sterile distilled water) plus 2 drops Tween 20 (100 ml<sup>-1</sup>) for 30 minutes under aseptic conditions.
- 6) Rinsed in 2 changes of sterile distilled water for 5 minutes each.
- 7) Surface decontaminated in 0.2% HgCl<sub>2</sub> solution (w:v) plus 2 drops Tween 20 (100 ml<sup>-1</sup>) for 10 minutes under aseptic conditions.
- 8) Rinsed in 3 changes of sterile distilled water for 5 to 10 minutes each.
- 9) Necrotic tissue trimmed off corm slices and 8 mm<sup>3</sup> explants removed from the cambial region for inoculation.

### **4.3.3 ENVIRONMENTAL CONDITIONS IN GROWTH ROOMS**

All cultures were maintained in growth rooms with a standard temperature of  $25 \pm 2^\circ\text{C}$  and light intensity of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  produced by cool-white fluorescent light tubes. The light regimes investigated were a 16 hour photoperiod, continuous light or continuous dark.

### **4.3.4 PREPARATION OF THE BASAL CULTURE MEDIUM**

The revised macro and micro nutrient basal medium (MS) of MURASHIGE and SKOOG

(1962) supplemented with 30 g l<sup>-1</sup> sucrose and 0.1 g l<sup>-1</sup> *myo*-inositol, and solidified with 10 g l<sup>-1</sup> agar at a pH of 5.8, was used routinely. The pH was adjusted with NaOH or HCl. When a clear gel was required the agar substitute, gelrite, was used at 3 g l<sup>-1</sup> to obtain the correct gel strength. If required other additives, such as polyvinylpyrrolidone (PVP) and activated charcoal, were added to the MS medium prior to adjusting the pH. For initiations, 10 ml of MS medium was dispensed into glass tubes (25 mm diameter) and autoclaved at a pressure of 103 kPa and a temperature of 121°C for 20 minutes. During routine subculturing thereafter plant tissue was transferred to the aseptic 250 ml glass bottles containing 40 ml of the MS medium required.

Optimum MS medium for seed germination was determined using full or half strength MS basal medium without PGRs. The effect of sugar on contamination was tested concurrently by omitting sucrose and *myo*-inositol from both media. Half of the tubes were placed under continuous light and the other half in the dark after inoculation.

#### **4.3.4.1 Determination of Plant Growth Regulator Requirement**

Optimum PGR requirement, or the cytokinin : auxin ratio, which will induce organogenesis or callus on the explant, is most accurately determined in grid trials in which the MS basal medium is supplemented with a factorial combination of a range of concentrations of a cytokinin and an auxin as shown in Table 8 (KYTE and KLEYN, 2001).

Once successful decontamination procedures had been finalized in this study, the optimum MS basal media and cytokinin : auxin ratios required to induce organogenesis, callus, multiple shoots and roots on the various explants were established using grid trials. The effect of the factorial combinations of the cytokinins, 6-benzylaminopurine (BA) or kinetin (K), and the auxin, 1-naphthaleneacetic acid (NAA) in the MS basal medium were examined for each explant as per Table 8. Ten tubes or bottles of medium were prepared for each combination of plant growth regulator tested in the grid unless stated to the contrary. The ideal photoperiod was determined by repeating each grid under different light regimes.

#### **4.3.5 CONTROL OF BROWNING, HYPERHYDRICITY AND CONTAMINATION**

Activated charcoal (at concentrations of 0.2 to 3.0%, w : v) is frequently added to media to adsorb phenolics and toxic compounds and thus prevent *in vitro* browning. It is also added to rooting media to adsorb residual cytokinins and root-inhibiting agents. In this study activated charcoal was therefore added to the MS basal multiplication and rooting media

**Table 8.** An example of a factorial grid which was used to determine the cytokinin : auxin ratio (BA : NAA or K : NAA) best suited to induce a response from either a primary or secondary *H. colchicifolia* explant *in vitro*. In the grid below the effect on the explant of 0, 1, 2, 3 and 5 mg l<sup>-1</sup> BA (or K) was tested either alone (zero NAA) or in combination with 0, 0.5 or 1 mg l<sup>-1</sup> NAA. (BA = 6-benzylaminopurine, NAA = 1-naphthaleneacetic acid, K = kinetin, 6-furfurylaminopurine.)

		Cytokinin concentration (mg l <sup>-1</sup> BA or K)				
		0	1	2	3	5
Auxin concentration (mg l <sup>-1</sup> NAA)	0	0 : 0	1 : 0	2 : 0	3 : 0	5 : 0
	0.5	0 : 0.5	1 : 0.5	2 : 0.5	3 : 0.5	5 : 0.5
	1	0 : 1	1 : 1	2 : 1	3 : 1	5 : 1

at a concentration of 10 g l<sup>-1</sup> (1%) to determine its value as an aid not only in the control of browning, but also to promote rooting *in vitro*.

The anti-oxidants, ascorbic and citric acid, have repeatedly been used to alleviate browning. Ascorbic (100 mg l<sup>-1</sup>) and citric (150 mg l<sup>-1</sup>) acid were therefore also added to the MS multiplication medium (supplemented with 2 mg l<sup>-1</sup> BA only) to determine their effect on browning and the regenerative capability of the meristemoid corm tissue used. Many vitamins are thermolabile, however, and should be filter sterilized (DODDS and ROBERTS, 1985) but whether these anti-oxidants were added to the basal medium before or after autoclaving was not specified in previous reports. These anti-oxidants were, therefore, not only filter sterilized and added to the MS basal medium after autoclaving, but also added to the MS medium prior to autoclaving as a control.

Contamination of *in vitro* cultures which are subject to browning is also a common problem. Benomyl (Benlate™), a broad spectrum fungicide, was therefore examined as a means to control fungal contaminants in the multiplication medium. Benomyl, 0.2% or 2 g l<sup>-1</sup>, was added to the MS medium, supplemented with 2 mg l<sup>-1</sup> BA only, either before autoclaving as a control or afterwards under aseptic conditions. However, because benomyl loses its effectiveness rapidly and could become toxic in the MS basal medium, a 0.2% benomyl solution was used as a fungicidal wash during subculturing. After washing, the secondary explant material was blotted to remove excess solution and the shoot bases flamed dry using an alcohol burner before transfer to fresh medium.

Polyvinylpyrrolidone (PVP), a high molecular weight (MW  $\pm$ 700 000), water soluble polymer, is also frequently added to basal media to control both browning and hyperhydricity, another common problem in tissue culture (PIERIK, 1987). The effect of PVP on the brown hyperhydric callus obtained from *H. colchicifolia* flower buds was therefore determined in a grid trial by supplementing the MS basal medium with a factorial combination of 0 and 0.5 mg l<sup>-1</sup> BA and 0, 0.5 and 1% (ie 0, 5 and 10 g l<sup>-1</sup>) PVP. Similarly the optimum quantity of PVP required to control browning and hyperhydricity of meristemoid corm explant tissue was determined in a factorial grid in which the multiplication medium was supplemented with 0 and 2 mg l<sup>-1</sup> BA (no auxin) and 0, 1 and 2% PVP.

#### **4.3.6 ACCLIMATIZATION OF REGENERATED PLANTLETS**

Only individual regenerated *H. colchicifolia* plantlets with leaves, a single corm and well developed roots were considered ready for pricking out and subsequent acclimatization. The acclimatization procedure followed for all treatments involved strengthening the regenerated plantlets by gradually exposing them to conditions of increased light intensity and decreased humidity. This included opening the culture bottles in the growth room to expose the rooted plantlets to the atmosphere under lights for 3 to 5 days. The plantlets were then removed from the culture vessels and all agar washed off the roots in water. The roots of the plantlets were then immersed in a 2 g l<sup>-1</sup> (0.2%) benomyl solution for 30 minutes, care being taken to not wet the leaves. The plantlets were then placed upright in glass bottles with their roots only immersed in distilled water while their corms and leaves were kept dry. These were left under an incandescent light source for 5 to 7 days. The distilled water was replenished when necessary.

When the corms became firm and the leaves more rigid, the plantlets were transplanted to 75 mm diameter plastic pots containing a potting medium of pre-enriched bark (Gromor®) and sand (2 : 1, v : v) and transferred to a greenhouse with an average maximum temperature of between 27°C to 30°C. To acclimatize to ambient temperature and humidity conditions the plantlets were initially placed under plastic sheeting and watered daily. Leaves were kept dry during watering. After 8 to 12 weeks the hardened plants were transferred to a 10% shadehouse without overhead irrigation. Plants were watered when necessary and transferred to 125 mm diameter plastic pots after 6 months. This procedure was followed for all *H. colchicifolia* plantlets regenerated *in vitro* unless stated to the contrary.

### 4.3.7 DIRECT MARKETING

It is advantageous for commercial purposes, however, to supply plantlets regenerated *in vitro* directly to the purchaser prior to acclimatization. To determine whether plantlets could survive cold storage, one hundred and twenty rooted plantlets were prepared for acclimatization as in Section 4.3.6 above. When ready for potting on, 30 control plantlets were immediately planted out into 125 mm diameter plastic pots and placed under the plastic cover in the greenhouse. The remaining 90 plantlets were placed upright in self-seal plastic packets (100 x150 mm), 5 per packet. The packets were sealed and stored in a cold room at 10°C in a light proof container. Six packets, ie 30 plantlets, were removed from the cold room after 4, 8 and 12 days respectively. The plantlets were transferred to potting medium in 125 mm plastic pots and acclimatized as described. Survival was determined after 6 months.

## 4.4 RESULTS AND DISCUSSION

### 4.4.1 DETERMINATION OF EXPLANT SOURCE

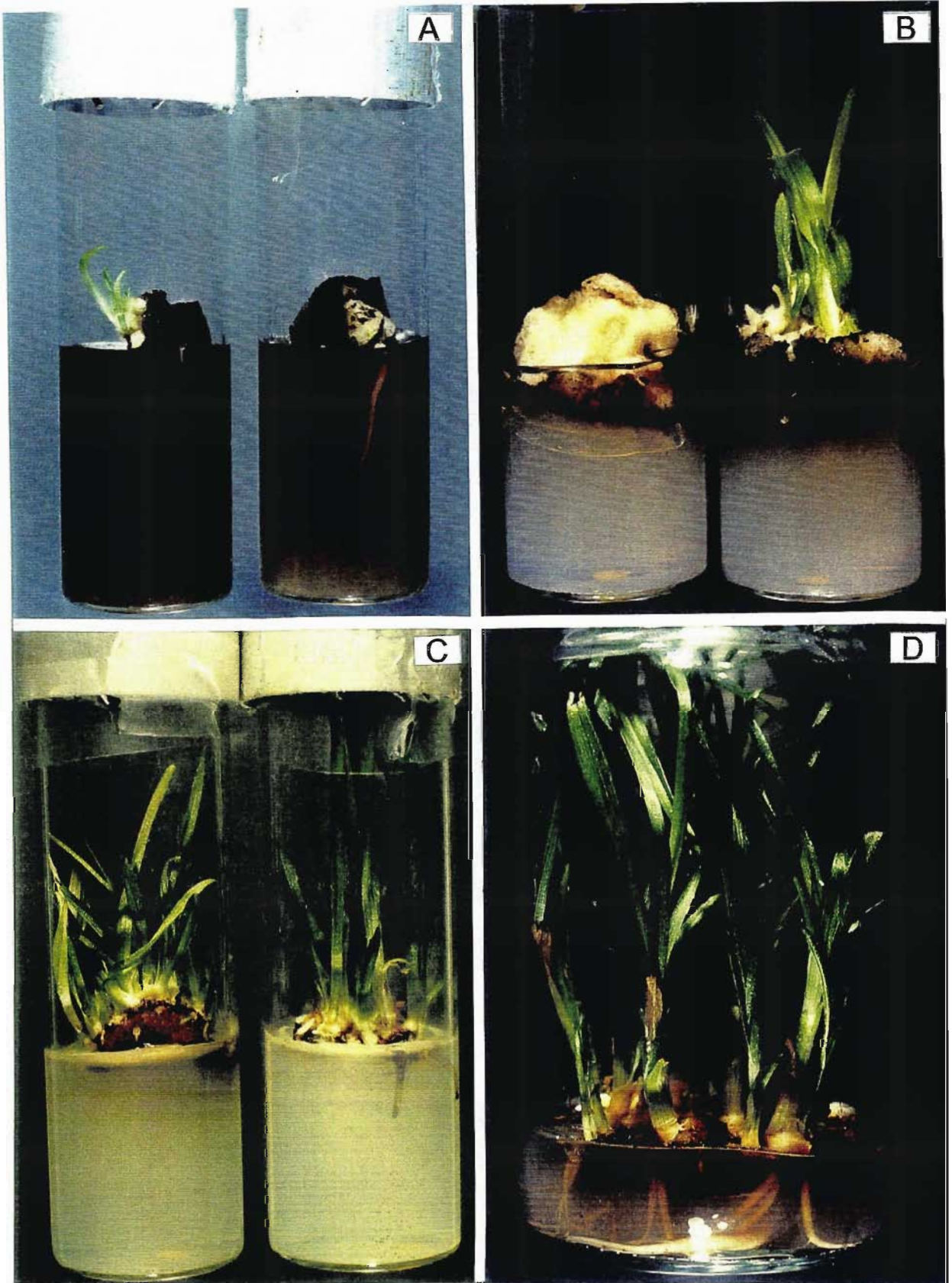
To obtain pathogen free *in vitro* culture material, explants should ideally be collected from disease-free mother plants. This proved difficult to achieve for the *H. colchicifolia* mother plants used in this study due to the continued presence of endogenous pathogens. Surface decontamination of explant material therefore remained a problem. The most successful decontamination procedures developed for each explant examined and which were subsequently used throughout this study are described in Section 4.3.2.

Initially only *H. colchicifolia* corms were evaluated as an explant source because regeneration of plants through direct organogenesis *in vitro* is preferable and had been achieved for other *Hypoxis* species from corm explants (PAGE, 1984; PAGE and VAN STADEN, 1984; VINESI *et al.*, 1990; APPLETON and VAN STADEN, 1995 a). These preliminary, and subsequent, attempts to obtain aseptic *H. colchicifolia* cultures were only partially successful, however, due to the presence of endogenous pathogens in the corms. At each inoculation attempt between 80% to 100% of the corm segments were lost to contamination (Table 9). In even the most successful inoculation, 94% of the primary corm explants were discarded. Of these, 80% were contaminated and 14% had turned black and necrotic due to the toxic effects of phenolics, browning and persistent internal pathogens identified as *Bacillus* and *Cladosporium* species.

As only 6% of the inoculated primary *H. colchicifolia* corm explants remained pathogen free *in vitro*, there was insufficient aseptic plant material available to conduct a factorial grid trial. Media requirements were therefore determined by transferring the aseptic corm explants to media on which other *Hypoxis* species had proliferated (APPLETON and VAN STADEN, 1995 a). After 18 weeks in culture the explants on MS medium supplemented with 1 mg l<sup>-1</sup> BA : 0.5 mg l<sup>-1</sup> NAA had produced meristemoids, shoots and roots. Once again phenolics caused the explants and surrounding media to turn brown but the meristemoids and shoots were not affected (Plate 6). On transfer to fresh medium of the same composition, however, malformed hairy roots were produced spontaneously at the expense of shoots. These roots were eliminated and a few shoots induced by transferring the compact meristemoidal material onto multiplication MS media with 1 or 2 mg l<sup>-1</sup> BA and no auxin. Ultimately, however, the cultures became hyperhydric and rooting was totally inhibited by the toxic effects of browning (Plate 6).

Similar results were experienced during the establishment of *in vitro* cultures of *C. orchioides*. Endogenous contaminants were a major problem when underground organs were used as explants (SURI *et al.*, 1999). Leaves were found to be the most suitable explant material, however, not because the most shoots were produced, but because they were more easily decontaminated and pathogen free cultures were obtained.

Pathogens were also not totally eliminated from the alternative primary *H. colchicifolia* explants examined, however, although levels of contamination were greatly reduced to 14% and 20% for young and mature leaf explants respectively, 10% for inflorescence peduncles and 8% for flower buds (Table 9). Subsequently all of the aseptic *H. colchicifolia* leaf and inflorescence peduncle explants turned black and necrotic *in vitro* due to the toxic effects of browning. Phenolic production and browning were not controlled by adding anti-oxidants, PVP or charcoal to the medium or by placing the cultures in the dark. The *H. colchicifolia* leaf and inflorescence peduncle explants also showed zero growth response *in vitro* regardless of the growth promoting supplements used. For these reasons their use as an explant source was not pursued. Although browning was not mentioned as being a problem, growth was also not induced on *H. hemerocallidea* leaf and peduncle explants *in vitro* (PAGE, 1984). It would appear therefore that the *in vitro* regeneration of plantlets from leaf explants is uncommon among *Hypoxis*, but not among the Hypoxidaceae as a method was developed to rapidly multiply *C. orchioides* plants *in vitro* through direct organogenesis and bulbil formation from leaf explants (SURI *et al.*, 1999).



**Plate 6.** Response of *H. colchicifolia* corm explants to *in vitro* culture. (A) All primary corm explants and media turned brown, inhibiting growth. Only 6% of explants produced shoots and roots directly from meristemoids. Tube diameter = 25 mm. (B) Further growth and rooting were inhibited by browning. (C) Multiple shoots and cormlets were induced after benomyl wash treatment. (D) Regenerated plantlets with roots in 250 ml glass jar.

**Table 9.** The response of the different *H. colchicifolia* explants to *in vitro* culture.

Explant	Inoculation medium		Explant brown (%)	Initiation medium		Multiplication medium		Corm and rooting medium	
	BA:NAA (mg l <sup>-1</sup> )	Contm (%)		BA:NAA (mg l <sup>-1</sup> )	% with shts / callus	BA:NAA (mg l <sup>-1</sup> )	% with shts / callus	BA:NAA (mg l <sup>-1</sup> )	% with corms / roots
Leaf, young	0 : 0	14	86	Grid	0	-	-	-	-
Leaf, mature	0 : 0	20	80	Grid	0	-	-	-	-
Infloresc peduncle	0 : 0	10	90	Grid	0	-	-	-	-
Flower bud	0 : 0	8	52	0 : 0 0.5 : 0 2 : 0 2 : 1	50	0.5 : 0  1 : 0	100 Vitr C Direct shts	0 : 0	100
Corm	0 : 0	80	14	1 : 0.5	6	2 : 0 1 : 0	97	0 : 0	100
Seed	0 : 0 - a - b - c - d	67 79 63 88	0	1 : 0.5 One Seed- ling	0	-	-	-	-

Cont = contamination, Infloresc = inflorescence, Shts = shoots, Vitr C = vitrified callus, a = ½ strength MS medium - sucrose, b = ½ strength MS medium + sucrose, c = full strength MS medium - sucrose, d = full strength MS medium + sucrose.

In view of the difficulties encountered in obtaining an aseptic *H. colchicifolia* culture, seeds were also inoculated in an attempt to obtain aseptic seedlings from which an aseptic culture could theoretically be initiated. *H. colchicifolia* seeds were also difficult to decontaminate with 63% and 67% contamination recorded for seed inoculated onto MS medium without sucrose compared to 79% and 80% for seed on MS medium with 30 g l<sup>-1</sup> sucrose (Table 9). After 13 weeks in culture only one seed had germinated on half strength MS medium without sugar. The seedling was used to initiate a culture, but again this was unsuccessful due to browning. No other seeds germinated. These results clearly indicated, however, that the factors controlling seed dormancy should first be determined for *H. colchicifolia* before whole seed can be used *in vitro*.

The most successful inoculations for *H. colchicifolia* were achieved using young flower buds as explants. Although on average 92% of the bud explants were successfully

**Table 10.** Response of *H. colchicifolia* flower bud explants to a cytokinin : auxin grid.  
 A = Shoots, corms and roots via direct organogenesis.  
 B = Shoots, corms and roots via an indirect callus phase.

		BA mg l <sup>-1</sup>			
		0	0.5	1	2
NAA mg l <sup>-1</sup>	0	A	A and B	-	B
	0.5	-	-	-	-
	1	-	-	-	A

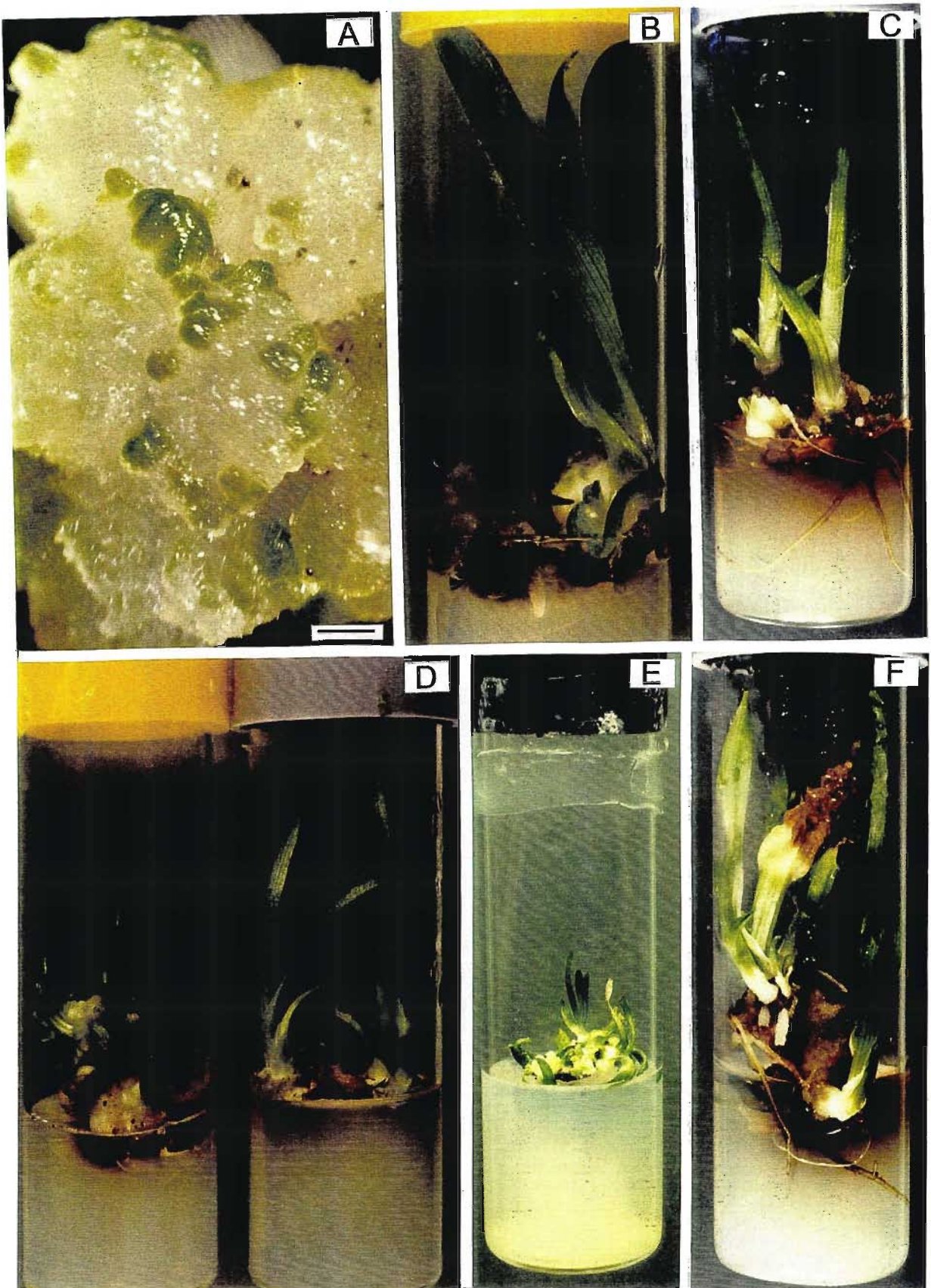
decontaminated, 52% of these subsequently used in a factorial hormone grid trial turned brown and necrotic due to phenolics. This demonstrated once again the negative role played by browning in the establishment of *H. colchicifolia* cultures (Table 9). Nevertheless multiple shoots, callus and roots were induced either directly via meristemoids or indirectly via an intervening callus phase from 50% of the remaining bud explants placed on various MS initiation media as outlined in Table 10 and illustrated in Plate 7.

Although browning of the resultant cultures remained a problem it was partially controlled by routinely subculturing the tissues at 4 week intervals onto media with the same composition as the MS initiation medium. Cultures established directly via meristemoids proliferated on MS multiplication medium supplemented with 2 mg l<sup>-1</sup> BA only, while those produced indirectly via intermediate callus proliferated on MS multiplication medium supplemented with 0.5 mg l<sup>-1</sup> BA. Corms and roots were then sequentially induced on MS medium without PGRs (Plate 7). Flower buds were also found to be a good explant source from which to initiate cultures of *H. hemerocallidea* (PAGE and VAN STADEN, 1986). Regenerated plantlets were only produced indirectly via a callus phase, however, and not directly via meristemoids as obtained for *H. colchicifolia*.

Corms and young flower buds were the most successful explant sources from which *in vitro* cultures of *H. colchicifolia* were eventually established in this study. The toxic effects of endogenous pathogens, phenolics and browning on the established cultures persisted, however, resulting in extensive losses of plant material.

#### 4.4.2 CONTROL OF PHENOLICS, BROWNING AND HYPERHYDRICITY

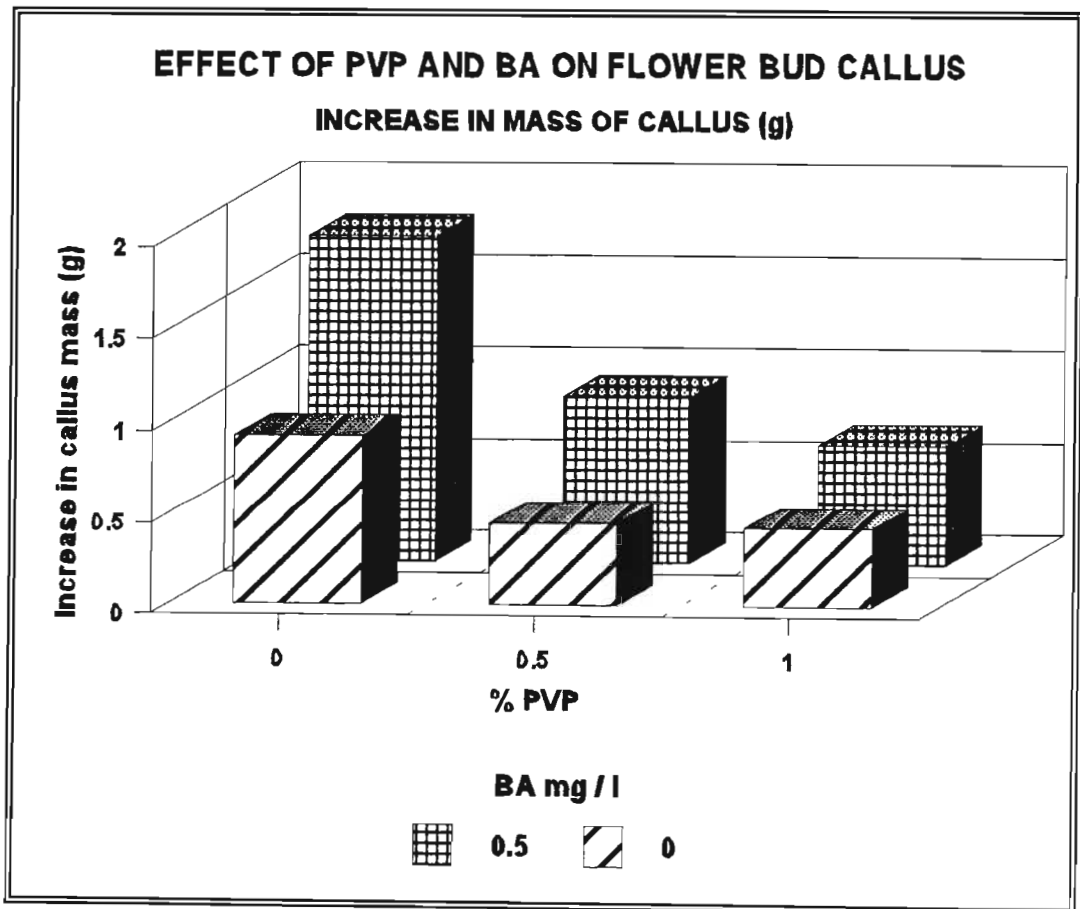
Attempts to control phenolics and browning by adding charcoal to the MS medium had the



**Plate 7.** Response of *H. colchicifolia* flower bud explants to *in vitro* culture. (A - C). Indirect regeneration of plantlets via an intermediate callus phase. (A) Callus with shoot primordia. Bar = 0.75 mm. (B) Callus and multiple shoots. (C) Rooted plantlets. (D - F) Direct regeneration from meristemoids. (D) Multiple shoots, browning evident. (E) Proliferating multiple shoots on maintenance medium. (F) Initiation of corms and roots. Tube diameter = 25 mm.

effect of extracting endogenous pathogens from the plant tissue resulting in 100% loss of all the *H. colchicifolia* plant culture material whether induced from corm or bud explants. Browning was reduced, however, when anti-oxidants or benomyl were added to the medium. Although this resulted in the plant material becoming white and healthy, these results were difficult to quantify as growth, and therefore mass, was inhibited by these additives. Eventually all the treated plant material became hyperhydric.

The alternate and more successful procedure was that in which plant tissue induced directly from compact meristemoids was washed in a 0.2% benomyl solution and then flamed dry to seal the cut surfaces and prevent leaching of phenolics into the medium. This resulted in the initial control of fungal contamination followed by a reduction in the incidence of browning. Subsequently the *in vitro* cultures proliferated and multiple shoots (4.75 shoots cm<sup>-3</sup> of explant) were produced on maintenance MS medium containing 2 mg l<sup>-1</sup> BA. Finally corms and roots were sequentially induced on MS media without PGRs resulting in the continuous regeneration of plantlets (Plate 6).



**Figure 7.** The effect of PVP in MS media supplemented with 0 or 0.5 mg l<sup>-1</sup> BA on browning and hyperhydricity evident in *H. colchicifolia* callus cultures.

In contrast browning and hyperhydricity of flower bud callus cultures was controlled by adding PVP to the MS medium. Results, as determined in a grid trial, however, clearly showed just the opposite which was that callus mass increased throughout on all media with a 0.5 mg l<sup>-1</sup> BA supplement (Figure 7). Contrary to expectation greatest callus proliferation was obtained on the MS medium supplemented with 0.5 mg l<sup>-1</sup> BA and to which no PVP had been added. Callus mass was also shown to decrease with an increase in PVP concentration in the MS medium regardless of the presence or not of BA, leading to the conclusion that the presence of PVP retarded callus growth.

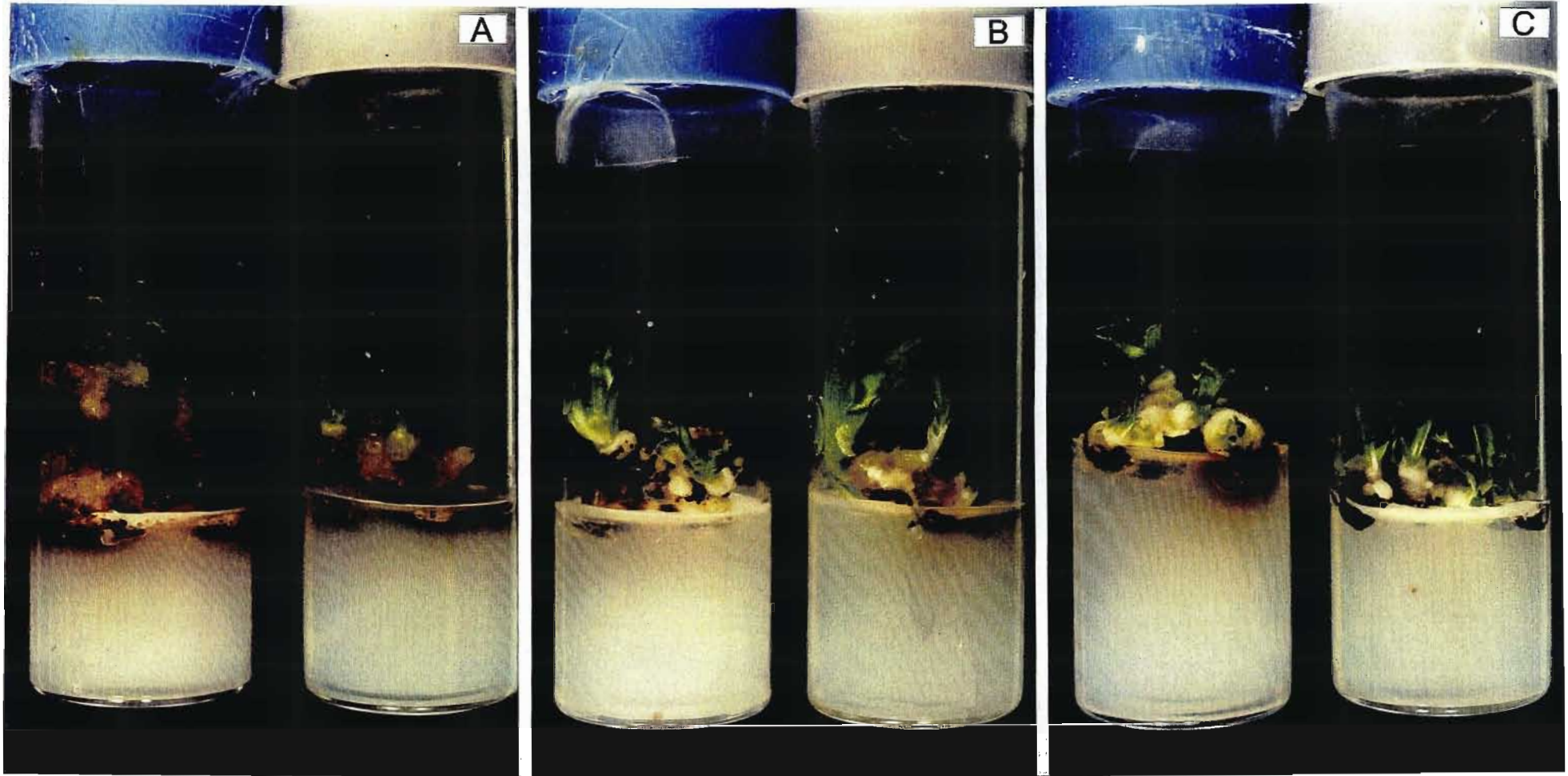
Figure 7, however, does not show that as the PVP concentration increased both browning and hyperhydricity were reduced in the callus cultures on all the media, both with and without a BA supplement (Plate 8). Although callus growth was inhibited, increased PVP concentration in the media also induced the formation of more shoots and corms. The callus cultures, however, ceased to proliferate if exposed to PVP supplemented medium over an extended period of time. The most effective way to maintain productive callus cultures was achieved thereafter by maintaining the callus on MS medium supplemented with 0.5 mg l<sup>-1</sup> BA and then transferring it when required to a medium with PVP in order to control browning and hyperhydricity or induce multiple shoots. Subsequent subculturing of the shoots onto MS media without PGRs resulted in the induction of corms which were separated and rooted individually.

#### **4.4.3 ACCLIMATIZATION OF REGENERATED PLANTLETS**

The success of an *in vitro* propagation system depends on the production of robust plantlets able to survive transplantation and acclimatization. The procedure described under Materials and Methods (Section 4.3.6) was developed for *H. colchicifolia*. Survival of plantlets regenerated via the improved methods outlined increased from less than 10% to between 70% in winter and 98% in summer using this procedure to acclimatize the plantlets.

#### **4.4.4 DIRECT MARKETING**

It is advantageous for commercial purposes to supply plantlets regenerated *in vitro* directly to the purchaser prior to acclimatization. All the rooted *H. colchicifolia* plantlets survived cold storage at 10 °C for up to 12 days prior to transplantation. After 6 months 100% of the plantlets stored for 8 days had survived acclimatization compared to 93.3% of the control plants and 86.6% of those stored for 4 days. At 76.6% survival was the lowest for plants



**Plate 8.** The effect of different concentrations of PVP (0, 0.5, 1%) on browning and hyperhydricity of flower bud callus on MS media supplemented with  $0.5 \text{ mg l}^{-1}$  BA (blue lids) or without PGRs (grey lids). (A) 0% PVP: Callus on MS medium with BA has doubled compared to callus on medium without PGRs, but browning and hyperhydricity are evident. (B) 0.5% PVP: and (C) 1% PVP: Browning, hyperhydricity and mass of callus have all been reduced with increased PVP concentration on MS media both with BA and without PGRs but number of multiple shoots has increased.

**Table 11.** Percentage survival, after 6 months, of *H. colchicifolia* plants regenerated *in vitro* and stored at 10°C for 4, 8 or 12 days prior to acclimatization procedures.

Treatment		No. of Plantlets		
Temperature	Storage time	Initial number	After 6 months	% Survival
1. Control 25°C	0 days	30	28	93.3
2. 10°C	4 days	30	26	86.6
3. 10°C	8 days	30	30	100
4. 10°C	12 days	30	23	76.6

stored for 12 days (Table 11). These results clearly indicated that plantlets regenerated using the protocol developed for *H. colchicifolia* were sufficiently robust to survive storage for at least 8 days before transfer to pots.

#### 4.5 CONCLUSION

Although plantlet regeneration is difficult to achieve for corm producing monocotyledons, a micropropagation system suitable for the continuous regeneration of plantlets was developed for *H. colchicifolia* by introducing several distinct sequential aseptic steps and different media into the procedure. Methods to control persistent pathogens and browning were also developed. Particular attention was paid to producing robust plants able to withstand cold storage and acclimatization. Plant survival increased from 10% initially to between 70% and 98% after implementing the procedures outlined.

In many of the early cultures of monocotyledons, plant regeneration was sporadic, of a short term nature and achieved via shoot morphogenesis. Recently *in vitro* cultures of cereals and grasses have been established via embryogenic suspension cultures (VASIL and VASIL, 1991). Micropropagation is extremely versatile and, although it is preferable to ultimately produce cloned plants regenerated from a single high yielding mother plant, when researching wild plants genetic diversity can also be maintained by using seed *in vitro* for mass plant propagation. Since *H. colchicifolia* plants are destroyed to obtain secondary products from their corms, it is also difficult to identify high yielding plants and thus produce cloned plants *in vitro*. In view of the difficulties encountered in initiating aseptic cultures from corm explants, it was decided to investigate whether plant regeneration for *H. colchicifolia* would be more successful *in vitro* from zygotic embryos via somatic embryogenesis.

## Chapter 5

# THE *IN VITRO* REGENERATION OF *H. COLCHICIFOLIA* PLANTLETS VIA EMBRYO CULTURE AND SOMATIC EMBRYOGENESIS

### 5.1 INTRODUCTION

Initial attempts to propagate *H. colchicifolia* plants *in vitro* via organogenesis met with varied success (APPLETON and VAN STADEN, 1995 a). Improved plantlet regeneration and greater uniformity between cultures were, however, achieved with the introduction of distinct sequential steps into the micropropagation procedure and the partial control of latent contaminants, phenolic leachates and browning (Chapter 4). Although a plant culture is regarded as established *in vitro* once it can be successfully subdivided for multiplication thereafter (KYTE and KLEYN, 2001), the difficulties encountered to establish and maintain the *H. colchicifolia* cultures as well as the length of time taken to regenerate plantlets *in vitro* indicated an inherent variability between its growth and development and that of other *Hypoxis* species. Variability between plant species has been encountered in previous studies leading to the remark that "it is not easy to give fast guidelines for organ regeneration *in vitro*" (PIERIK, 1987).

The early *in vitro* cultures of monocotyledonous species were also reported to have been far more difficult to induce than those of dicotyledons (KRIKORIAN and KANN, 1986). Attributed to a lack of meristematic cambial regions, this may have contributed to the limited success with the *in vitro* initiation of *H. colchicifolia*. Plant regeneration from the earlier organogenic cultures of the Gramineae was also reported to be inefficient, sporadic and of a short-term nature. Since the 1980s, however, reliable, long-term plant regeneration has been achieved via *in vitro* embryogenic cultures for cereals and grasses (VASIL and VASIL, 1981; 1991), the daylily (*Hemerocallis* spp.) (KRIKORIAN and KANN, 1981) and the oil palm (*Elaeis guineensis*) (TEIXEIRA et al., 1993). Many of these have been included in a list of representative examples from 11 families of monocotyledons in which unambiguous evidence of somatic embryo formation has been published (KRISHNARAJ and VASIL, 1995). The *in vitro* regeneration of *Hypoxis* plants via somatic

embryogenesis has not been reported. In view of the more recent success achieved with other monocotyledons, the feasibility of using the *in vitro* techniques of somatic embryogenesis and embryo culture to regenerate *H. colchicifolia* plants were therefore investigated in this section of the present study.

## **5.2 IN VITRO TECHNIQUES**

Organogenesis and somatic embryogenesis are the two principal *in vitro* techniques by means of which viable plantlets can be regenerated from an isolated explant in response to specific culture conditions. Embryo culture, although an older established technique, does not appear to enjoy the same popularity as a means to regenerate plantlets *in vitro* as the former.

### **5.2.1 ORGANOGENESIS**

In organogenesis, the regeneration of viable plantlets is achieved by the sequential induction of shoots, rootstock and roots either directly from the explant or indirectly via an intermediate callus phase and usually in response to changes in the cytokinin : auxin ratio. An inherent feature of organogenesis is the presence of vascular connections between the regenerating organs or plantlets and the mother tissue (HACCIUS, 1978). Adventitious shoots and roots are also a common feature of these cultures. The application of organogenesis as a method to propagate *H. colchicifolia* plants was reported in Chapter 4. The cytokinin, BA, applied exogenously was shown to induce shoots in these organogenic cultures while removal of exogenous plant growth regulators (PGRs) stimulated root formation.

### **5.2.2 SOMATIC EMBRYOGENESIS**

Somatic embryogenesis, however, is generally defined throughout the literature reviewed as a process in which a bipolar structure or somatic embryo can develop from a somatic cell through an orderly sequence of embryological stages of development characteristic of zygotic embryos, but without the fusion of gametes (Yeung, 1995; JIMÉNEZ, 2001). These non-zygotic structures or somatic embryos have closed radicular ends typical of zygotic embryos. HACCIUS (1978) therefore described both zygotic and somatic embryos as being "a new individual arising from a single cell and having no vascular connection with maternal tissue". Furthermore the embryo-like structures produced *in vitro* are only judged

authentic somatic embryos if there is no indication of pre-existing vascular strands, adventitious shoots or roots. They must also be capable of direct growth along the bipolar shoot-root axis to produce a plantlet, "seedling" or "embryogenic propagule" (KRIKORIAN, 1982).

To date a wide range of plant species have been shown to have the potential or capacity for somatic embryogenesis. Somatic embryos can develop both *in vivo* and *in vitro* from vegetative cells. Furthermore somatic embryos have been shown to differentiate *in vitro* either directly from the surface of an explant or indirectly via an intermediate callus phase (THORPE, 1995). Historically it has been reported that direct somatic embryogenesis takes place from pre-embryogenically determined cells (PEDCs) without a callus phase and indirect somatic embryogenesis from induced embryogenically determined cells (IEDCs) via proliferation of redifferentiated callus (WILLIAMS and MAHESWARAN, 1986). It has recently been pointed out, however, that there is no clear distinction between direct and indirect somatic embryogenesis since in reality the callus formed is either embryogenic or not (VON ARNOLD *et al.*, 2002). Embryogenic callus is composed of proembryonic masses (PEMs), however, which distinguishes it from non-embryogenic callus. It is still not known, though, whether the first formed PEM is an authentic embryo which has deviated from normal embryo development and proliferated in response to PGRs or not. It is accepted, however, that somatic embryogenesis is a multi-step regeneration process beginning with the induction of PEMs followed by somatic embryo formation, maturation, desiccation and plant regeneration.

At this point, however, it should be emphasized that the *in vitro* technique in which the entire process of somatic embryogenesis from initiation to somatic embryo formation, germination and the regeneration of plantlets, is also widely referred to as *in vitro* "somatic embryogenesis". By definition, however, embryogenesis is "the phase of ontogeny concerned with the progressive division of the zygote to fabricate the embryo" and that "successful completion of (zygotic) embryogenesis is the basis for the production of seeds and fruits in plants" (RAGHAVEN and SHARMA, 1995). Strictly speaking embryogenesis terminates with the formation of the embryo, either zygotic or somatic. In practice, however, complete plantlet regeneration is an integral part of the *in vitro* protocol.

The *in vitro* technique of somatic embryogenesis has nevertheless played an invaluable role in experimental embryology to elucidate not only the physical and morphological patterns of embryo development in many angiosperms and gymnosperms, but also some of the biological mechanisms, physiological, biochemical or genetic, that control cell

differentiation and the processes of embryogenesis. Although many of these mechanisms are still unclear, VON ARNOLD *et al.* (2002) have suggested that by constructing fate maps in which an adequate number of known morphological and molecular markers specifying critical stages in somatic embryo development are represented, a model of the whole process of somatic embryogenesis can be created for each plant species examined.

The most valuable application of somatic embryogenesis over the years, however, has been in the development of *in vitro* techniques for the large-scale production of somatic embryos for the commercial propagation of cloned plants for agriculture, forestry and horticulture. Although the commercial value of somatic embryogenesis has long been recognised (KRIKORIAN, 1982; MERKLE *et al.*, 1990) and is still promoted (VON ARNOLD *et al.*, 2002), progress towards implementing this goal has been slow (PARROTT *et al.*, 1991). Improved *in vitro* techniques have, however, been developed for the mass production of somatic embryos of various grasses and cereals (VASIL and VASIL, 1991). In the horticultural industry, advances in the micropropagation of *Ranunculus asiaticus* clones via somatic embryogenesis have also been reported (BERUTO, 2002). Progress in this field is well documented in THORPE (1995), a text which can also serve as an excellent bibliography on somatic embryogenesis.

Of the more than 1000 articles on micropropagation published annually (BROWN *et al.*, 1995), a large number are on somatic embryogenesis. Major texts and reviews referred to for the purposes of this study include those listed in Chapter 4, and others by STREET and WITHERS (1974); RAGHAVEN (1976); TISSERAT *et al.* (1979); KRIKORIAN (1982); AMMIRATO (1983); EVANS *et al.* (1986); WILLIAMS and MAHESWARAN (1986); PARROTT *et al.* (1991); EMONS (1994); THORPE (1995); HARTMAN *et al.*, (1997); JIMÉNEZ (2001) and VON ARNOLD *et al.* (2002).

Historically, the papers by STEWARD (1958), STEWARD *et al.* (1958 a, b) and REINERT (1958) have been cited as being the first reports of somatic embryo formation *in vitro*. These were observed in cell suspension cultures of carrot, *Daucus carota*. More recently, however, HALPERIN (1995) questioned whether STEWARD *et al.* (1958 b) had actually observed somatic embryos in their cultures. He further suggested that this fact had been overlooked and not corrected because the original paper had merely been cited over and over again without actually being read and critically reviewed. Different views on these papers are presented in KRIKORIAN (1982) and HALPERIN (1995). With hindsight, Harry Waris was also belatedly credited for his pioneering work in the late 1950s on somatic embryo formation (KRIKORIAN and SIMOLA, 1999). Waris was reported to have made the

observation that vegetative cells sloughed off aseptic seedlings of *Oenanthe aquatica* (Umbelliferae) in liquid medium, were able to produce embryos which developed into structures he termed neomorphs.

In the intervening years the heterogeneous nature of *in vitro* embryogenic cultures was reported. The many anomalous embryo-like structures observed were variously described as daughter structures, pseudo-embryos, embryoids, and neomorphs. These structures have recently been collectively termed "embryo mutants" (VON ARNOLD *et al.*, 2002).

Initially somatic embryo development and the factors controlling somatic embryogenesis were investigated using a few species of dicotyledons only. Subsequently, however, the formation of somatic embryos *in vitro* has been described for many plant species, including monocotyledons (THORPE, 1995). Although the patterns of somatic embryo development are reported to be similar for all angiosperms, information pertinent to monocotyledons is very limited. An explanation given for this is that somatic embryogenesis has been examined in great detail in the Gramineae, but in only 18 genera of other herbaceous (KRISHNARAJ and VASIL, 1995) and 3 genera of woody (YEUNG, 1995) monocotyledons.

#### **5.2.2.1 Somatic Embryogenesis in Monocotyledons**

The principle features of, and important factors affecting, somatic embryogenesis in monocotyledons are also summarised in the above mentioned reviews. These showed that the nutrient medium most commonly used is that of MURASHIGE and SKOOG (1962), in either solid or liquid form. Addition of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) to the medium promoted the induction of embryogenic tissues in monocotyledons. Of the cytokinins, BA was only occasionally used during the regeneration phase. Other combinations of auxins and cytokinins were investigated with various results. The addition of abscisic acid (ABA) to the medium prior to embryo maturation prevented precocious germination of somatic embryos in some species of Gramineae (VASIL and VASIL, 1991).

Abscisic acid has also been used to regulate the maturation process of somatic embryos of dicotyledons *in vitro*. Following its usual role in senescence and inhibition of stem growth, ABA was shown to suppress the development of abnormal somatic embryos in embryogenic cell cultures of caraway. This resulted in the formation of normal embryos and led to the suggestion that inhibitors could play a role in zygotic embryo development (AMMIRATO, 1974). An inverse correlation between ABA content and embryo

germinability has also been demonstrated during the development of the embryo of *Phaseolus vulgaris* both *in vivo* and *in vitro* (PREVOST and LE PAGE-DEGIVRY, 1985). During natural seed development, however, ABA levels increase to between 1 and 10  $\mu\text{M}$  at the mid-stage of embryogenesis and prior to any water loss from the seed. Levels then decrease as the seed dehydrates (HETHERINGTON and QUATRANO, 1991). Since these changes in the concentration of ABA occur concurrently with the programmed dehydration of the seed, it was suggested that ABA plays a major role in promoting the maturation pathway in the seed and thereby assisting the embryo to acquire desiccation tolerance (KERMODE, 1990). A recent suggestion is that the mode of action of ABA may be through the regulation of certain genes involved in the desiccation and maturation phases of embryogenesis (JIMÉNEZ, 2001) and thus in the reduction of aberrant or mutant embryos. This supports the suggestion that the phases of embryo development are independently genetically regulated as summarised in the review by VON ARNOLD *et al.* (2002).

Embryogenic cultures of monocotyledons are generally induced and maintained on solid media although both liquid and agar-solidified nutrient media are used. Suspension cultures have, however, been established for some of the Gramineae and Amaryllidaceae. Commercially, embryogenic suspension cultures have not yet been established for the date (*Phoenix dactylifera* L.) or coconut palms (*Cocos nucifera* L.) (BLAKE, 1983; TISSERAT, 1991). Improved embryogenic suspension culture methodology is being developed for the oil palm (*Elaeis guineensis* Jacq) (RIVAL *et al.*, 1997).

As in the case of organogenic *in vitro* cultures of monocotyledons, the type of explant selected ultimately determines whether an embryogenic culture can be established or not. In monocotyledons several explants have been used including meristems, bulb scales, leaf bases, seeds and embryos. Important factors to consider are the stage of development and the physiological condition of the explant. The greatest success has been achieved using excised zygotic embryos, both immature and mature, as they contain many undifferentiated, meristematic cells. These cells are still developmentally uncommitted and can therefore be induced to divide to form embryogenic callus (KRISHNARAJ and VASIL, 1995). Little information, however, is available concerning the structural, physiological and biochemical changes during the initiation of an embryogenic culture from zygotic embryo explants. This information would provide a better insight into the early events of embryogenic culture initiation (YEUNG, 1995).

Once induced the embryogenic cells develop into somatic embryos following the same developmental sequence as a zygotic embryo. In the literature reviewed the stages of

development observed for somatic embryos are generally described in terms of dicotyledons namely as proembryonic, globular, torpedo and heart-shaped. However, only the first stages of embryo development, from single cell to globular, are similar for both dicotyledons and monocotyledons, but the later stages and mature embryos are quite different (RAGHAVEN, 1976; RAGHAVEN and SHARMA, 1995). Furthermore, even though the mature zygotic embryos of the Gramineae are complex and clearly different from other herbaceous monocotyledons as illustrated by RAGHAVEN (1976), the sequence and morphological changes of embryo development in cereals and grasses are often used to describe the events of somatic embryogenesis in herbaceous monocotyledons in general. The pathways and changes in morphology reported for daylily (SMITH and KRIKORIAN, 1991), date palms (DEMASON *et al.*, 1991) and oil palms (TEIXEIRA *et al.*, 1993) are, however, far more typical of monocotyledons. These differences observed indicate that it would be advantageous to have some basic knowledge on the embryology of the plant under investigation prior to the use of zygotic embryo explants for the induction of an embryogenic culture.

#### **5.2.2.1.1 Zygotic Embryo and Seedling Development in the Hypoxidaceae**

Known embryological and general floral, seed and fruit characteristics of the Hypoxidaceae are presented in DAHLGREN and CLIFFORD (1982). Much of this information is based on the earlier work of DE VOS (1948; 1949) and WILSENACH and WARREN (1967). Later information confirmed these earlier observations (ZIMUDZI, 1993, 1994 b). The embryo is classified as an Asterad type which is common among the monocotyledons. It is linear in shape, being several times longer than broad, lies embedded in the endosperm and is attached to a small nucellar cap by a short suspensor. Endosperm formation is helobial. Twin embryo-sacs were shown to be a common feature of *H. rooperii* (= *H. hemerocallidea*) (WILSENACH and WARREN, 1967). It would appear that apomixis could occur in nearly all South African polyploid species of *Hypoxis*. Phytomelan, an inert, brittle, charcoal-like substance rich in carbon, is produced in the seed coats and is a distinctive feature of the Hypoxidaceae due to its thickness. The phytomelan layer develops in the seed of members of the Asparagales within 8 to 44 days after pollination (DAHLGREN and CLIFFORD, 1982).

#### **5.2.3 EMBRYO CULTURE**

Embryo culture is an established *in vitro* technique by means of which complete viable plantlets are obtained directly from excised zygotic embryos and has been practised in

plant breeding for nearly a century. Embryo culture has also been widely used as an aid to decipher and understand the various aspects of embryo morphogenesis (SANDERS and ZIEBUR, 1963; BHOJWANI and RAZDAN, 1983). Using this method excised embryos are individually isolated and germinated *in vitro* to provide one plant per embryo. Embryo culture is often not considered further, however, because it does not result in the rapid regeneration of large numbers of plantlets so characteristic of other micropropagation techniques (GEORGE, 1993).

At the Royal Botanic Gardens, Kew, however, clonal plants for horticultural purposes are regenerated *in vitro* from vegetative explants, but plants propagated for conservation purposes are often regenerated from seeds in order to maintain genetic diversity (FAY and GRATTON, 1992). In this way plants of different genotypes are raised, although the species obtained at Kew using this method were not specifically mentioned. Thereafter more than one plant can be regenerated per seedling from the *in vitro* culture if necessary. Although micropropagation from seed lots is not deemed to be acceptable for clonal propagation, embryo culture should perhaps be more widely considered as a viable *in vitro* method for the mass production of plants for conservation purposes, or to maintain the genetic diversity of medicinal plants until a high yielding variety is identified for further clonal regeneration. There are no records of embryo culture of any of the Hypoxidaceae.

## **5.3 MATERIALS AND METHODS**

### **5.3.1 SELECTION AND PREPARATION OF EXPLANT MATERIAL**

#### **5.3.1.1 Coatless Seeds**

Mature *H. colchicifolia* seeds were collected in late summer (February) from an area adjacent to sites 1 and 2 in Cleland Road, Hayfields (Chapter 3). Viable and non-viable seeds were separated as per the method used for *H. hemerocallidea* (HAMMERTON, 1985). Viable seeds were decontaminated using the protocol outlined in Chapter 4, Section 4.3.2.3, except that in step 5 the seeds were immersed in a 0.1% mercuric chloride solution for 5 minutes instead of sodium hypochlorite, and step 7 was omitted. After the final wash, the decontaminated seeds were immersed in sterile distilled water for one hour to imbibe after which the testa was removed from each seed without damaging the underlying tissues. The coatless seeds (ie. embryo plus endosperm) were inoculated singly onto basal medium in glass tubes (25 mm x 100 mm).

### **5.3.1.2 Excised Zygotic Embryos**

To investigate whether immature or mature zygotic embryos would be the most suitable explant material from which to induce somatic embryogenesis, a range of zygotic embryos were dissected from seed harvested from stock *H. colchicifolia* mother plants from the Botanical Gardens, University of Natal, Pietermaritzburg for use in this study.

The mother plants flowered in spring between September and October. Intact fruit capsules were harvested in summer (November) and separated into 3 broad categories to obtain seeds at 3 different stages of development. The oldest capsules at the base of each inflorescence contained between 24 to 30 seeds (ie. 8 to 10 seeds per locule) with the largest embryos, the intermediate 12 to 15 seeds, and the youngest at the apex between 0 and 6 seeds with the most immature embryos. The intact capsules were surface-decontaminated, imbibed and their testa removed using the same procedure as described for coatless seeds (Section 5.3.1.1). The coatless seeds were washed in sterile distilled water and dried on autoclaved filter paper prior to dissecting out each embryo. Undamaged excised embryos were then more accurately separated into 3 groups using length and developmental stages as criteria as follows:

- 1) globular, immature embryos less than 0.5 mm in length;
- 2) elongated, late globular to intermediate embryos between 0.5 mm and 1.25 mm in length (Plate 9A); and
- 3) linear, mature embryos between 1.25 mm and 2 mm in length.

Excised zygotic embryos were placed horizontally, 5 per Petri dish, on 15 ml of experimental MS basal medium in 65 mm diameter clear plastic Petri dishes (Labotec) which were sealed with Parafilm® before incubation. Each embryo was placed with its micropylar end orientated towards the centre of the dish.

### **5.3.2 CULTURE MEDIA, PROCEDURES AND CONDITIONS**

The revised MS basal medium of MURASHIGE and SKOOG (1962) supplemented with 3% sucrose and 0.01% myo-inositol, solidified with 1% agar (Associated Chemical Enterprises) and adjusted to pH 5.8, was used routinely unless stated to the contrary. All media and culture vessels were autoclaved at 121°C for 20 minutes.

### **5.3.2.1 Coatless Seeds**

#### **5.3.2.1.1 *Initiation of Embryogenic Culture: Plant Growth Regulator Treatments***

The optimum cytokinin : auxin ratio required to initiate somatic embryogenesis was evaluated in a grid trial in which the coatless seed explants were inoculated onto MS basal medium supplemented with a factorial combination of 0, 0.5, 1 and 2 mg l<sup>-1</sup> BA with 0, 1, 2, 3 and 5 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D). In total there were 20 treatment combinations, each with 10 replicates of glass tubes containing 10 ml of medium and inoculated with one coatless seed explant. The tubes were sealed with Parafilm® and placed in a growth room at 25 ± 2°C. Five replicates from each treatment were maintained under a 16 / 8 hour photoperiod with light supplied by Phillips cool white fluorescent tubes at a light intensity of 50 μmol m<sup>-2</sup> s<sup>-1</sup> while the remaining 5 replicates were placed in the dark. Once initiated the cultures were subcultured at 8 week intervals onto MS media with the same original combination of plant growth regulator treatments.

### **5.3.2.2 Excised Zygotic Embryos**

#### **5.3.2.2.1 *Initiation of Embryogenic Culture: Plant Growth Regulator Treatments***

The suitability of immature, intermediate (Plate 9A) and mature zygotic embryos as explants from which to induce somatic embryogenesis and the optimum cytokinin : auxin ratio requirements were determined in a grid trial in which the MS basal medium was supplemented with plant growth regulators in a factorial combination of 0 and 0.5 mg l<sup>-1</sup> BA with 0, 0.5, 1 and 2 mg l<sup>-1</sup> 2,4-D. After autoclaving, 15 ml aliquots of the MS basal media were hot dispensed under aseptic conditions into 65 mm diameter, sterile, clear plastic Petri dishes. Fifteen Petri dishes of medium were prepared for each combination of PGRs tested. The grid consisted of a total of 8 treatment combinations per group of zygotic embryos with 5 Petri dishes of medium allocated to each of the 3 groups.

Each Petri dish contained 5 excised zygotic embryos placed horizontally on the MS medium with their micropylar ends orientated towards the centre of the dish. Due to damage equal numbers of immature, intermediate and mature embryos were not always obtained from the decontaminated seed lots dissected, however, with the result that there were fewer than 5 viable embryos in some of the Petri dishes. Results were, therefore, expressed as a percentage of the total number of undamaged embryos finally inoculated

per treatment. The excised zygotic embryos were incubated at  $25 \pm 2^\circ\text{C}$  in the dark and viewed weekly.

### **5.3.2.2.2 Subculture Treatments**

Embryo Culture: Six weeks after the initial PGR treatments, the germinating zygotic embryos were subcultured to glass tubes containing 10 ml of fresh MS medium supplemented with  $0.5 \text{ mg l}^{-1}$  BA, and transferred from dark to light conditions. The embryos were incubated at  $25 \pm 2^\circ\text{C}$  in the light provided by Phillips cool white fluorescent tubes with a light intensity of  $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$  and a 16 / 8 hour photoperiod. The transfers were subcultured thereafter at 6 week intervals if necessary. Roots were induced on an MS rooting medium without PGRs.

Differentiation of Friable, Embryogenic and Non-embryogenic Callus Cultures: Three months after the zygotic embryos were inoculated onto the initial PGR treatments, primary explants with developing callus were transferred to fresh media containing the PGR combinations of 0 or  $0.5 \text{ mg l}^{-1}$  BA and 0, 0.5 or  $1 \text{ mg l}^{-1}$  2,4-D. The callus cultures were subcultured at 8 week intervals thereafter onto MS media with the same composition as these media. Cultures were maintained in the dark at  $25 \pm 2^\circ\text{C}$  in sealed Petri dishes.

Maturation of Somatic Embryos: Also 3 months after the initial PGR treatments, nodular embryogenic callus with somatic embryos at the proembryo, globular and early cotyledonary stages was transferred to MS maturation medium supplemented with either  $0.5$  or  $1 \text{ mg l}^{-1}$  abscisic acid (ABA) to promote maturation of the somatic embryos. Aqueous stock solutions of ABA were filter sterilized through  $0.22 \mu\text{M}$  Millipore filters under aseptic conditions and added to warm MS media which were then dispensed in 15 ml aliquots into 65 mm diameter sterile Petri dishes. The nodular callus cultures were returned to the dark to proliferate and mature, while those cultures with globular and early cotyledonary stage somatic embryos were immediately placed in the light. The dark incubated cultures were subcultured again after 3 months and at 6 to 8 week intervals thereafter. At each subculture any cultures with maturing somatic embryos were transferred to the same MS medium with ABA but moved from dark conditions to a light regime with a 16 / 8 hour photoperiod and light intensity of  $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ .

Maturation of Malformed Embryo-like Structures: Non-embryogenic nodular callus with malformed embryo-like structures induced on media with both BA and 2,4-D was also subcultured on to maturation medium with ABA as above.

Rooting of Somatic Embryos: Somatic embryos were placed on MS rooting medium without PGRs to induce roots.

Origin and Development of Round Embryo-like Structures: Primary round pseudo-embryonic structures produced from excised zygotic embryos were separated and inoculated individually onto solid MS medium with  $1 \text{ mg l}^{-1}$  2,4-D in 65 mm diameter Petri dishes, incubated in the dark and their development monitored. When fully developed the secondary round structures were subcultured and either returned to MS medium with 2,4-D in the dark, or transferred to MS maturation medium with 0.5 or  $1 \text{ mg l}^{-1}$  ABA and placed under lights with a light intensity of  $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$  and a 16 / 8 hour photoperiod. Rooting was induced on medium without PGRs. Developmental stages were monitored visually and recorded photographically.

Acclimatization of Rooted Zygotic and Somatic Embryos: Rooted zygotic and somatic embryos were acclimatized as per the procedures outlined in Chapter 4, Section 4.3.6.

## 5.4 RESULTS

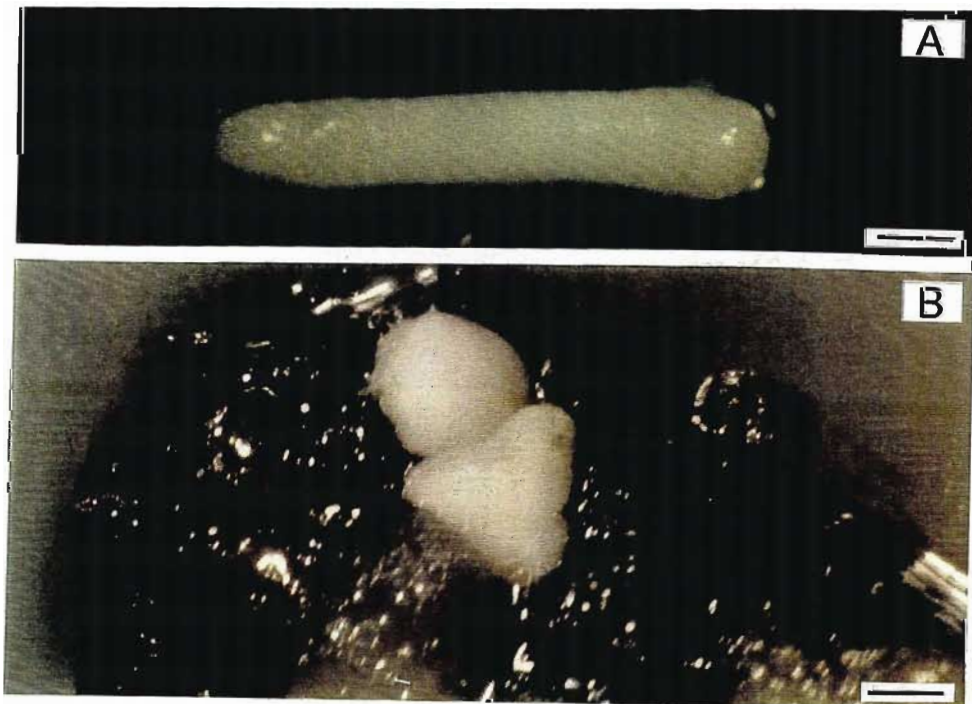
### 5.4.1 COATLESS SEEDS

Contaminants destroyed 52% of the coatless *H. colchicifolia* seed explants within the first 12 days after inoculation. After 4 weeks a further 38.5% of the explants were discarded due to secondary contamination, necrosis and the toxic effects of phenolic leachates and browning. After 8 weeks, 94.5% of the coatless seeds had been discarded. The negative effects of latent contaminants, phenolics and browning which occurred using corm and bud explants were not avoided using coatless seeds as explants. No responses were recorded from the original coatless seed explants placed in the light at the time of inoculation.

However, 3 weeks after inoculation, swelling was observed in the micropylar end of 7 of the remaining coatless seeds maintained in the dark. After 16 weeks in culture a translucent callus had developed on the swollen endosperm of these seeds. These responses were random, being recorded from several treatments regardless of the PGR composition of the MS medium, namely  $0 \text{ mg l}^{-1}$  BA +  $3 \text{ mg l}^{-1}$  2,4-D;  $0.5 \text{ mg l}^{-1}$  BA +  $2 \text{ mg l}^{-1}$  2,4-D; and  $2 \text{ mg l}^{-1}$  BA + 0, 3 or  $5 \text{ mg l}^{-1}$  2,4-D. The endosperm and callus were removed at the next subculture, but further response from the cleaned, swollen seed explants was very slow.

During the next 16 weeks, round structures covered by a translucent mucilage developed within the swollen micropylar ends of 2 of these seed explants which were cultured on MS medium supplemented with  $0.5 \text{ mg l}^{-1}$  BA +  $2 \text{ mg l}^{-1}$  2,4-D. After 14 months in culture individual white embryo-like structures with a few hairs were observed within the cluster of enlarged round structures of one of these final 2 surviving seed explants (Plate 9B). On transfer to the light each embryo-like structure produced a rudimentary radicle with hairs but no shoots. They did not germinate and plantlets were not obtained.

These results, however, show that embryo-like structures can be induced from coatless seed explants. Although this occurred on MS medium supplemented with  $0.5 \text{ mg l}^{-1}$  BA +  $2 \text{ mg l}^{-1}$  2,4-D, the optimum concentration and combination of PGRs required to initiate this response could not be conclusively determined due to the random and low response achieved throughout from coatless seed explants. It would appear, however, that the round structures initially formed, and therefore the embryo-like structures that followed, might have originated from the underlying zygotic embryo because the endosperm and callus had been removed from the explant in the interim. This showed that somatic embryos could possibly be induced from excised zygotic embryos of *H. colchicifolia*. This aspect was therefore investigated.



**Plate 9.** (A) An intermediate size zygotic embryo excised from an *H. colchicifolia* seed showing the slightly swollen micropylar end. (Bar = 0.17 mm) (B) White embryo-like structures with hairs produced within the cluster of enlarged round structures formed in the swollen micropylar end of a coatless seed explant (Bar = 0.83 mm).

## 5.4.2 EXCISED ZYGOTIC EMBRYOS

### 5.4.2.1 Decontamination

The decontamination procedure was successful with 98% of the excised zygotic embryos remaining pathogen free after inoculation. Some of the surviving zygotic embryos were eventually discarded, however, due, not to contamination, but to their complete lack of response *in vitro*.

### 5.4.2.2 Response of Zygotic Embryos to Plant Growth Regulator Treatments

Because only 2% of the undamaged embryos discarded overall was as a result of contamination, for the purposes of this study the total percentage of embryos discarded per treatment for each group was taken to represent the percentage of embryos from which a response *in vitro* was not obtained. Using the results presented in Table 12, therefore, the percentage of embryos discarded per group of excised embryos is indicative of whether the immature, intermediate or mature zygotic embryos are suitable primary explants from which to induce an *in vitro* culture of *H. colchicifolia*.

Results show that 100% of the globular stage immature zygotic embryos less than 0.5 mm in length were discarded and therefore did not respond to any of the treatments in the PGR grid trial. Responses were, however, obtained from both the intermediate, 0.5 mm to 1.25 mm, and mature, 1.25 mm to 2 mm, zygotic embryos, but these varied between treatments. Overall, however, higher percentages of the intermediate embryos were discarded per treatment compared to the mature embryos indicating that the former were less responsive to the *in vitro* treatments than the mature embryos. Results indicated therefore that immature globular stage zygotic embryos are not, but intermediate and mature stage zygotic embryos are suitable as primary explant sources.

The percentage embryos discarded in each treatment as presented in Table 12 can also be used to indicate the effect of the different PGR treatments on the overall responses of the zygotic embryos *in vitro*. In the treatment without PGRs, the high percentages of 86.4% and 56% recorded for the number of intermediate and mature zygotic embryos discarded respectively, show that a low response was obtained from these explants on MS media without PGRs. In all the other treatments, with the exception of one, the percentage of discarded intermediate and mature embryos recorded was 47.6% or less. This would indicate that zygotic embryo response *in vitro* was stimulated in the presence of PGRs. The

**Table 12.** *In vitro* responses, after 12 weeks, of cultured immature globular stage (< 0.5 mm in length), intermediate (0.5 mm to 1.25 mm) and mature (1.25 mm to 2 mm) zygotic embryos of *H. colchicifolia*, to PGR treatments with the factorial combinations of 0 and 0.5 mg l<sup>-1</sup> BA and 0, 0.5, 1 and 2 mg l<sup>-1</sup> 2,4-D. Cultures were maintained in dark conditions. (NEmb = number of undamaged zygotic embryos remaining per treatment, NR = no response, Ger = zygotic embryo germination, C = callus, FC = friable non-embryogenic callus, ENC = embryogenic nodular callus, MNod C = malformed nodular callus, Tot C = total percentage of embryos with callus, Disc = zygotic embryos discarded).

Treatments		BA mg l <sup>-1</sup>					
		0			0.5		
		Excised zygotic embryos			Excised zygotic embryos		
		immature (< 0.5 mm)	intermediate (0.5 to 1.25 mm)	mature (1.25 to 2 mm)	immature (< 0.5 mm)	intermediate (0.5 to 1.25 mm)	mature (1.25 to 2 mm)
2,4-D mg l <sup>-1</sup>	0	10 NEmb NR  100% Disc	22 NEmb 13.6% Ger 0% C 86.4% Disc	25 NEmb 44% Ger 0% C 56% Disc	10 NEmb NR  100% Disc	21 NEmb 33.4% Ger 19% FC 0% ENC 19% Tot C 47.6% Disc	25 NEmb 84% Ger 12% FC 0% ENC 12% Tot C 4% Disc
	0.5	20 NEmb NR  100% Disc	25 NEmb 0% Ger 20% FC 36% ENC 56% Tot C 44% Disc	24 NEmb 0% Ger 54.2% FC 33.3% ENC 87.5% Tot C 12.5% Disc	20 NEmb NR  100% Disc	20 NEmb 0% Ger 50% FC 10% M Nod C 60% Tot C 40% Disc	25 NEmb 4% Ger 80% FC 4% M Nod C 84% Tot C 12% Disc
	1	20 NEmb NR  100% Disc	25 NEmb 0% Ger 20% FC 44% ENC 64% Tot C 36% Disc	25 NEmb 0% Ger 28% 36% ENC 64% Tot C 36% Disc	18 NEmb NR  100% Disc	25 NEmb 0% Ger 48% FC 8% M Nod C 56% Tot C 44% Disc	25 NEmb 0% Ger 72% FC 12% M Nod C 84% Tot C 16% Disc
	2	16 NEmb NR  100% Disc	24 NEmb 0% Ger 0% FC 33.3% ENC 33.3% Tot C 66.7% Disc	23 NEmb 0% Ger 30% FC 13% ENC 43.5% Tot C 56.5% Disc	16 NEmb NR  100% Disc	20 NEmb 0% Ger 65% FC 10% M Nod C 75% Tot C 25% Disc	20 NEmb 0% Ger 65% FC 10% M Nod C 75% Tot C 25% Disc

only exception was the treatment with 2 mg l<sup>-1</sup> 2,4-D in which 66.7% of the intermediate and 56.5% of the mature zygotic embryos were discarded suggesting that embryo response might have been slightly inhibited in the presence of higher concentrations of 2,4-D.

Furthermore results presented in Table 12 and Figure 8 also show the effect of the different PGR treatments on the type of growth response, as well as the percentage and type of callus induced on the intermediate and mature zygotic embryos after 12 weeks in culture. By this stage 13.6% and 33.4% of the intermediate, and 44% and 84% of the mature zygotic embryos had germinated in the treatments without PGRs or supplemented with 0.5 mg l<sup>-1</sup> BA respectively. A small percentage (4%) of mature embryos had also germinated in the treatment with 0.5 mg l<sup>-1</sup> BA + 0.5 mg l<sup>-1</sup> 2,4-D. Embryo germination was not induced in any of the other treatments in this grid trial.

In contrast, callus was not obtained from either group of zygotic embryos in the treatment without PGRs, but was induced in all the other treatments (Figure 8). Overall 52% of the intermediate and 64% of the mature embryos produced callus. Friable non-differentiated callus was the most common type of callus induced, particularly from the treatments in which both BA and 2,4-D were combined. Other developing callus was either non-embryogenic nodular callus with malformed embryo-like structures or embryogenic with nodular callus and potential globular and somatic embryos.

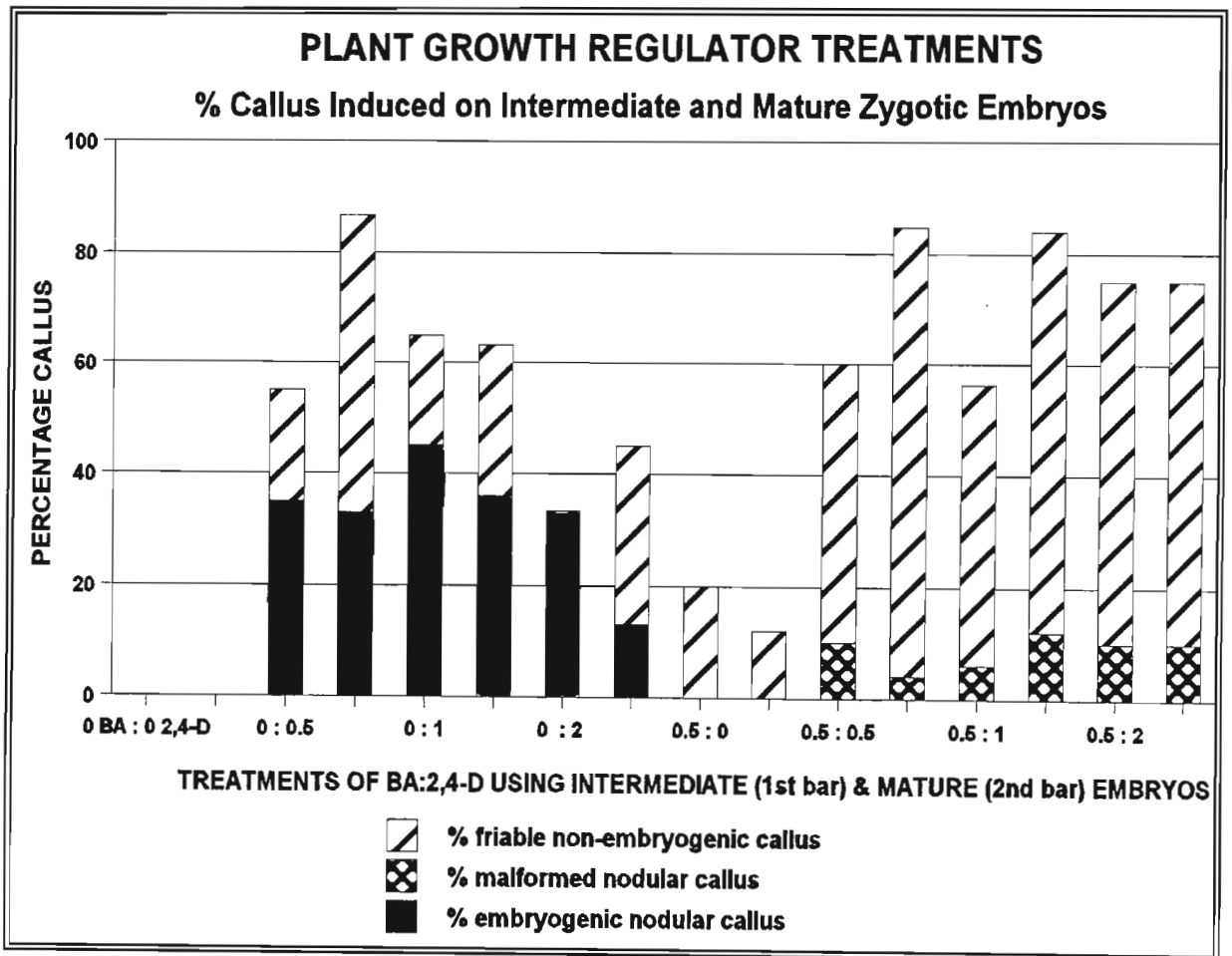
When the individual treatments were examined after 12 weeks in culture, embryogenic nodular callus had been induced on the embryos from the treatments supplemented with 0.5, 1 and 2 mg l<sup>-1</sup> 2,4-D only. This embryogenic nodular callus could also be easily distinguished from the non-embryogenic nodular callus induced in the treatments with the combined supplements of 0.5 mg l<sup>-1</sup> BA + 0.5, 1 and 2 mg l<sup>-1</sup> 2,4-D due to the presence of malformed embryo-like structures in the latter treatments. In the treatment with 0.5 mg l<sup>-1</sup> BA and no auxin, however, neither embryogenic nor malformed nodular callus was produced, only friable non-embryogenic callus (Figure 8). These results clearly show therefore that for *H. colchicifolia*, embryogenic nodular callus can be induced from both intermediate and mature zygotic embryos on MS media containing various concentrations of 2,4-D only.

Results also clearly show that the immature globular stage zygotic embryos less than 0.5 mm in length are a poor explant source as they did not respond *in vitro* at all. Although a higher percentage of mature than intermediate zygotic embryos remained viable and responded *in vitro*, the percentage of embryos from which embryogenic nodular callus was

obtained was not significantly higher for either group. Zygotic embryos longer than 0.5 mm can therefore be used as primary explants from which to induce an embryogenic culture of *H. colchicifolia*. Mature zygotic embryos greater than 1.25 mm, however, were the most suitable explant source from which to induce embryo germination for the direct production of plantlets.

### 5.4.2.3 Development of *In Vitro* Culture Systems

By monitoring the different growth responses of the zygotic embryos during the PGR grid trial, as well as the time frames in which the subsequent developmental and morphological changes were induced, several distinct *in vitro* culture systems were established.



**Figure 8.** The effect of the factorial combinations of 0 and 0.5 mg l<sup>-1</sup> BA with 0, 0.5, 1 and 2 mg l<sup>-1</sup> 2,4-D on the percentage and type of callus induced on intermediate (0.5 to 1.25 mm) and mature (1.25 to 2 mm) zygotic embryos represented by the first and second bar respectively of each pair of bars per treatment.

#### 5.4.2.3.1 *Embryo Culture: Initiation and subculture treatments*

Within 7 days of inoculation, several of the dark incubated intermediate and mature zygotic embryos on MS medium with 0.5 mg l<sup>-1</sup> BA began to swell at their micropylar end. After 21 days in culture the swollen embryos had grown to approximately 5 mm in length and produced hairs on the radicular end. Embryos from the treatment without PGRs also enlarged but developed at a slower rate than those on medium with BA. After 28 days in culture the swollen embryos on MS medium with 0.5 mg l<sup>-1</sup> BA began to germinate followed a week later by those on PGR free medium (Plate 10A a). The germinating embryos produced shoots and roots spontaneously in the light and rooted “seedlings” on PGR free medium after 12 weeks in culture (Plate 10A b). After 6 months 78% of the plantlets produced had survived the acclimatization process. Compared to regeneration via organogenesis the direct production of seedlings *in vitro* via embryo culture proved to be a quick method to propagate *H. colchicifolia* plantlets particularly as the culture was not affected by contamination or browning.

Furthermore, a secondary organogenic callus culture was also generated from the embryo culture (Plate 10B a - c). Callus, first observed at the bases of some of the germinating embryos placed in the light after 8 to 10 weeks in culture (Plate 10B a), proliferated and produced multiple shoots on MS medium with 0.5 mg l<sup>-1</sup> BA (Plate 10B b). After 14 weeks, shoots rooted spontaneously or were rooted on a PGR free MS medium (Plate 10B c). Browning was observed but did not adversely affect the callus culture. Overall 85% of the rooted callus derived plantlets survived acclimatization. These results therefore clearly show that embryo culture in which seedlings are induced either directly from excised zygotic embryos or indirectly from callus, is a viable *in vitro* system for the regeneration of *H. colchicifolia* plants.

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**Plate 10.** Regeneration of *H. colchicifolia* plantlets via embryo culture (A) directly from germinating excised zygotic embryos and (B) indirectly from callus produced from germinating embryos. (A a) Germinating zygotic embryo with first formed leaves (Bar = 0.64 mm). (A b) Rooted “seedling” from zygotic embryo. (B a) Callus and multiple shoots proliferated at base of “seedlings” *in vitro* to produce (B b) an organogenic callus culture from which (B c) plantlets rooted spontaneously.

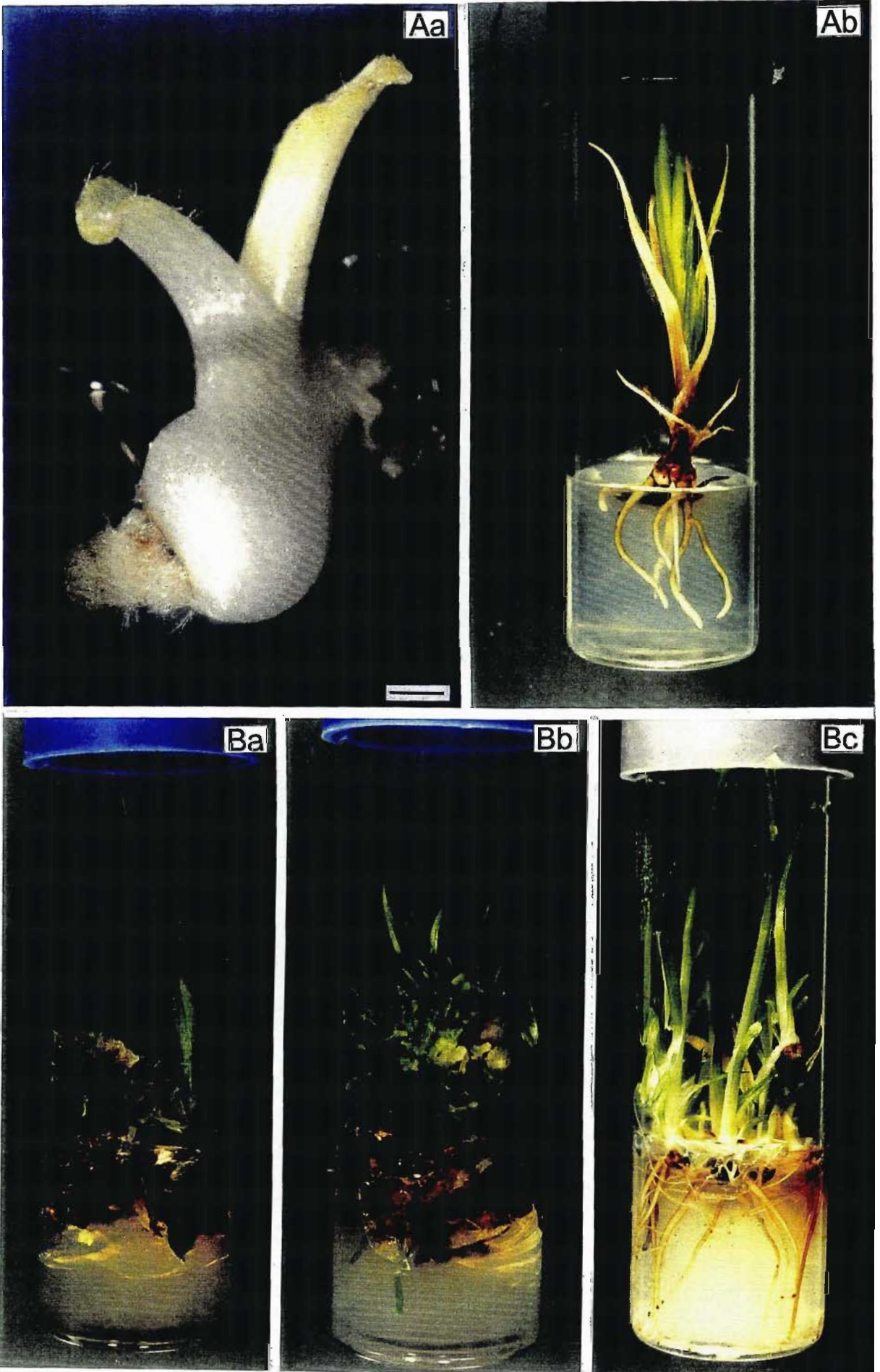


Plate 10

#### 5.4.2.3.2 *Initiation of Callus and Embryogenic Cultures*

In contrast to embryo culture above, the excised zygotic embryos which did not germinate but showed a response *in vitro*, produced callus in one of the following two ways.

- 1) Between 7 and 14 days after inoculation, a translucent friable non-embryogenic callus developed on those embryos which did not produce a nodular type of callus (Table 12 and Figure 8). This callus was produced in all of the treatments in the grid trial in which the MS medium contained 2,4-D and / or BA, but was not induced in the treatment without PGRs. Friable callus was eventually induced on all these embryos and proliferated but did not develop further.
- 2) Within the same time frame a mucilagenous covering developed over the swollen micropylar ends of those embryos (Plate 11A a) from which a nodular type of callus was eventually induced. The nodular type of callus continued to develop along several pathways which were monitored.

Nodular Callus Development: Six weeks after inoculation, clusters of nodular callus extruded through the mucilagenous covering which had become crusty and darker in appearance (Plate 11A b). A translucent friable callus, similar to the non-embryogenic friable callus produced on many of the embryos, had also developed on some of these embryos but at the opposite non-micropylar end.

The effect of the PGRs used in the grid trial on the overall induction of nodular callus are presented in Figure 8 as discussed. Nodular callus was induced on zygotic embryos in all of the treatments in which the MS media contained 2,4-D, either with or without BA, but did

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**Plate 11.** Initiation of (A) somatic and (B) malformed embryos. (A a) Excised zygotic embryo with mucilagenous covering over swollen micropylar end (Bar = 0.32 mm). (A b) Embryo at 6 weeks with proembryonic nodular callus cluster extruded from dark crust at micropylar end, and friable callus at opposite end (Bar = 0.43 mm). (A c) Embryogenic nodular callus with opaque globular and cotyledonary stage somatic embryos. Plumule visible in depression between hypocotyl and cotyledon (Bar = 0.5 mm). (B a) Non-embryogenic nodular callus with malformed embryos (Bar = 0.4 mm). (B b) Shoots produced from malformed embryos confirm their unipolar orientation and organogenic origin (Bar = 1.44 mm).

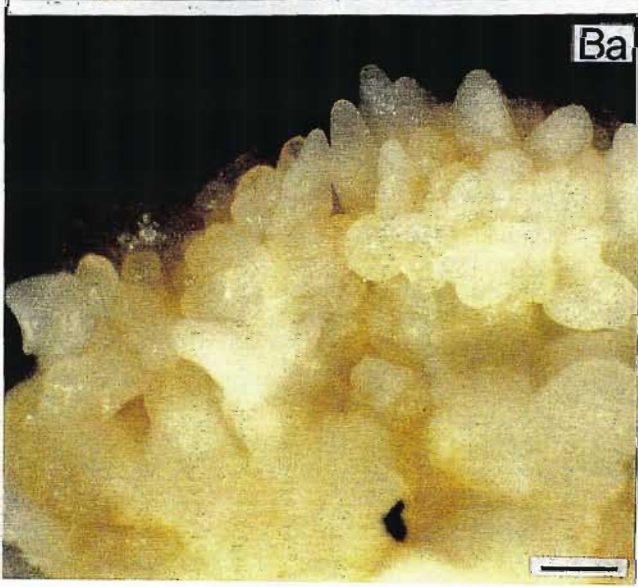
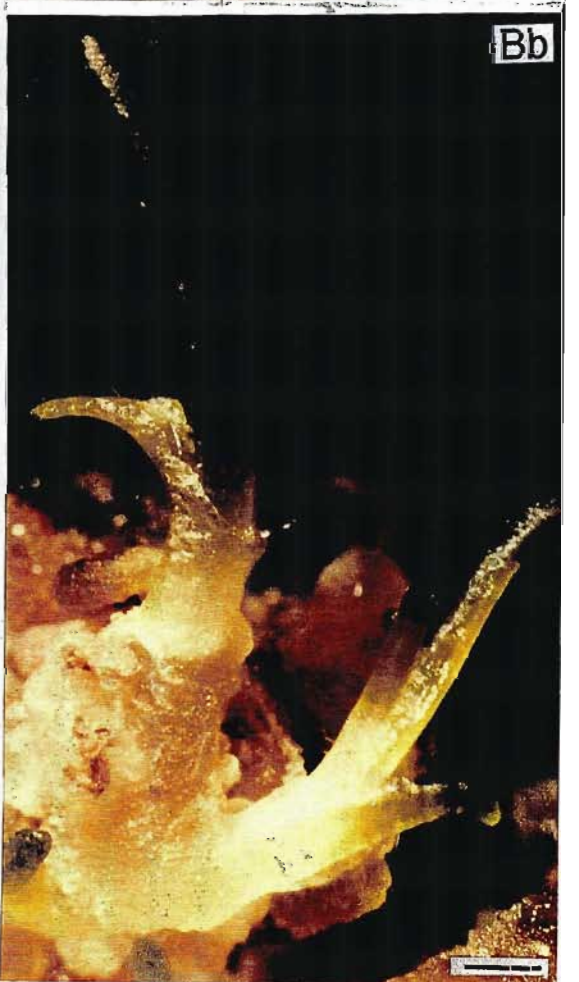


Plate 11

not develop in the treatments without PGRs or with BA only. At an early stage of development the nodular callus was indistinguishable between treatments, but during the tenth week it could be separated into 2 distinct culture systems, namely:

- i) An embryogenic culture in which opaque globular proembryos and cotyledonary stage somatic embryos were evident on the nodular callus which had been induced in the treatments with MS media containing 2,4-D only (Plate 11A c). At this stage the plumule was visible in a depression at the junction of the cotyledon and hypocotyl in a number of the bipolar cotyledonary stage somatic embryos; and
- ii) A non-embryogenic culture in which unipolar malformed embryos were evident on the nodular callus on media containing both 2,4-D and BA (Plate 11B a).

Three months after inoculation these calli were separated into three culture lines, namely the "friable callus", "embryogenic nodular" and "malformed non-embryogenic nodular" cultures.

#### **5.4.2.3.3 Friable Callus Culture: Maturation**

Although the soft friable non-embryogenic callus proliferated on subculturing, neither shoots nor roots were induced regardless of the PGR treatments. It will not be discussed further.

#### **5.4.2.3.4 Malformed Non-embryogenic Culture: Maturation and Rooting**

The malformed embryo-like structures from the non-embryogenic nodular callus turned green when transferred to maturation medium containing ABA and from dark to light conditions. Meristematic regions with green shoot primordia also developed in the callus. Leaves, but no radicles, were produced from the malformed embryos which remained attached to the callus confirming their unipolar orientation and organogenic origin (Plate 11B b). The nodular callus induced in treatments with media supplemented with both 2,4-D and BA was therefore non-embryogenic. Since the culture developed was organogenic and the malformed embryos were not bipolar, roots were induced on rooting medium 7 months after inoculation. On average 85% of the plantlets survived acclimatization proving this to be a successful *in vitro* method by means of which to regenerate *H. colchicifolia* plants.

#### 5.4.2.3.5 **Embryogenic Culture: Maturation and Rooting of Somatic Embryos**

Four weeks after nodular embryogenic callus with globular proembryos and cotyledonary stage somatic embryos was transferred to MS maturation medium supplemented with either 0.5 or 1 mg l<sup>-1</sup> ABA and placed in the light, the cotyledonary stage somatic embryos had turned green and meristemoid callus with green primordia had developed (Plate 12A a). Clusters of white hairs were also produced among the callus. The bipolar nature of the somatic embryos was evident (12A b). As the cotyledonary stage somatic embryos matured plumules emerged from the depression at the junction of the cotyledon and hypocotyl (Plate 12A c) and radicles were produced (Plate 12A d). At this stage the germinating bipolar somatic embryos were easily dislodged from the culture.

Germinating somatic embryos (Plate 12A c, A d) developed into “seedlings” (Plate 12B a) on transfer to MS rooting medium without PGRs. Rooted plantlets were produced within 6 to 8 weeks (Plate 12B b). An average of 84% of these plantlets survived acclimatization.

Due to the small quantity and heterogeneous nature of the embryogenic cultures established, factors often encountered during *in vitro* studies, it was difficult to quantitatively assess or calculate the numbers of somatic embryos, at the same stage of development, present at any given time. Overall observations of the embryogenic culture showed, however, that although authentic bipolar somatic embryos proliferated in culture they were produced in small numbers when the culture was maintained on solid MS medium with 2,4-D. In addition, somatic embryos already present matured on transfer to maturation medium with ABA, but globular proembryos ceased to proliferate and additional somatic embryos were not induced. Instead root-like protuberances with hairs developed under both dark (Plate 13A a) and light (Plate 13A b) conditions (ie on media with ABA).

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**Plate 12.** Embryogenic Culture - (A) Maturation and (B) Rooting of Somatic Embryos. On MS maturation medium and in the light, (A a) meristemoid callus was produced and the cotyledonary stage somatic embryos turned green (Bar = 0.71 mm), (A b) the bipolar somatic embryos were easily dislodged (Bar = 0.3 mm), and (A c) the plumule (Bar = 0.41 mm) and (A d) radicle began to emerge (Bar = 0.36 mm). On MS rooting medium, (B a) the radicle and first leaf of the “seedling” elongated (Bar = 1 mm), and (B b) rooted plantlets were obtained.

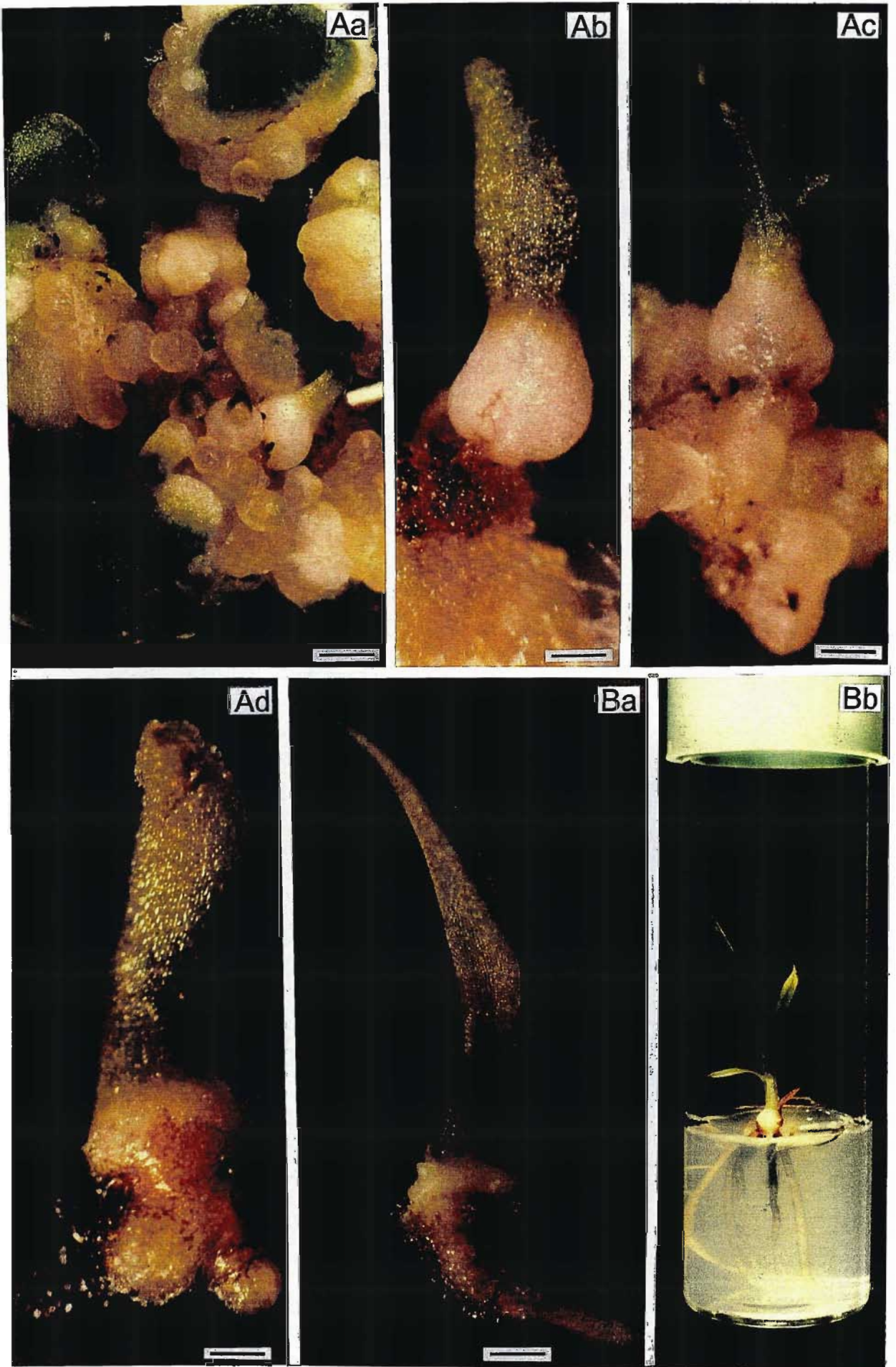


Plate 12

**Table 13.** Response of the *H. colchicifolia* embryogenic nodular callus cultures with somatic embryos to treatments in which the MS basal medium was supplemented with a factorial combination of 0 and 0.5 mg l<sup>-1</sup> BA with 0, 0.5 and 1 mg l<sup>-1</sup> ABA. (ENC = embryogenic nodular callus)

	Treatments	BA mg l <sup>-1</sup>	
		0	0.5
ABA mg l <sup>-1</sup>	0	ENC proliferation inhibited. Germinating somatic embryos and plantlets rooted spontaneously.	ENC has become organogenic with apical meristemoids. Multiple shoots and roots induced. Maturation of all somatic embryos inhibited.
	0.5	ENC remained embryogenic initially. Shoot development inhibited. Hairy protruberances produced. Maturation of somatic embryos not inhibited but slow.	ENC has become organogenic. Shoot development promoted. No hairy protruberances produced. Somatic embryo maturation slow. Maturation of young somatic embryos inhibited.
	1	ENC remained embryogenic. Shoot development inhibited. Hairy protruberances produced. Maturation of somatic embryos not inhibited and rapid.	ENC now partially organogenic with meristemoids. Long shoots induced. Hairy protruberances produced. Somatic embryo maturation slow. Maturation of young somatic embryos inhibited.

#### 5.4.2.3.6 *Origin and Development of Hairy Root-like Protuberances*

The effect of ABA, with BA as a control, on the embryogenic potential of the nodular callus and the formation of these hairy root-like protuberances was therefore examined in a grid trial in which the MS basal medium was supplemented with a factorial combination of 0 and 0.5 mg l<sup>-1</sup> BA with 0, 0.5 and 1 mg l<sup>-1</sup> ABA. Several interesting aspects (Table 13) emerged from this trial, the most notable being the ease with which the embryogenic callus became organogenic with green meristematic tissue and multiple shoots and roots, when exposed to BA regardless of the presence of ABA or not. In the treatments with ABA only, however, bipolar somatic embryos already present developed to maturity but new somatic embryos were not produced. These results indicated therefore that ABA at concentrations of 0.5 and 1 mg l<sup>-1</sup>, appears to promote not only the maturation of somatic embryos already present in the nodular callus but also the production of large hairy root-like protuberances, which

dwarf the somatic embryos. At these concentrations of ABA and without the auxin, 2,4-D, the initiation of new embryos, shoots and roots was also inhibited and the embryogenic potential of the nodular callus appeared to be diminished.

To determine the origin of the hairy root-like protuberances (Plate 13A a, b), the sequential stages of their development on ABA supplemented media were monitored. Initially round structures, transferred to maturation medium with the embryogenic nodular callus clusters, were observed to enlarge and produce hairy protuberances which dwarfed the somatic embryos (Plate 13B a). When these round pseudoembryonic structures (Plate 13B a) were dissected out and cultured separately, they appeared to “germinate”. The hairy protuberances elongated to form a radicle followed sequentially by leaves (Plate 13B b). These large structures developed into plantlets on PGR free rooting medium (Plate 13B c), 85% of which survived acclimatization.

These results have shown that two different somatic embryo-like structures capable of independent germination on maturation media supplemented with ABA were ultimately obtained from excised zygotic embryos inoculated originally on media with 2,4-D. These are the bipolar somatic embryos which originated from the embryogenic nodular callus and the large round pseudoembryonic structures of unknown origin from which hairy root-like protuberances emerged and which were also always associated with embryogenic nodular callus.

#### **5.4.2.3.7      *Origin and Development of the Round Pseudoembryonic Structures***

To determine the origin and development of the round pseudoembryonic structures which produced the hairy protruberances, the swollen micropylar ends of 10-week-old zygotic embryos with a dark crusty covering and embryogenic nodular callus, were examined. As the ends enlarged they had divided to produce round structures imbedded in mucilage.

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**Plate 13.**      Origin and development of (A) large hairy protuberances on MS maturation medium under (A a) dark (Bar = 1 mm) and (A b) light conditions (Bar = 1.25 mm). (B) These were observed to develop from (B a) the single round pseudoembryonic structures which dwarfed the somatic embryos (Bar = 1 mm) and (B b) appeared to “germinate” to form a radicle and leaves (Bar = 0.85 mm) and finally (B c) rooted plantlets.

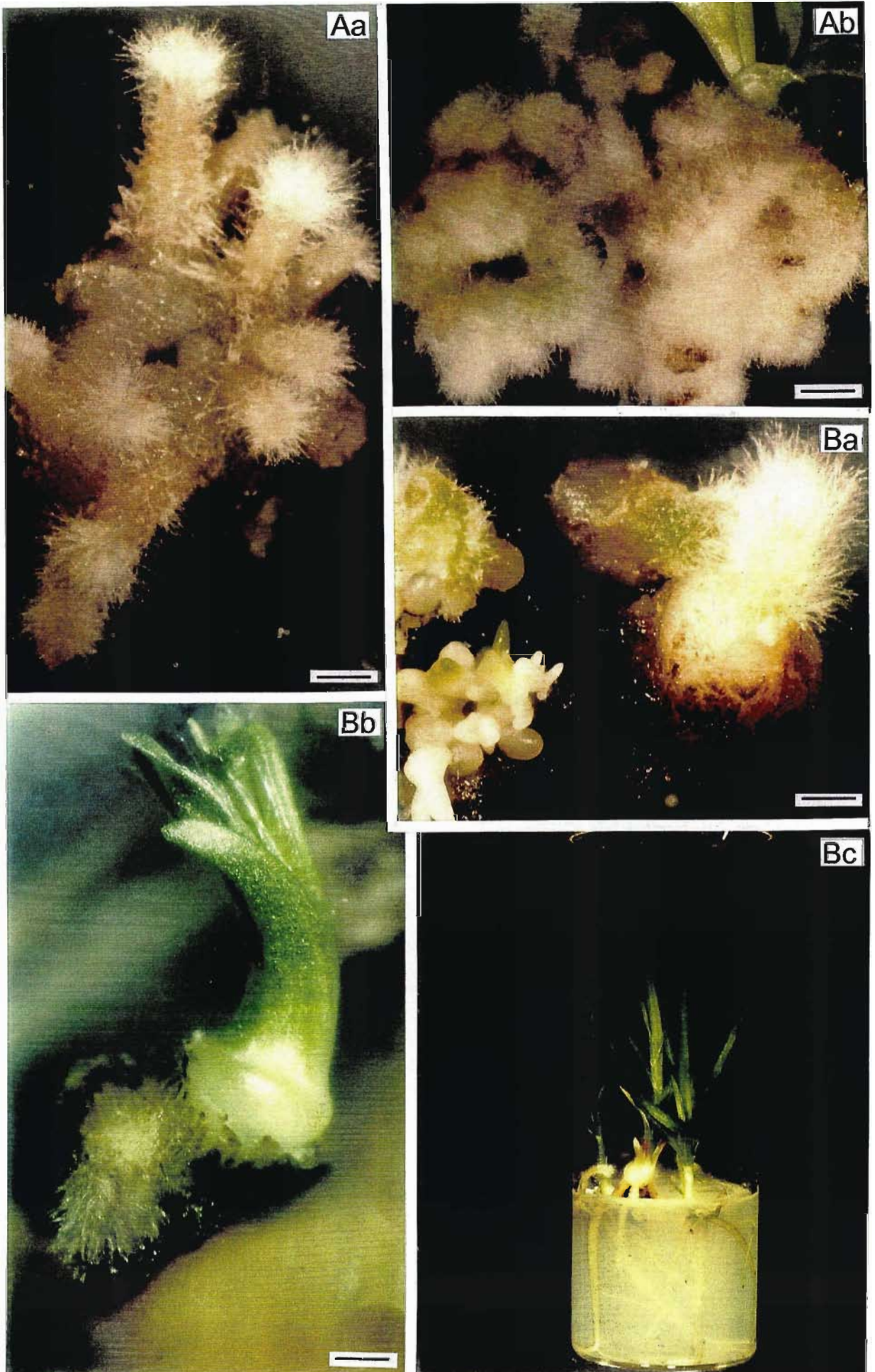


Plate 13

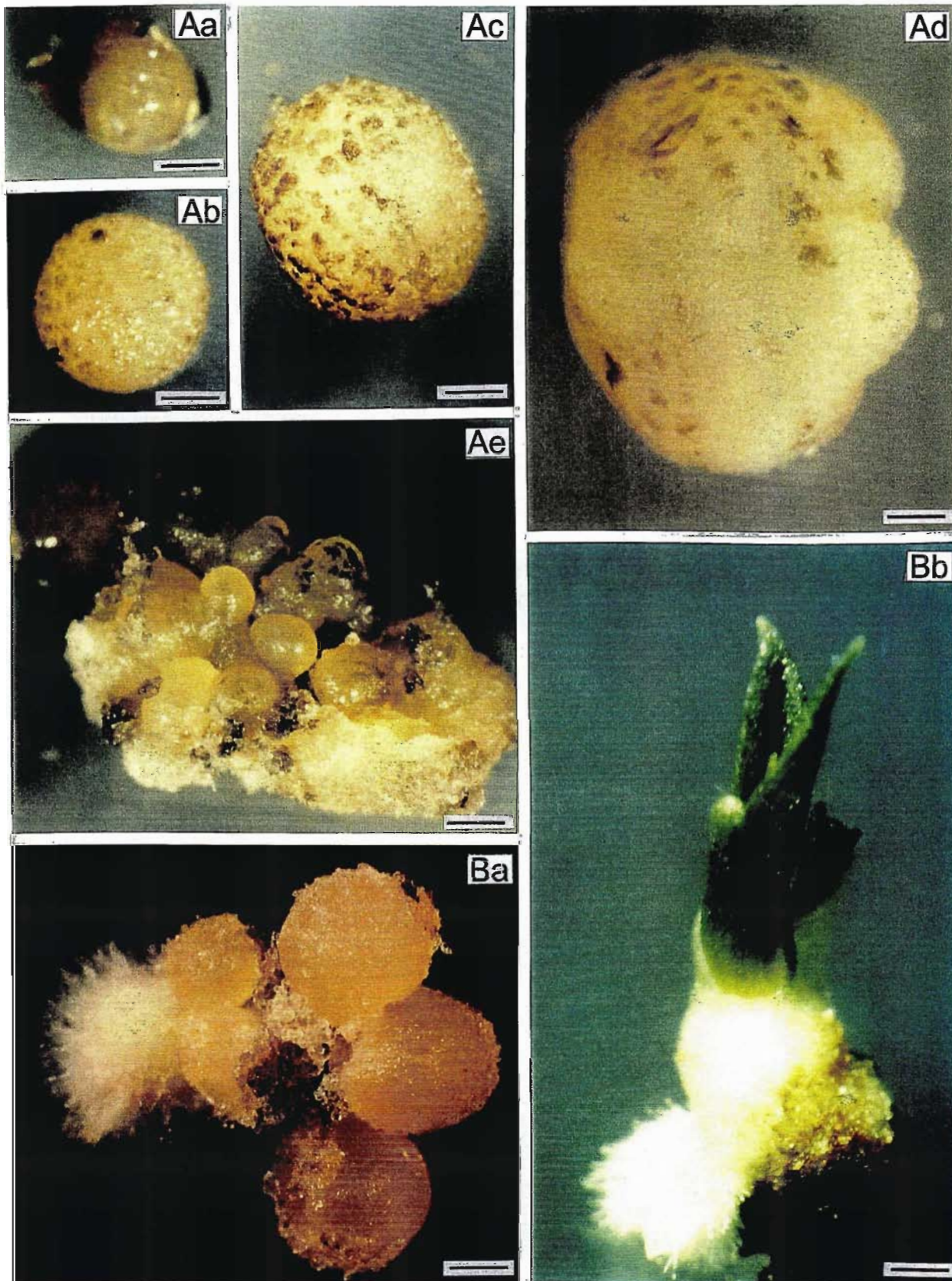
Cultured separately these primary round structures (Plate 14A a) initially developed a crusty covering (Plate 14A b, c) and then divided (Plate 14A d) to produce a number of smaller, independent secondary round structures which were exposed when the crusty covering was removed (Plate 14A d). In turn these secondary round structures, subcultured individually or in small groups onto MS maturation medium supplemented with 0.5 or 1 mg l<sup>-1</sup> ABA and placed in the light, produced a hairy protuberance (Plate 14B a) as originally observed (refer Plate 13B a). On transfer to PGR free medium these structures then “germinated” to produce a plant (Plate 14B b).

#### **5.4.2.3.8 Confirmation of Development of Round Pseudoembryonic Structures**

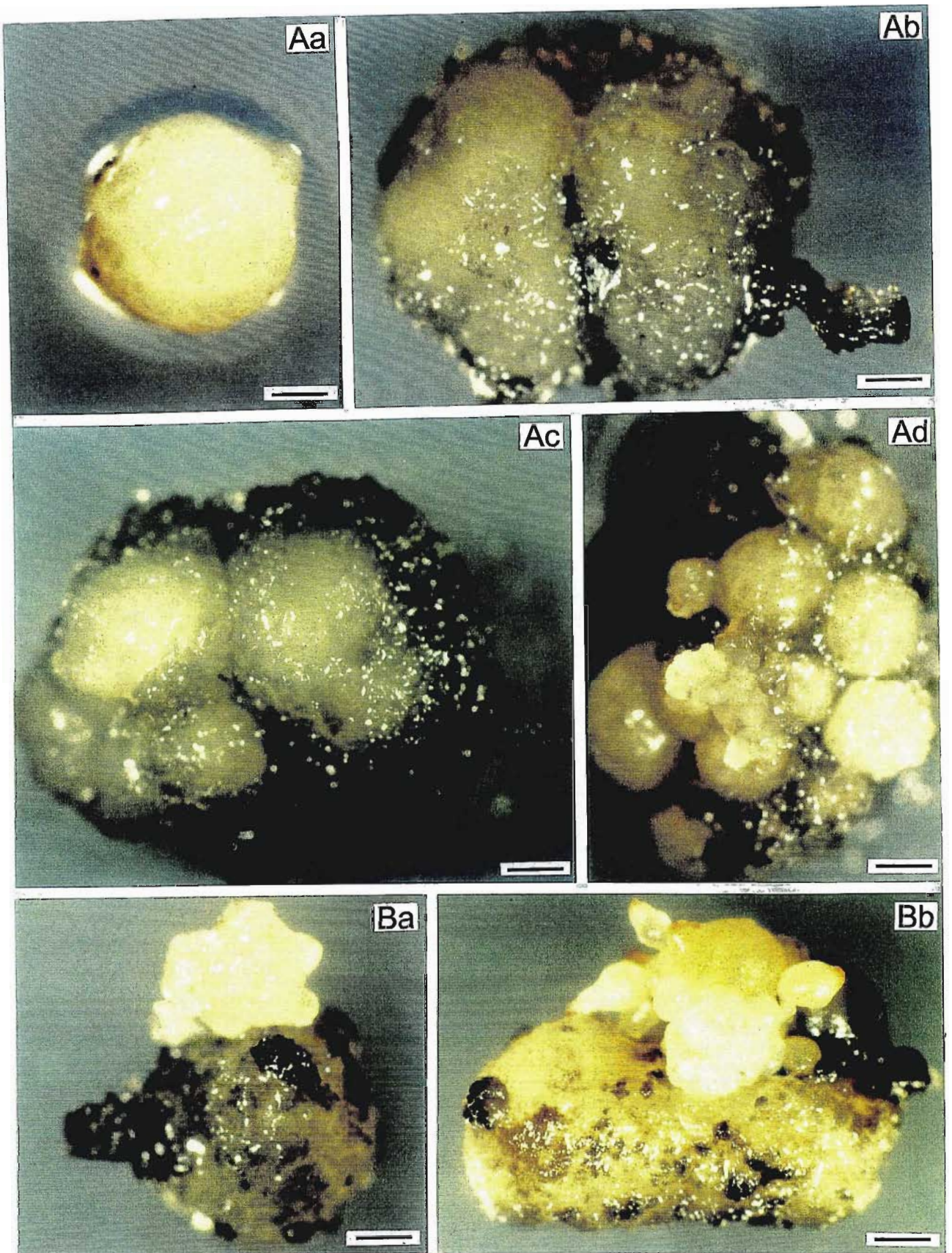
To confirm the developmental stages just described, primary round structures were again dissected from the swollen micropylar end of zygotic embryos, placed individually onto MS medium supplemented with 1 mg l<sup>-1</sup> 2,4-D and incubated in the dark. Each primary round structure (Plate 15A a) developed a dark crusty covering which was removed to facilitate monitoring the next developmental stages shown to be cleavage (Plate 15A b), and repeated subdivisions (Plate 15A c) to ultimately form secondary round structures imbedded in mucilage (Plate 15A d). These would normally have been in a cluster covered by a crusty layer, but were exposed in this case as the crusty covering had been removed.

Individual secondary round structures removed from a cluster, placed back onto MS medium with 2,4-D and incubated in the dark, in turn formed a crusty covering and divided as per the sequence described for the primary round structures. Other secondary round structures, however, produced clusters of embryogenic nodular callus (Plate 15B a) from which bipolar somatic embryos were produced (Plate 15B b). By repeating these procedures at each subculture, both the embryogenic culture and production of somatic embryos could be scaled-up and formed the basis for the development of a micropropagation system via embryogenesis.

At the same time, however, a number of the secondary round structures were subcultured individually onto MS maturation medium supplemented with 0.5 or 1 mg l<sup>-1</sup> ABA and placed in the light, and these showed a different pattern of development. Some “germinated” to produce a large protuberance or radicle covered in white hairs as already demonstrated (Plate 14B a). Others, however, elongated to produce long root-like structures (Plate 16A) which did not develop further until transferred to medium without PGRs or with BA only and incubated under lights. After 4 weeks callus (Plate 16B a) and / or somatic embryo-like structures (Plate 16B b) developed at the apical end of these root-like structures. These



**Plate 14.** Development of round pseudoembryonic structures. (A) On MS medium with 2,4-D in dark (A a) primary round structures from swollen zygotic embryos (Bar = 1 mm) (A b, c) developed a crusty covering (Bar = 1 mm) and (A d) divided (Bar = 0.64 mm) to (A e) form individual secondary round structures (Bar = 2 mm) which (B) produced (B a) a hairy protuberance on medium with ABA (Bar = 1 mm) and (B b) a plantlet on PGR free medium (Bar = 0.7 mm).



**Plate 15.** Developmental stages (A) were confirmed by monitoring (A a) a primary round structure (Bar = 0.37 mm) which (A b) divided by cleavage (Bar = 0.46 mm) and (A c) repeated divisions (Bar = 0.55 mm) to (A d) produce individual secondary round structures (Bar = 0.75 mm) which (B a) produced embryogenic nodular callus clusters (Bar = 0.41 mm) and (B b) bipolar somatic embryos (Bar = 0.5 mm).

embryo-like structures were not authentic somatic embryos, however, as they did not “germinate” along a bipolar axis (Plate 16B c) and the formation of twinned or multiple plantlets was not an uncommon feature. Approximately 80% of these plantlets, rooted on PGR free medium (Plate 16B d), survived acclimatization.

## 5.5 OVERALL DISCUSSION AND CONCLUSION

While the main objective of the current study is to develop a micropropagation technique for the large scale regeneration of *H. colchicifolia* plantlets, *in vitro* somatic embryogenesis has not been reported for *Hypoxis* before and merits further discussion.

Generally the factors controlling embryogenesis and organ regeneration are not known. The most important factor contributing to the success or failure to induce somatic embryogenesis in monocotyledons, however, has been attributed to explant choice. It has been argued that very young tissues and immature zygotic embryos are ideal explants because the cells of monocotyledons differentiate early and rapidly lose their mitotic and morphogenetic ability thereafter (KRISHNARAJ and VASIL, 1995). Somatic embryos have been induced *in vitro* from immature zygotic embryos of oil palm (TEIXEIRA *et al.*, 1993) and immature inflorescences for many species of Gramineae (VASIL and VASIL, 1991), but what defines immaturity is unclear. Results from the present study show, however, that both coatless seeds and excised globular stage or “immature” zygotic embryos < 0.5 mm long are unsatisfactory explants from which to induce an embryogenic culture of *H. colchicifolia*. On the other hand, round “pseudoembryonic” structures and authentic bipolar somatic embryos induced via embryogenic nodular callus, were obtained from excised intermediate and mature stage zygotic embryos between 0.5 and 2.0 mm in length.

Although the stage of development of the explant was important for the induction of somatic embryogenesis, results also showed that the type of PGR added to the nutrient medium ultimately determined the nature of the response induced from the zygotic embryos of *H. colchicifolia*. The presence of the auxin, 2,4-D, alone was essential for the induction of embryogenic callus, somatic embryos and maintenance of the embryogenic

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**Plate 16.** Some of the secondary round structures on medium with ABA elongated to form (A) root-like structures (Bar = 1 mm) which (B) on PGR free medium produced at the apical end (B a) callus (Bar = 0.83 mm) and / or (B b) somatic embryo-like structures (Bar = 0.6 mm) which (B c) “germinated” (Bar = 1.3 mm) to produce (B d) rooted plantlets.

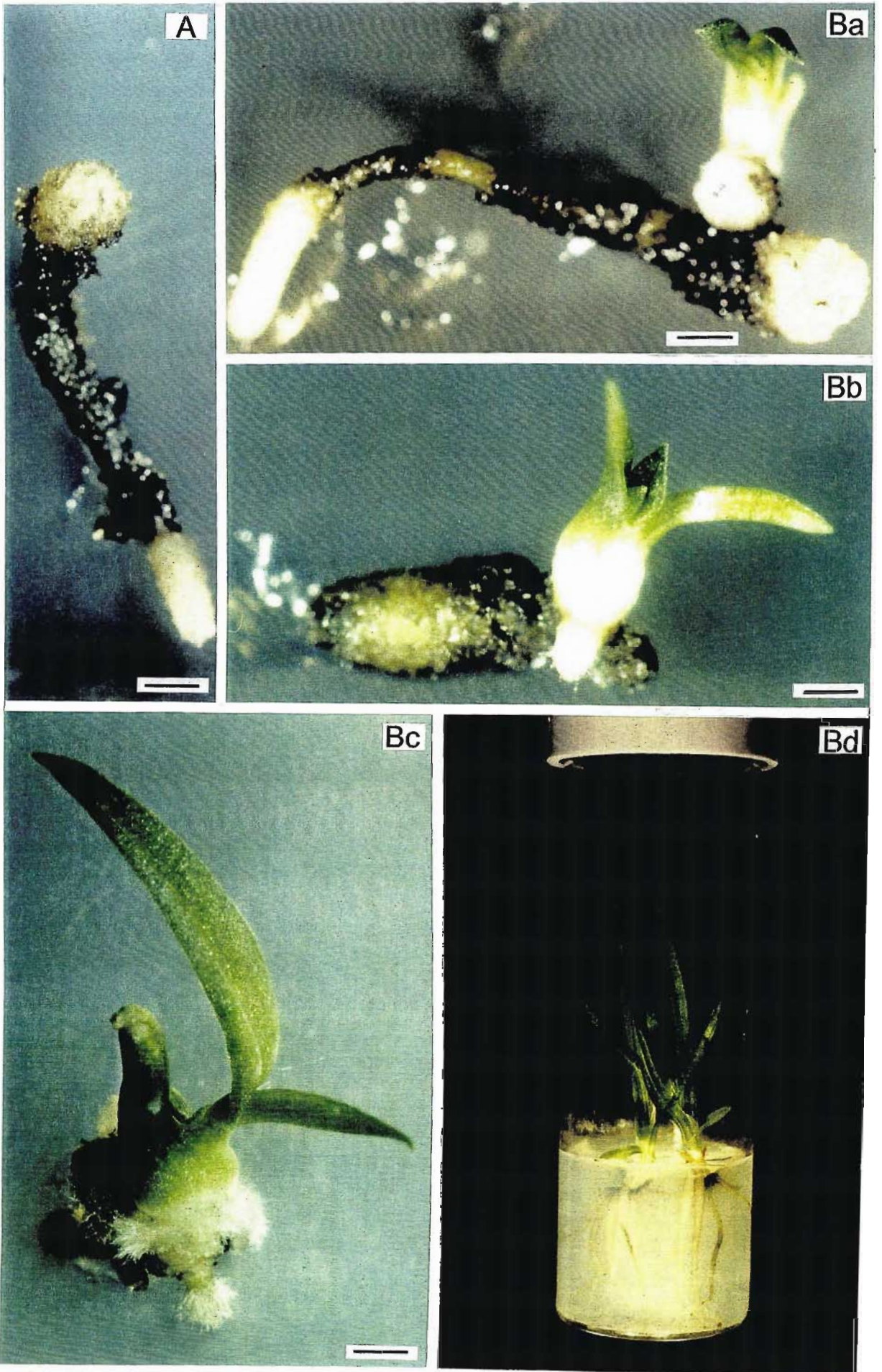


Plate 16

culture, whereas the presence of the cytokinin, BA, either without or in conjunction with 2,4-D, resulted in the induction of non-embryogenic callus and malformed embryos. In cultures without PGRs or BA only, however, germination was induced in up to 84% of the mature zygotic embryos.

The important role of exogenously applied PGRs in the induction and regulation of the processes of embryogenesis in these cultures was further emphasized when the addition of ABA to the medium was shown to be essential in the final development or maturation of the authentic somatic embryos, but inhibitory to the further development of early stage proembryos in the culture. The presence of ABA also promoted the precocious germination of the large round primary and secondary pseudoembryonic structures shown to be associated with the embryogenic nodular callus, as well as their development into root-like structures. Furthermore the final developmental stage was induced and phenotypically normal rooted plantlets obtained when all PGRs were removed from the medium.

The non-identification of factors controlling organ regeneration has also been attributed to a lack of published histological information showing whether preexisting primordia in the tissue were induced to develop when the PGR was removed or added to the medium, or whether new primordia were initiated by the treatment (HALPERIN, 1995). To histologically follow the development of a single primordium is impossible, however, although many histological studies have been published showing globular stage somatic embryos formed directly from epidermal cells, for example *yanhusuo* (*Corydalis yanhusuo*), a tuberous Chinese medicinal herb, (KUO *et al.*, 2002). In many studies, however, the effect of added PGRs on the induction and development of somatic embryos has been determined visually by monitoring morphological changes using techniques such as transfer of organs between liquid and solid media (TORRES *et al.*, 2001) and cell tracking (TOONEN *et al.*, 1996).

In the present study it is also significant that a translucent friable non-embryogenic callus was induced initially from both the intermediate and mature zygotic embryos in all of the induction treatments with added PGRs regardless of their combination. Since callus was not induced in any of the PGR free treatments, it would appear that the response was initiated only in the presence of PGRs. Although absolute evidence is lacking these results indicate that during the induction of embryogenesis some cells must have remained unresponsive to the PGRs (2,4-D) which induced embryogenesis in other cells. It could be argued therefore that certain vegetative cells of *H. colchicifolia* zygotic embryos are neither preembryonically determined cells (PEDC) nor have the capacity to become embryogenic,

but differentiate to produce non-embryogenic friable callus in the presence of the PGRs used in the treatments in this study.

A recent suggestion is that the mode of action of the PGRs in the induction and regulation of embryogenesis, may be through the regulation of certain genes. For ABA, these genes would be involved in the desiccation and maturation phases of somatic embryogenesis (JIMÉNEZ, 2001). From literature reviewed it was also concluded that the heterogeneous nature of *in vitro* cultures can be explained on the basis that different classes of mutant forms are arrested at different stages of embryo development and that this is under genetic control. Although many of the mechanisms controlling cell differentiation and the processes of embryogenesis are still unclear, genetic analysis of unnamed *in vitro* embryo mutants led to the conclusion that the three basic phases of embryo development, namely pattern formation, morphogenesis and cytodifferentiation, are regulated independently (VON ARNOLD *et al.*, 2002).

It was therefore suggested that by constructing fate maps, in which an adequate number of known morphological and molecular markers specifying critical stages in somatic embryo development are represented, a model of the process of somatic embryogenesis could be created for each plant species investigated (VON ARNOLD *et al.*, 2002). Fate maps could then be used to identify factors controlling organ regeneration and somatic embryogenesis. Defined protocols for the different phases of *in vitro* somatic embryogenesis are not readily available for monocotyledons, however (TEIXEIRA *et al.*, 1993). Neither are photographic records of the morphological changes of the embryogenic tissues coinciding with these phases. Similarly records of the regeneration of plantlets from somatic embryogenic cultures of monocotyledons are sparse as listed in the review by KRISHNARAJ and VASIL (1995). Therefore the establishment of defined protocols for each species studied in which all the phases of *in vitro* somatic embryogenesis up to plantlet regeneration, as well as the morphological changes of all the components are outlined, would assist not only in the construction of fate maps, but in the identification of relevant molecular markers.

While developing protocols in the present study, the process of somatic embryogenesis from excised zygotic embryos of *H. colchicifolia* was shown to be complex. The *in vitro* cultures established were also heterogeneous because the differentiation of the component cells and tissues was not uniformly regulated. By carefully subdividing and monitoring the stages of development of the individual structures produced, however, the origin of all the embryo-like structures formed in the embryogenic cultures could be traced back to either the large round primary pseudoembryonic structures induced in the swollen

micropylar ends of the zygotic embryos or those induced in the secondary and later cycles. Repetitive cycles of embryogenic tissue were also obtained from which a continuously regenerating system was produced. Phenotypically normal plantlets which survived acclimatization were regenerated from the embryo-like structures whichever developmental pathway was followed.

The many anomalous embryo-like structures observed in embryogenic cultures, variously described as embryoids, pseudo-embryos, daughter structures and neomorphs, have been collectively termed embryo mutants (VON ARNOLD *et al.*, 2002). However, by revisiting the publications on neomorphs by Waris in 1958, KRIKORIAN and SIMOLA (1999) stated that the “new” plant forms Waris described were not mutants and that “We now know beyond doubt that neomorphs are somatic embryos formed under conditions that are not optimal for normal development”. Proof of this statement was not provided at the time. The large root-like structures produced in the embryogenic cultures of *H. colchicifolia* (Plate 16), however, are identical to the structures in photographs published of not only Waris’ neomorphs (KRIKORIAN and SIMOLA, 1999), but also the root-like carrot structures (STEWART *et al.*, 1958b), free-floating plantlets from *Atropa* suspension cultures (THOMAS and DAVEY, 1975), the pseudobulbils from embryogenic cultures of *Citrus sinensis* (BUTTON *et al.*, 1974), and the abnormal structures termed neomorphs produced in suspension cultures of daylily (KRIKORIAN and KANN, 1981). Unusual structures, morphologically different from the original, these could also be equated with “neomorphs”.

Although quantitative evidence is lacking, the developmental phases determined for the root-like structures or neomorphs in the present study and the photographic evidence can, however, provide proof of the above statements. Not only was their origin conclusively traced back to the large round primary pseudoembryonic structures induced in the swollen micropylar ends of the zygotic embryos, but these root-like structures were induced when the large round pseudoembryonic structures were prematurely exposed to ABA, which is now known to act through the regulation of certain genes controlling the different stages of development of somatic embryos. A similar mode of action was observed if the large round pseudoembryonic structures were transferred to ABA supplemented media at a slightly later stage of development and precocious germination was induced. It would therefore appear that the round “pseudoembryonic” structures induced in the embryogenic cultures of *H. colchicifolia* could in fact be somatic embryos from which somatic embryogenesis was directly induced.

The induction of similar round structures, proembryoids or spherical pseudobulbils, in nucellar isolates of the Washington navel orange was reported by BUTTON and BORNMAN (1971). In a later study embryogenesis was induced from ovules of the Shamouti orange from single cells on the periphery and within these proembryoids (BUTTON *et al.*, 1974). These proembryoids, surrounded by greatly thickened cell walls, were shown to divide to give rise to globular proembryoids which were freed from the thick walls as they grew and the walls degenerated. The pseudobulbils developed later from enlarged proembryoids of *Citrus*. These phases as described are almost identical to the highly organised stages of development recorded for *H. colchicifolia* and support the suggestion that two types of somatic embryos are induced in embryogenic cultures of *H. colchicifolia*, namely the large globular primary embryos ("single rounds") and small bipolar secondary embryos.

The successful induction of somatic embryogenesis in *Citrus* species is often presented as a unique embryogenic system of regeneration and is attributed to their inherent ability to produce more than one embryo per seed, or polyembryony. These adventive embryos are produced from nucellar tissue adjacent to the developing zygotic embryo (DUNSTAN *et al.*, 1995). Bi-nucleate, twin embryo-sacs of nucellar origin are also commonly found in the large *Hypoxis* species, *H. hemerocallidea* and *H. costata* (WILSENACH and WARREN, 1967). Apomixis also often occurs in *Hypoxis* species (ZIMUDZI, 1994 b). Twinned plantlets were also often obtained from embryos produced from the embryogenic cultures of *H. colchicifolia* in this study. Although twin-embryo sacs have not been demonstrated or observed for *H. colchicifolia*, these features could explain the formation of the large primary globular somatic or "adventive" embryos during induction of the embryogenic culture and the similarities observed between the developmental pathways of somatic embryogenesis in *Citrus* and *H. colchicifolia*.

Furthermore, in the embryogenic *Citrus* cultures, the large round proembryoids were shown to have greatly thickened cell walls. In the *H. colchicifolia* cultures, a mucilaginous covering developed around the primary pseudoembryonic structures in a similar manner. With time, however, this covering became dark, often black, and crusty in appearance. In the Hypoxidaceae a very thick phytomelan crust is produced around developing seeds *in vivo* within 8 to 44 days after pollination (DAHLGREN and CLIFFORD, 1982). It is suggested therefore that the black crust formed *in vitro* around the pseudoembryonic structures produced by cleavage in the micropylar ends of the excised zygotic embryos might be, or emulate, the initial formation of a phytomelan crust.

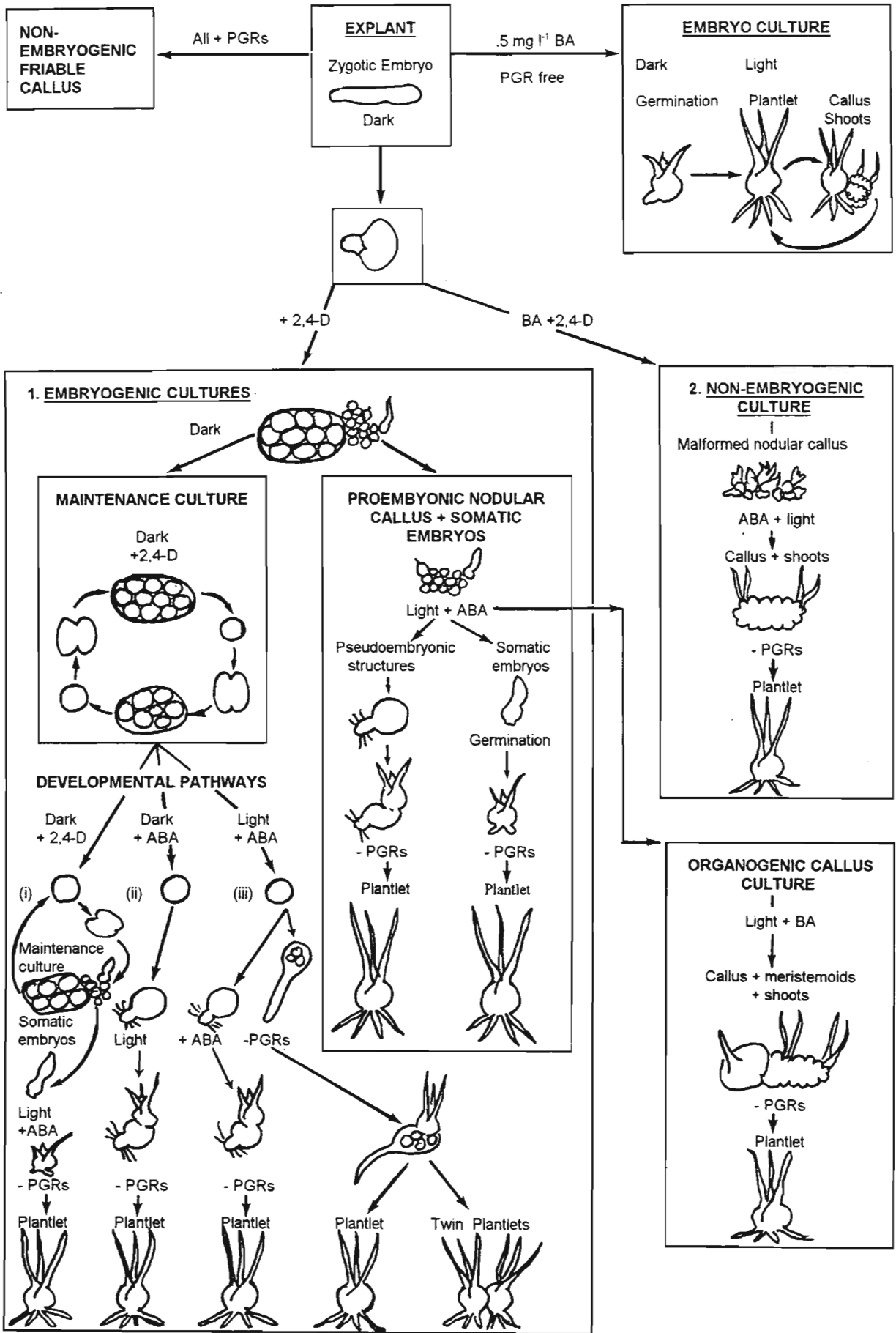
## 5.6 SUMMARY

As a result of the highly organised stages of development obtained for *H. colchicifolia*, regeneration protocols could be developed from not only the embryogenic tissues but all of the tissues and organs induced from the zygotic embryos on solid nutrient media. The ease with which the embryogenic cultures became organogenic once transferred from maintenance medium also resulted in highly regenerative callus cultures. Distinct micropropagation systems were therefore established via embryo culture, organogenesis and somatic embryogenesis from zygotic embryo explants. Phenotypically normal plantlets were recovered from all of the cultures.

A diagrammatic overview of the micropropagation protocols developed for *H. colchicifolia* from zygotic embryo explants showing these pathways, as illustrated in Plates 9 to 16, is presented in Figure 9. This clearly shows how the different pathways are interlinked and can be manipulated by the addition or removal of plant growth regulators. Of these, embryo culture proved to be the most efficient *in vitro* system developed overall because the regenerants remained free of contaminants, phenolics and browning, and directly produced "seedlings" were available for greenhouse cultivation within 3 months of inoculation. Furthermore a secondary organogenic callus culture was induced from the seedlings thus increasing plantlet regeneration.

Two distinct cultures were also obtained from the nodular callus extruded from the swollen micropylar end of the excised zygotic embryos depending on the presence or absence of the cytokinin, BA, in combination with 2,4-D. These were the embryogenic cultures from which somatic embryos were obtained, and the non-embryogenic culture with malformed embryos. Both of these culture lines also remained free of contaminants, phenolics and browning without the addition of charcoal. In comparison to embryo culture, however, the first plantlets regenerated from both of these cultures were only available for greenhouse cultivation after 8 to 9 months.

By carefully separating out and culturing individual entities or daughter structures produced in the embryogenic cultures on solid nutrient medium several developmental pathways were identified. The repetitive nature of these pathways and the repeated formation of the large round pseudoembryonic structures and neomorphs in the different cultures suggests that these structures are basal to and form an integral part of the somatic embryogenesis pathway. Not only were the maintenance cultures established from these structures but the



**Figure 9.** Diagrammatic pathways showing *H. colchicifolia* plantlet regeneration via somatic embryogenesis on solid media.

nodular callus and somatic embryos were extruded from them. This is clearly illustrated in the flow diagram presented in Figure 9.

VON ARNOLD *et al.* (2002) suggested constructing fate maps for individual plant species in order to identify different morphological stages of development and genetic markers to identify controlling factors in somatic embryogenesis. The diagrammatic pathways showing all the steps and morphological stages of development identified for *H. colchicifolia* during the process of initiating somatic embryogenesis *in vitro* would be an ideal system to test this suggestion as the different structures can be isolated along the various pathways and their genotype investigated.

The objective of the current study, however, was to develop micropropagation protocols for the mass regeneration of *H. colchicifolia* plantlets. Although *in vitro* somatic embryogenesis and the protocols developed in this section of the study were highly successful, the rate of production of the somatic embryos was too slow for mass propagation. Since these cultures were established on solid media and somatic embryogenesis is often generated in liquid medium, the effect of liquid culture on the productivity of these cultures was investigated further.

## *Chapter 6*

# THE REGENERATION OF *H. COLCHICIFOLIA* PLANTLETS IN LIQUID CULTURES

## 6.1 INTRODUCTION

The regeneration of *H. colchicifolia* plantlets via embryo culture and *in vitro* somatic embryogenesis was achieved on agar solidified MS medium from excised zygotic embryos (Chapter 5). While the establishment of a micropropagation system through embryo culture was very successful, the production and subsequent proliferation of authentic somatic embryos via *in vitro* somatic embryogenesis was slow. Since the use of zygotic embryos as explants reduced the occurrence of widespread contamination and browning that occurred with other explants of *H. colchicifolia*, it would be appropriate to use the same type of explant or the cultures already induced from them, to determine an alternative method by means of which plant regeneration can be scaled up. The prime objective of this section, therefore, was to determine, firstly, whether an *in vitro* embryogenic liquid or cell suspension culture of *H. colchicifolia* could be established and, secondly, whether the production of somatic embryos could be scaled up sufficiently in liquid thereafter to increase plantlet regeneration over and above that achieved on solid media.

## 6.2 LIQUID AND CELL SUSPENSION CULTURES

The use of liquid media for culturing plant cells and organs *in vitro* is well known. Increased growth rates of callus and organs can often be achieved in liquid as opposed to solid nutrient media because a greater surface area of the tissue is in contact with the medium thus allowing more efficient uptake of nutrients and dispersal of toxic metabolites from the vicinity of the tissue. Stationary liquid cultures are often used to culture large pieces of tissue but these often require support such as glass wool, rafts or a filter paper bridge. Although referred to as suspension cultures, intermediate size plant tissues, callus and organs maintained in liquid media are not easily kept in suspension unless continually agitated and aerated on a rotary platform shaker. In contrast a plant cell suspension culture consists of more evenly dispersed single cells and small cell aggregates which continue to grow and multiply while suspended, through agitation, in a liquid nutrient medium (PIERIK,

1987; HALL, 1991). Whether the culture grown in liquid nutrient medium is referred to as a liquid or suspension culture therefore often depends on the plant species and its potential to produce callus clumps or single cells.

Contrary to expectation, both callus and cell suspension cultures are morphologically and / or cytogenetically heterogeneous, however (PIERIK, 1987). Although cell suspension cultures are useful in that they can be selectively subcultured to obtain greater homogeneity, it has also been argued that a less uniform culture with larger cell aggregates is advantageous in preserving the totipotent character of suspension cultures, for example of the Gramineae. Because of this ease of manipulation, cell suspension cultures are useful experimentally. Cell suspension cultures also have a characteristic growth curve consisting of an initial lag phase, exponential growth, a linear phase and a stationary phase all of which are easily monitored. The growth curve in turn is dependant on the concentration of the culture since a small inoculum results in a greatly extended lag phase, and a large inoculum in a shorter lag phase (HALL, 1991).

Cell suspension cultures have therefore been used to great advantage in embryological studies and to produce somatic embryos. In the latter it was shown that the uniformity of the somatic embryo populations could be improved by filtering the liquid medium during subculturing to selectively separate embryos at different stages of development (GEORGE, 1993). At each subculture the next phase of somatic embryo formation could then be initiated by selective manipulation of the PGRs added to the liquid medium as described for solid media in Chapter 5. In these cultures, plantlet regeneration was, however, not usually induced while still in liquid. As a result isolated somatic embryos were induced to germinate and produce plantlets on another, usually solid, medium (DODDS and ROBERTS, 1985).

The factors used to control and manipulate somatic embryogenesis in liquid culture are generally the same as those discussed in Chapter 5 for cultures on solid media. It was, however, not always easy to immediately recognise from the literature reviewed whether the culture under discussion was a suspension culture or not. However, the method generally used to obtain somatic embryos in suspension was to inoculate callus, previously induced on an explant cultured on solid media, into a liquid medium. An embryogenic cell suspension culture was then progressively induced by manipulation of the PGRs.

Somatic embryo formation in suspension cultures was first described in carrot (STEWART *et al.*, 1958b) and in many dicotyledons since then (THORPE, 1995). In monocotyledonous

species, the induction of somatic embryogenesis and the formation of somatic embryos in suspension cultures were reported much later. These processes were initially reported for the grasses and cereals (VASIL and VASIL, 1981; VASIL, 1985) and the daylily (KRIKORIAN and KANN, 1981), but have been expanded to include several other herbaceous and woody species since then (THORPE, 1995). However, neither somatic embryogenesis nor plantlet regeneration from liquid or cell suspension cultures has previously been demonstrated for *Hypoxis*.

Since the aim of this study was to regenerate *H. colchicifolia* plantlets on a large scale, the feasibility of using suspension cultures to regenerate somatic embryos was investigated. To achieve this the best choice of explant or inoculant to initiate a self-perpetuating embryogenic culture in liquid had first to be determined. Furthermore optimum media and culture conditions, growth rates and whether the somatic embryos produced could be transferred back to solid medium to maintain proliferation of nodular callus needed to be established. For the purposes of this study all the cultures established are referred to as liquid cultures because of their heterogeneity. An exception is the "single cell suspension culture" which was initially more homogeneous in nature as it was composed of single cells and small cell aggregates.

## **6.3 MATERIALS AND METHODS**

### **6.3.1 EXPLANT MATERIAL, CULTURE MEDIA, CONDITIONS AND PROCEDURES**

#### **6.3.1.1 Initiation and Establishment of Liquid Cultures**

##### **6.3.1.1.1 *Inocula***

The explant material or inoculum used to establish the initial liquid cultures was obtained from aseptic, 30-week-old maintenance cultures which were already embryogenic and had been induced from excised *H. colchicifolia* zygotic embryos as described in Chapter 5. These cultures had been maintained on MS basal medium supplemented with 30 g l<sup>-1</sup> sucrose, 0.1 g l<sup>-1</sup> myo-inositol and 1 mg l<sup>-1</sup> 2,4-D, adjusted to pH 5.8 and solidified with 10 g l<sup>-1</sup> agar. Cultures were also kept in the dark at a temperature of 25 ± 2°C in 65 mm diameter clear plastic Petri dishes (Labotec) which were sealed with Parafilm®.

Initially two types of *in vitro* plant material were selected as experimental inocula. These were, firstly, the secondary round pseudoembryonic structures enclosed in a dark crusty covering, which had been produced by cleavage from a primary round pseudoembryonic structure and, secondly, the clusters of proembryonic nodular callus with somatic embryos extruded from them. Both inocula were obtained from a single explant and used to initiate two separate liquid culture lines namely an "ex single round" line and an "ex nodular callus" line. Since the embryogenic capacity of the 30-week-old maintenance cultures had been declining, 5 explant replicates were initially selected to inoculate 5 culture flasks for each of the two culture lines.

#### **6.3.1.1.2      *Initiation of the "Ex Single Round" Liquid Culture***

The aseptic inoculum used to initiate the "ex single round" liquid culture was prepared by removing the dark crusty covering and mucilage surrounding the round pseudoembryonic structures (8 to 10 per explant, and each 1 mm to 1.5 mm in diameter). Since only one explant ( $\pm 2.5 \times 3 \times 2$  mm) was used per flask, a total of 8 to 10 single round pseudoembryonic structures, still surrounded by scattered mucilaginous cells, was therefore used to inoculate 30 ml of liquid MS medium per 100 ml Erlenmeyer flask. The liquid medium consisted of the revised nutrient medium of MURASHIGE and SKOOG (1962) supplemented with 30 g l<sup>-1</sup> sucrose, 0.1 g l<sup>-1</sup> myo-inositol, 1 mg l<sup>-1</sup> 2,4-D and adjusted to pH 5.8. Each Erlenmeyer flask containing one explant of 8 to 10 single round structures, was replicated 5 times. The flasks were closed with an aseptic cotton wool bung. The cultures were then maintained at a temperature of  $25 \pm 2^\circ\text{C}$  under dark conditions while being agitated on a platform rotary shaker set at a constant speed of 120 rotations per minute.

To obtain optimum conditions for adequate aeration, it is generally recommended that suspension cultures should occupy 20% of the total volume of a 250 ml Erlenmeyer flask and the speed range of the platform shaker should be set at between 100 and 120 rotations per minute (DODDS and ROBERTS, 1985). In this study, however, conditions precluded altering the speed settings of the platform shakers used. Phenolic production was also more readily controlled if the liquid cultures were maintained in 100 ml rather than in 250 ml flasks. The effect of the smaller flasks and higher speed of 120 rotations per minute on the shearing of the culture was compensated for by increasing the volume of the liquid medium by 10% to 30 ml per flask. This volume was always used unless stated to the contrary.

### **6.3.1.1.3      *Initiation of the “Ex Nodular Callus” Liquid Culture***

The clusters of proembryonic nodular callus ( $\pm 3 \times 2 \times 2$  mm) with somatic embryos which were removed from the explant used to inoculate a flask of the “ex single round” liquid culture as described above, were teased apart and placed into a 100 ml Erlenmeyer flask containing 30 ml of liquid MS medium with  $1 \text{ mg l}^{-1}$  2,4-D. Each of the 5 flasks prepared was closed, agitated on a rotary shaker and incubated in the dark (refer Section 6.3.1.1.2).

### **6.3.1.2      *Initial Subcultures and Procedures***

Both the “ex single round” and the “ex nodular callus” liquid cultures were subcultured into 30 ml of fresh liquid MS maintenance medium per 100 ml Erlenmeyer flask after 52 days, followed by three subcultures at 15 day intervals. Successive subcultures were performed every 10 days thereafter unless stated to the contrary. The procedure followed during subculturing was to first allow the culture to settle in the flask, decant off the supernatant (old medium) and wash the settled culture in sterile, half strength, liquid MS medium without PGRs. The cleaned plant culture was then finally transferred to a clean, aseptic flask containing 30 ml of fresh, full strength MS maintenance medium.

The different stages of development were monitored for each liquid culture by removing aliquots of dispersed culture at regular intervals for examination. Between 9 and 10 weeks after inoculation the composition of the components in each flask was fully representative of either the “ex single round” or the “ex nodular callus” liquid culture. After 14 weeks, both cultures had grown to the extent that additional flasks could be prepared as follows.

The washed plant culture in each flask of the “ex single round” liquid culture was subdivided equally into 3 x 100 ml flasks, each containing 30 ml of fresh liquid MS medium with  $1 \text{ mg l}^{-1}$  2,4-D. In contrast, the total volume of the more slowly growing “ex nodular callus” liquid culture from all replicate flasks was subcultured into 8 x 100 ml flasks, 6 of which contained 30 ml of fresh liquid MS medium with  $1 \text{ mg l}^{-1}$  2,4-D while the other 2 flasks contained 30 ml of liquid MS medium supplemented with  $1 \text{ mg l}^{-1}$  ABA. The latter 2 flasks were transferred to liquid MS medium without PGRs after 2 weeks. The cultures were all incubated in the dark at a temperature of  $25 \pm 2^\circ\text{C}$  on a platform rotary shaker set at a constant speed of 120 rotations per minute. At this stage (14<sup>th</sup> week) both cultures had proliferated to the point where secondary cultures were established and further subculture treatments implemented.

## 6.3.2 SECONDARY LIQUID CULTURES AND SUBCULTURE TREATMENTS

### 6.3.2.1 The “Ex Single Round” Liquid Culture

#### 6.3.2.1.1 *Maturation of the Embryo-like Daughter Structures from the “Ex Single Round” Liquid Culture*

As the single and clustered embryo-like daughter structures in the “ex single round” cultures enlarged, samples of both were transferred to 100 ml flasks containing 30 ml of liquid MS medium without PGRs and exposed to a 16 / 8 hour photoperiod. Light was supplied by Phillips cool white fluorescent tubes at an intensity of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ . After 2 weeks, the daughter structures were transferred to MS medium solidified with  $10 \text{ g l}^{-1}$  agar to mature. The MS medium was either PGR free or supplemented with  $0.5 \text{ mg l}^{-1}$  ABA. Cultures were incubated at  $25 \pm 2^\circ\text{C}$  in the light as described in Chapter 5.

#### 6.3.2.1.2 *Initiation and Development of a “Single Cell” Suspension Culture*

Between 10 and 12 weeks after inoculation, the principal “ex single round” liquid cultures turned grey-white and dense as a result of the rapid proliferation of single cells and small cell aggregates. These single cells were then separated from any residual inoculum and daughter structures in the principal culture and used to initiate a “single cell” suspension culture during the 16<sup>th</sup> week. The filtered single cells in a small volume of filtrate were divided into 6 x 1 ml portions which were used to inoculate 6 x 100 ml Erlenmeyer flasks each containing 30 ml of liquid MS medium supplemented with  $1 \text{ mg l}^{-1}$  2,4-D. The flasks were closed, placed on a rotary shaker and incubated at  $25 \pm 2^\circ\text{C}$  in the dark. The cultures were subcultured at 10 day intervals thereafter at which time small samples of the suspension culture were removed, stained with acetocarmine to highlight meristematic regions, and examined to monitor cell development. The acetocarmine stain, prepared by extracting 4% w:v carmine in 50% v:v acetic acid for 24 hours and filtering the extract (SUNDERLAND and DUNWELL, 1977), was diluted with distilled water 2:1 (v:v) before use.

#### 6.3.2.1.3 *Maturation of Daughter Structures from the Newly Initiated “Single Cell” Suspension Culture*

As daughter structures developed in the principal culture, samples were removed and transferred to flasks containing either liquid MS maintenance medium, auxin free or ABA

supplemented MS medium to promote maturation. Mature structures were transferred to PGR free MS medium solidified with 10 g l<sup>-1</sup> agar and the light to germinate. Regenerated plantlets, rooted on PGR free medium, were acclimatized as outlined in Chapter 4.

### **6.3.2.2 The “Ex Nodular Callus” Liquid Culture**

#### **6.3.2.2.1 *Re-initiation of an Embryogenic Nodular Callus Culture on Solid Medium***

To determine whether an embryogenic callus culture with somatic embryos could be re-initiated on solid medium from the scaled up 14-week-old “ex nodular callus” liquid culture, small round single structures which had developed in the liquid medium were transferred back onto MS maintenance medium supplemented with 1 mg l<sup>-1</sup> 2,4-D and solidified with 10 g l<sup>-1</sup> agar in Petri dishes which were incubated in the dark at 25 ± 2°C. Morphological changes were monitored at weekly intervals.

#### **6.3.2.2.2 *The Effect of ABA on the Maturation of the Proembryo-like Daughter Structures from the “Ex Nodular Callus” Liquid Culture***

To determine the effect of ABA on the maturation of the proembryo-like daughter structures produced in the cultures, 2 ml settled volume of “ex nodular callus” which had been in liquid MS maintenance medium for 18 weeks, was inoculated into each of 8 x 100 ml Erlenmeyer flasks. The MS liquid medium was supplemented with 0.5 mg l<sup>-1</sup> ABA in 4 flasks, and with 1 mg l<sup>-1</sup> ABA in the remaining 4 flasks. The cultures were agitated on a rotary shaker at a speed of 120 rotations per minute and incubated in the dark at 25 ± 2°C. After 14 days in ABA pulsed medium all the cultures were transferred into 30 ml of MS medium without PGRs. All subcultures were at 10 day intervals thereafter.

At each subculture the old liquid medium was decanted off and discarded. The remaining culture was washed in half strength liquid MS medium before being transferred to a clean flask with 30 ml of full strength liquid MS medium without PGRs. When the volume of settled culture in a flask exceeded 30 ml, the culture was subdivided into ± 5 ml portions which were transferred to new flasks. As the cultures proliferated space on the rotary shakers became a limiting factor, however, and the morphological changes in and growth of only one culture line could therefore be monitored satisfactorily.

### **6.3.2.2.3      *Somatic Embryo Germination and Plantlet Regeneration in Liquid Culture***

After this culture line had been in PGR free medium for 12 weeks, the MS liquid medium was reduced to a minimal amount (5 ml initially and then 2.5 ml) to induce partial desiccation in an attempt to promote germination. Germinating embryo-like structures were exposed to light conditions before their final transfer to PGR free MS medium solidified with 10 g l<sup>-1</sup> agar to induce shoots and roots. Rooted plantlets were acclimatized as per the procedure outlined in Chapter 4.

### **6.3.2.3      *Determination of Growth Rates of Three Principal Liquid Cultures***

#### **6.3.2.3.1      *Establishment of the Cultures for Determination of Cell Growth***

The rate of cell growth was determined for the three principal liquid cultures using the settled cell volume technique. About 18 weeks after the first liquid cultures were initiated four experimental liquid and suspension cultures were prepared from existing cultures as follows.

Settled cell volume and therefore cell growth could not be determined for the “ex single round” liquid culture due to the presence of clusters of large embryo-like structures. Only the newly established “single cell” culture was therefore used from this culture line. Two experimental “single cell” suspension cultures were initiated by adding 20 ml settled volume of the “single cell” culture to 480 ml liquid MS medium supplemented with 1 mg l<sup>-1</sup> 2,4-D and 20 ml to 480 ml medium without PGRs. Each stock culture was then subdivided into 5 x 100 ml replicates which were transferred to 5 x 500 ml side-arm suspension culture flasks. The flasks were closed using a cotton wool bung, covered with aluminium foil to provide dark conditions, agitated on a rotary shaker at a speed of 120 rotations per minute and incubated at 25 ± 2°C. Settled cell volumes were measured after 24 hours and at the same time at 2 day intervals thereafter.

The experimental “ex nodular callus” liquid cultures, however, were initiated from ± uniform nodular callus culture established at the same time that the “single cell” suspension culture was initiated. Initially a stock culture was prepared by adding 20 ml of the ± uniform nodular callus to 480 ml liquid MS medium supplemented with 1 mg l<sup>-1</sup> 2,4-D. This stock culture was then subdivided into 5 x 100 ml replicates which were transferred to 5 x 500 ml side-arm

suspension culture flasks and treated as described for the experimental "single cell" suspension cultures.

The third experimental liquid culture used, the "ABA pulsed ex nodular callus" culture, was also established from an "ex nodular callus" liquid culture which had been transferred to an MS liquid medium supplemented with  $1 \text{ mg l}^{-1}$  ABA after 14 weeks in culture, and then to an MS liquid medium without PGRs 2 weeks later. The stock liquid culture was first prepared by adding 20 ml of the culture to 480 ml liquid MS medium without PGRs, and then subdivided into 5 x 100 ml replicates which were transferred to 5 x 500 ml side-arm suspension culture flasks and treated as described above.

#### **6.3.2.3.2      *Establishment of a Concentrated "Single Cell" Suspension Culture for Determination of Cell Growth***

To obtain a more representative growth curve of the "single cell" suspension culture, it was necessary to not only increase the concentration of the inoculum but use a smaller volume side-arm flask. The stock culture was prepared by adding 50 ml of inoculum to 200 ml MS liquid medium supplemented with  $1 \text{ mg l}^{-1}$  2,4-D. This was then subdivided into 5 x 50 ml replicates which were transferred to 5 x 250 ml side-arm suspension culture flasks and treated as described above for the experimental "single cell" suspension cultures.

#### **6.3.2.3.3      *Determination of Settled Cell Volumes***

Settled cell volumes were measured for the 5 experimental cultures with the aid of the side-arm flasks. Firstly the side-arm was filled with a representative portion of the liquid medium and suspended cells. The flasks were then clamped so that the upper angle of the side-arm was perpendicular to allow the cells to settle. After a period of 5 minutes the flask was tipped again so that the lower half of the side-arm was perpendicular and the cells could settle finally into the tip of the side-arm. After another 5 minutes the depth of the settled cells in the arm was measured using a vernier caliper. The settled cell volume was measured every second day at the same time for the first 4 cultures and daily for the concentrated culture. Results were expressed as a percentage of the total length of the side-arm.

## **6.4 RESULTS AND DISCUSSION**

### **6.4.1 INITIATION AND ESTABLISHMENT OF THE LIQUID CULTURES**

Establishment of both the "ex single round" and the "ex nodular callus" liquid cultures was slow due to the small size of the inocula. The cultures were therefore only subcultured for the first time after 52 days, followed by three subcultures at 15 day intervals. Leaching of phenolics resulting in the discolouration of the liquid medium occurred as the cultures proliferated, however, but could be controlled by washing the plant culture and performing successive subcultures every 10 days. During the 9<sup>th</sup> week after inoculation aliquots of dispersed culture removed at regular intervals for examination were fully representative for each culture line. It was only after the 14<sup>th</sup> week, however, that the cultures had proliferated sufficiently to be subdivided. The "ex single round" liquid culture in each flask could be subcultured equally into 3 x 100 ml flasks at this stage and the total "ex nodular callus" liquid culture into 8 x 100 ml flasks.

### **6.4.2 DEVELOPMENTAL STAGES OF THE LIQUID CULTURES AND SUBCULTURE TREATMENTS**

#### **6.4.2.1 Development and Proliferation of the "Ex Single Round" Liquid Culture**

The different stages of development exhibited by the "ex single round" liquid culture during the first 10 weeks of its establishment all occurred in liquid MS maintenance medium with 2,4-D and are illustrated in Plate 17. Initially the single secondary round structures used as the inoculum divided to produce groups of opaque white tertiary daughter structures surrounded by translucent mucilage (Plate 17A) which developed into a dark crusty covering (Plate 17B). With continuous agitation in liquid MS medium these groups, which varied in size from 10 to 45 mm<sup>3</sup>, separated into smaller groups of maturing white structures (Plate 17C). Finally the individual tertiary round daughter structures between 0.5 and 1.5 mm in diameter, were released into the surrounding liquid medium and a mucilagenous covering, which turned black later, was produced around each structure (Plate 17D). Further development then proceeded along 2 separate pathways. The released tertiary daughter structures either divided (Plate 17D) to produce a group of quaternary round daughter structures which developed further as discussed and illustrated for the initial round structures (Plate 17 A - C) thus perpetuating the culture cycle, or they

matured and elongated to form either individual (Plate 17E) or clusters (Plate 17F) of bipolar pseudoembryo-like structures.

#### **6.4.2.1.1     *Maturation of the Single and Clustered Embryo-like Daughter Structures from the “Ex Single Round” Liquid Culture***

On transfer to PGR free liquid medium and exposure to light, the large single and clustered pseudoembryo-like daughter structures produced root-like protrusions at their distal end. Some turned black as a result of increased phenolic production but did not germinate (Plate 18A). Further development only occurred after their transfer to PGR free solid MS medium. A radicle with white hairs emerged from the root-like protrusions and callus with false somatic embryos developed at the opposite end of each structure (Plate 18B). Unipolar embryos with leaves and meristemoids with shoot or root primordia also developed from the callus (Plate 18C). The presence of ABA in the solid MS medium did not promote maturation or germination of these structures. A successful organogenic *in vitro* culture was ultimately obtained from the induced callus on PGR free solid MS medium. Regenerated rooted plantlets (Plate 18D) were acclimatized (Chapter4) and an 80 % survival rate was achieved.

#### **6.4.2.1.2     *Initiation and Development of a “Single Cell” Suspension Culture from the “Ex Single Round” Liquid Culture***

Approximately 10 weeks after inoculation, single cells and small cell aggregates had rapidly proliferated in the principal “ex single round” liquid culture which turned thick and grey-white as a result. Although the origin of these cells was not known, they were separated out and used to initiate the “single cell” suspension culture (Section 6.3.2.1.2).

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**Plate 17.**     Sequential stages of development exhibited by the secondary round pseudoembryonic structures during the initiation of the “ex single round” liquid culture. Initially each divided to produce a group of separate white tertiary daughter structures surrounded by (A) translucent mucilage (Bar = 0.37 mm) which (B) developed into a dark covering (Bar = 0.4 mm). These groups separated into (C) smaller groups (Bar = 0.3 mm) from which the individual tertiary round structures were released as shown in (D) a sample of the 9-week-old culture (Bar = 1 mm). Tertiary daughter structures (D) divided to repeat the culture cycle or elongated to form (E) individual (Bar = 0.4 mm) or (F) clusters of pseudoembryo-like structures (Bar = 1.5 mm).

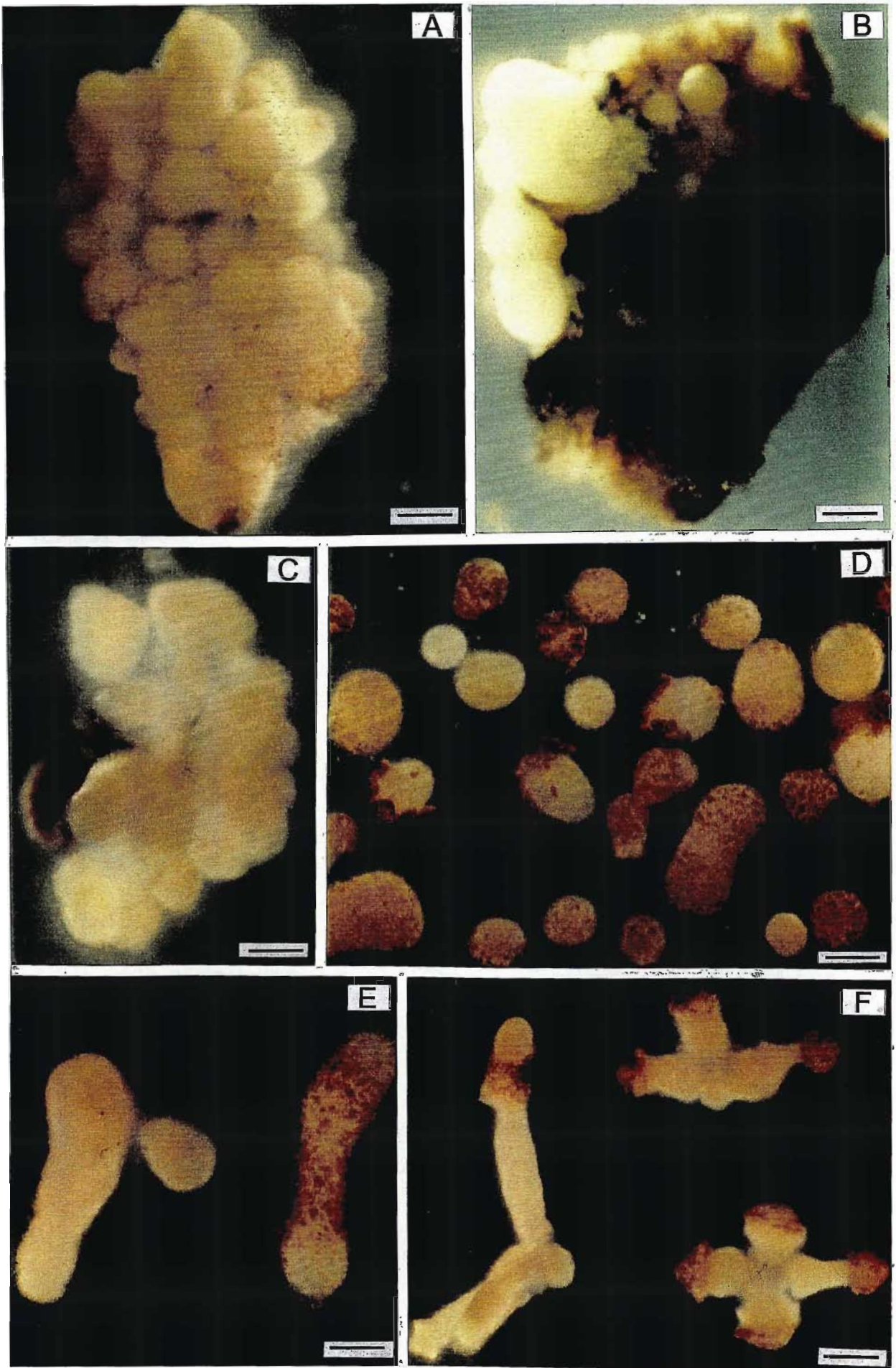
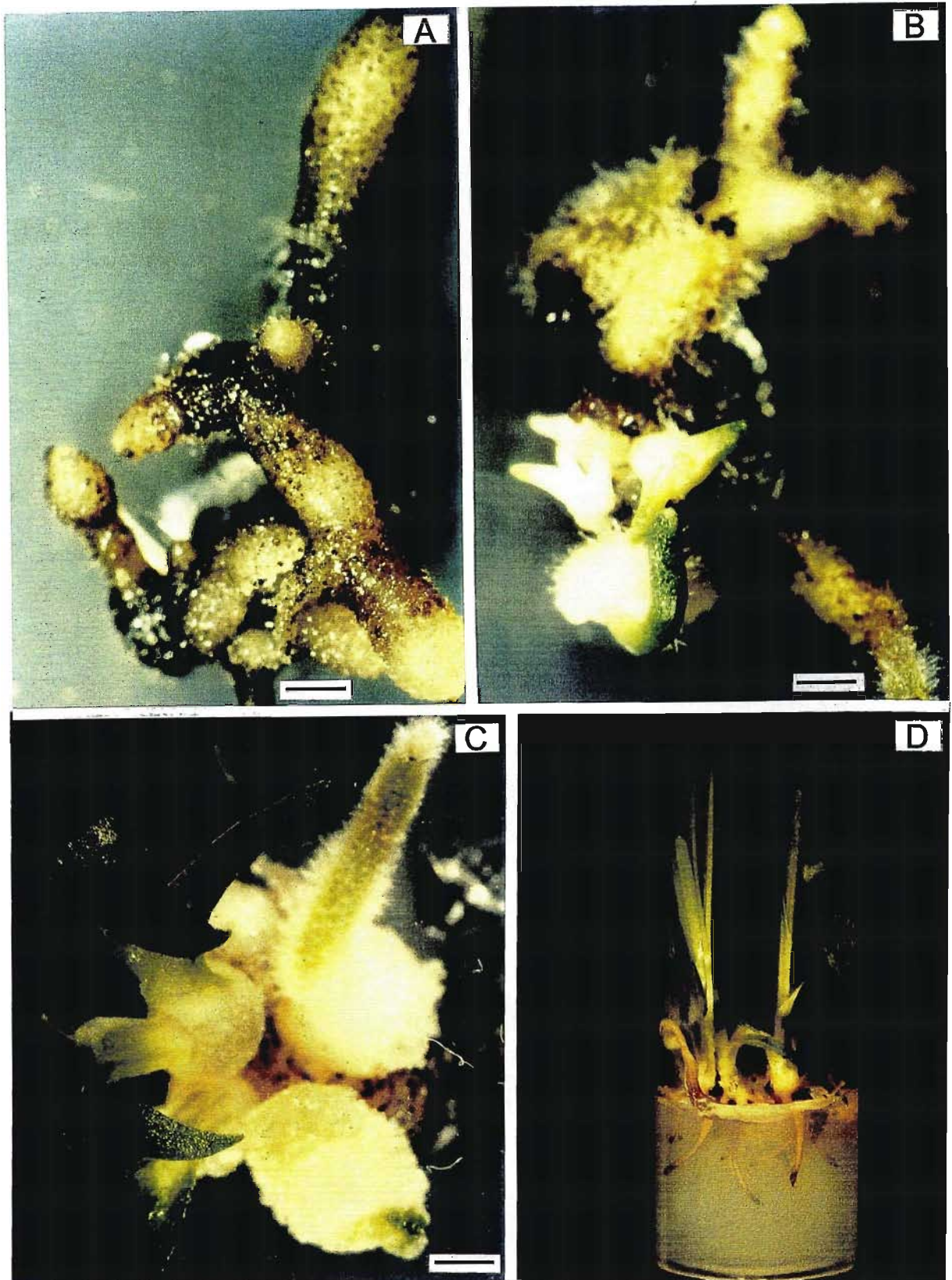


Plate 17

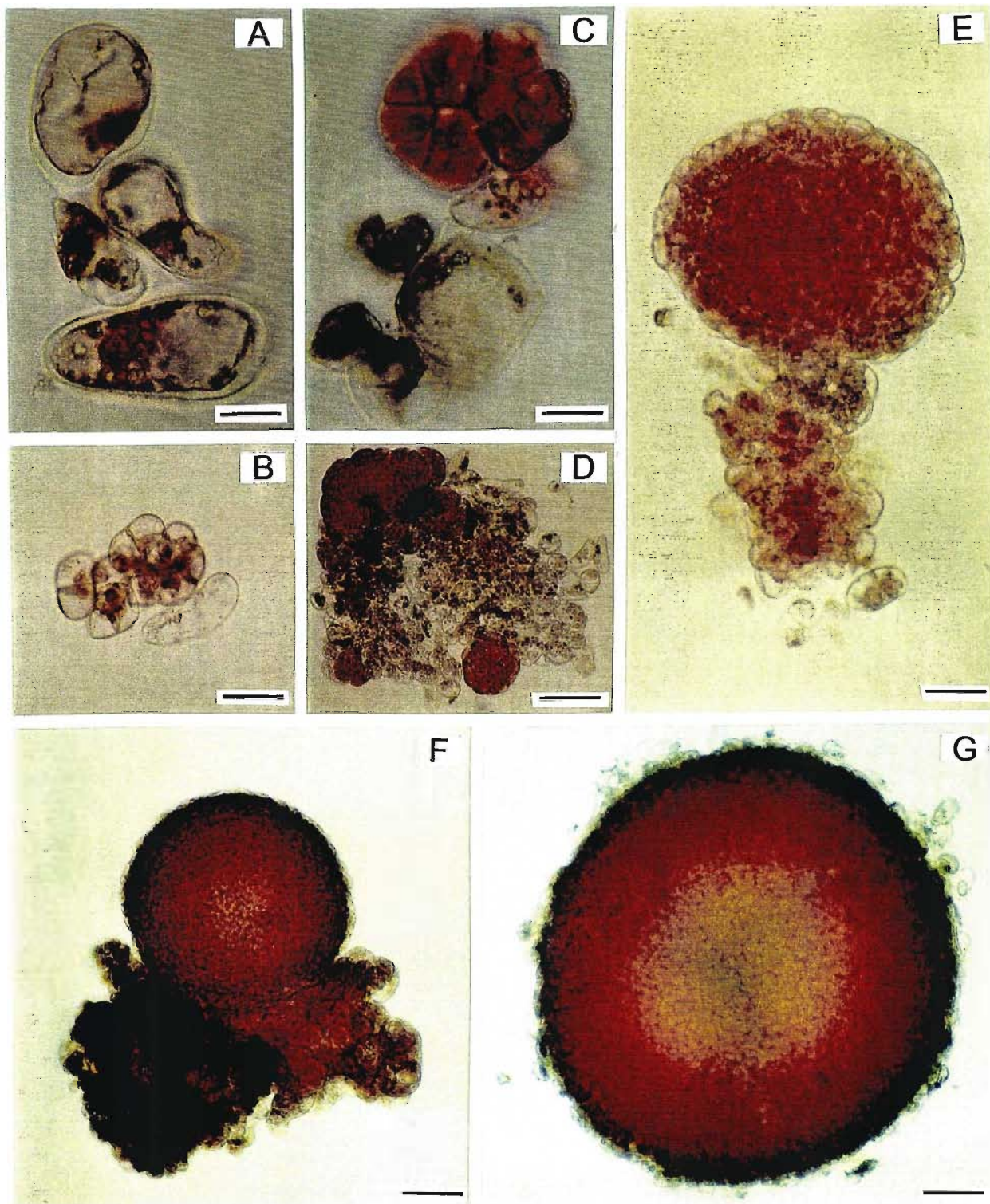


**Plate 18.** Plantlet regeneration from clusters of pseudoembryo-like daughter structures (A) from the "ex single round" liquid culture. (A) In PGR free liquid medium a root-like protrusion developed at the distal end but no germination occurred (Bar = 0.8 mm). (B) On PGR free solid medium a radicle with white hairs emerged, while callus and unipolar somatic embryos developed at the proximal end (Bar = 0.9 mm). (C) Leaves and meristemoids with shoot or root primordia developed (Bar = 1.2 mm). (D) Rooted plantlets were obtained.

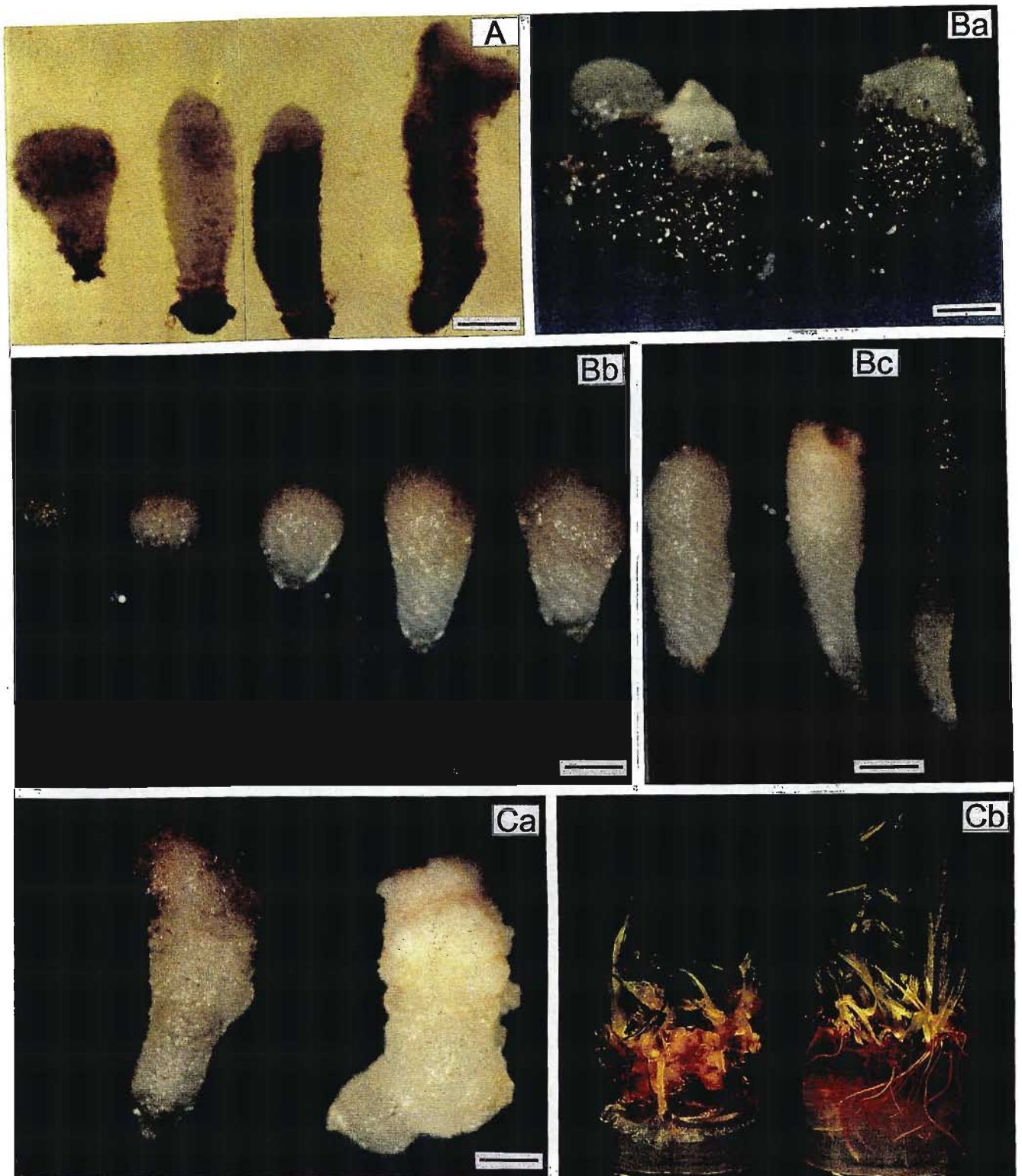
The embryogenic potential and cell development of this pale yellow suspension culture was easily monitored since being highly active with a rapid rate of cytoplasmic synthesis, these regenerative cells stained densely and uniformly with the general stain, acetocarmine (SUNDERLAND and DUNWELL, 1977). This was in direct contrast to the lightly staining non-embryogenic cells with slow rates of cytoplasmic synthesis and large vacuoles, and those which were inactive and highly vacuolate. Some of the non-embryogenic cells, however, had developed starch grains and therefore also stained densely with acetocarmine, but they could be distinguished from the embryogenic cells which generally lacked starch and therefore stained more lightly.

Aliquots of dispersed "single cell" suspension culture stained with acetocarmine showed that the suspension consisted of single, densely staining, regenerative, cytoplasmic cells (Plate 19A) which divided actively (Plate 19B) to produce clusters of cells resembling various stages of early (Plate 19C - D) and late globular proembryos (Plate 19D). In contrast to these dark pink to red embryogenic cells, other inactive, highly vacuolate cells (Plate 19B), slightly active, cytoplasmic cells with large vacuoles which stained a light brown (Plate 19C - D) and clusters of highly active, non-embryogenic cells with starch grains which stained a dark brown (Plate 19F) were also present in the suspension culture. These cells did not develop further, but the embryogenic globular stage proembryos matured to form a later stage globular proembryo in which an outer epidermal layer and small suspensor were evident (Plate 19E). At this stage the globular proembryos were still surrounded by aggregates of embryogenic (Plate 19E) and non-embryogenic cells with starch grains (Plate 19F). The large later stage globular somatic proembryos (Plate 19F) then broke away completely from the cell aggregates to develop independently in the liquid medium (Plate 19G).

Freely suspended late stage globular proembryos removed from the suspension culture at this stage and cultured independently in liquid MS maintenance medium produced a range of distinctly bipolar embryo-like structures (Plate 20A) which did not germinate or differentiate into shoots and roots. To induce maturation, aliquots of culture containing embryogenic cell aggregates with globular proembryos at various stages of development were transferred to PGR free liquid MS medium (Plate 20B a). The cell aggregates ceased to proliferate and died in the absence of 2,4-D, but the free-floating proembryos matured to form early to late globular (Plate 20B b) and bipolar cotyledonary (Plate 20B c) stage somatic embryos. Although these somatic embryos matured in liquid medium in the absence of auxin, they did not produce shoots and roots, but a crystalline callus. When transferred to PGR free MS medium solidified with 10 g l<sup>-1</sup> agar, the cotyledonary stage



**Plate 19.** Sequential development of the "single cell" suspension culture. The dispersed cell culture consisted of (A) regenerative, cytoplasmic cells (Bar = 0.01 mm) which (B) divided actively (Bar = 0.025 mm) to produce (C) densely staining (Bar = 0.012 mm) (D) early stage (Bar = 0.04 mm) and (E) later stage globular proembryos with an outer epidermal layer and small suspensor surrounded by embryogenic cell aggregates (Bar = 0.02 mm). (F) Large globular stage somatic proembryos (Bar = 0.06 mm) broke away and (G) developed independently (Bar = 0.043 mm). Also present were non-embryogenic (B) inactive, vacuolate cells, (C) lightly stained cytoplasmic cells, and (F) non-embryogenic cells with starch grains.



**Plate 20.** Further stages of development of globular proembryos from the "single cell" suspension culture. (A) In liquid MS maintenance medium these produced a range of bipolar embryo-like structures which did not germinate (Bar = 0.4 mm). (B) In PGR free liquid MS medium (B a) cell aggregates disintegrated and the proembryos (Bar = 0.4 mm) released matured to produce a range of (B b) early to late globular (Bar = 1.05 mm) and (B c) cotyledonary bipolar somatic embryos (Bar = 1 mm). (C) On PGR free solid MS medium the somatic embryos produced (C a) a crystalline callus (Bar = 1 mm) from which (C b) shoots and plantlets were regenerated.

somatic embryos still failed to germinate and again produced a crystalline callus (Plate 20 C a) from which an organogenic callus culture with multiple shoots (Plate 20C b) was induced within 8 weeks. Regenerated plantlets rooted spontaneously on the same medium (Plate 20C b).

#### **6.4.2.2 Development and Proliferation of the “Ex Nodular Callus” Liquid Culture**

Initially the clusters of proembryonic nodular callus used as the inoculum separated into smaller clusters and single round daughter structures, or proembryos, during agitation in liquid MS maintenance medium. By monitoring the development of isolated round entities, it was determined that a dark brown to black crusty covering developed initially around the enlarging proembryos (Plate 21A a) which began to divide when 1.8 mm to 2 mm in diameter. The black covering then split to extrude secondary clusters of proembryonic nodular callus which were released into the liquid medium. All these developmental stages were represented in an aliquot of 9-week-old “ex nodular callus” liquid culture (Plate 21 A b). At this stage the components of this and the 9-week-old “ex single round” liquid culture (Plate 17 D) were completely different. Also at no time were small single cells and cell aggregates produced in the “ex nodular callus” liquid culture as occurred in the “ex single round” culture and from which the “single cell” suspension culture was initiated. The “ex nodular callus” culture was successfully established and scaled-up as described, but the proembryos produced failed to develop further in the MS maintenance medium supplemented with 2,4-D.

##### **6.4.2.2.1 *Re-initiation of an Embryogenic Nodular Callus Culture on Solid Medium***

When replated back onto solid MS maintenance medium, individual free-floating globular

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**Plate 21.** (A) In liquid maintenance medium (A a) proembryos developed a dark covering which split to extrude nodular callus (Bar = 0.55 mm) as shown among (A b) components of a 9-week-old “ex nodular callus” liquid culture (Bar = 1 mm). (B) On solid maintenance MS medium an embryogenic callus culture was re-initiated from (B a) single round proembryos (Bar = 0.33 mm) which produced (B b) proembryonic nodular callus clusters (Bar = 0.38 mm) which enlarged and produced (B c) globular proembryos (Bar = 0.7 mm) and (B d) early cotyledonary stage somatic embryos (Bar = 0.82 mm).

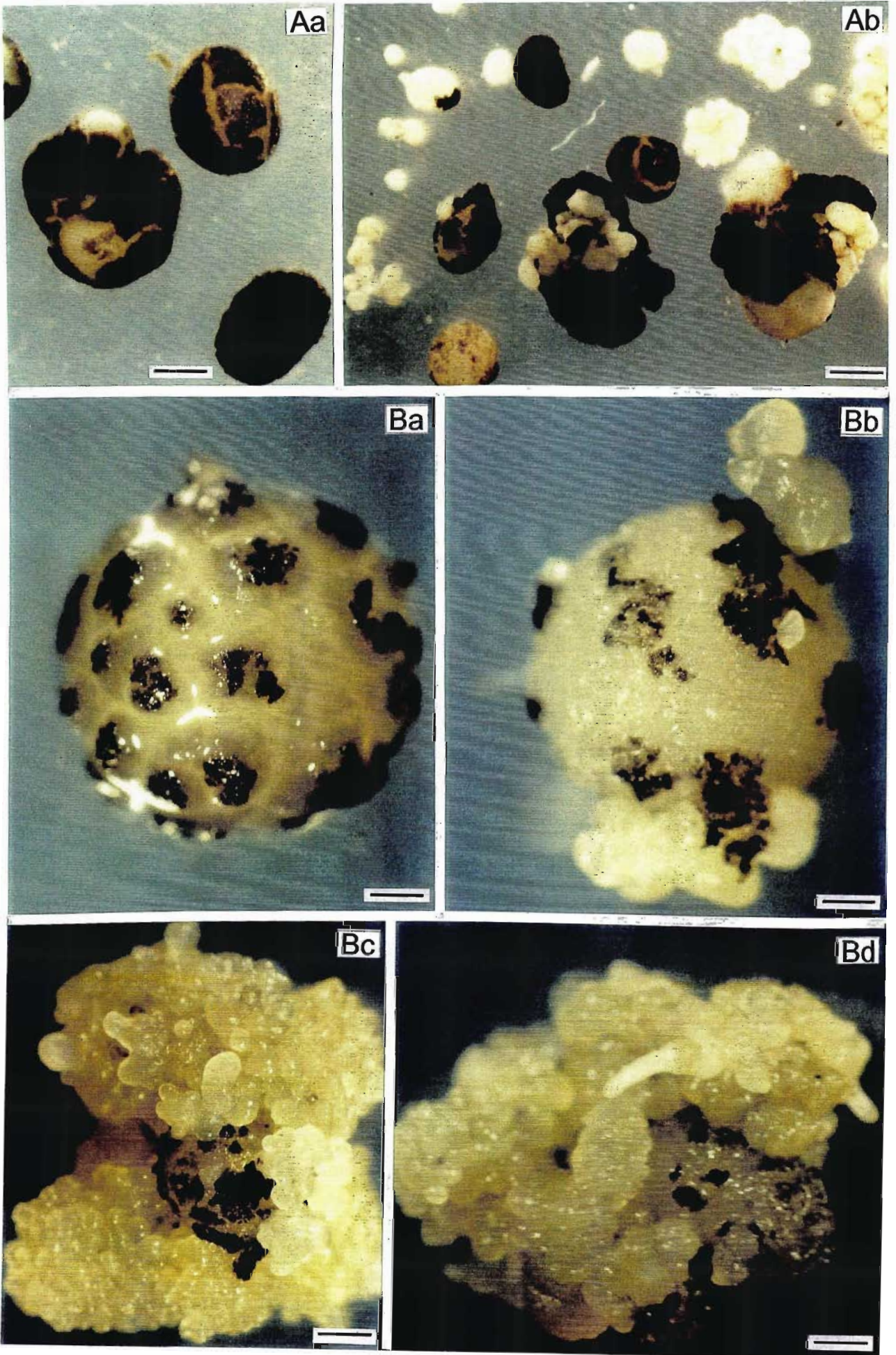


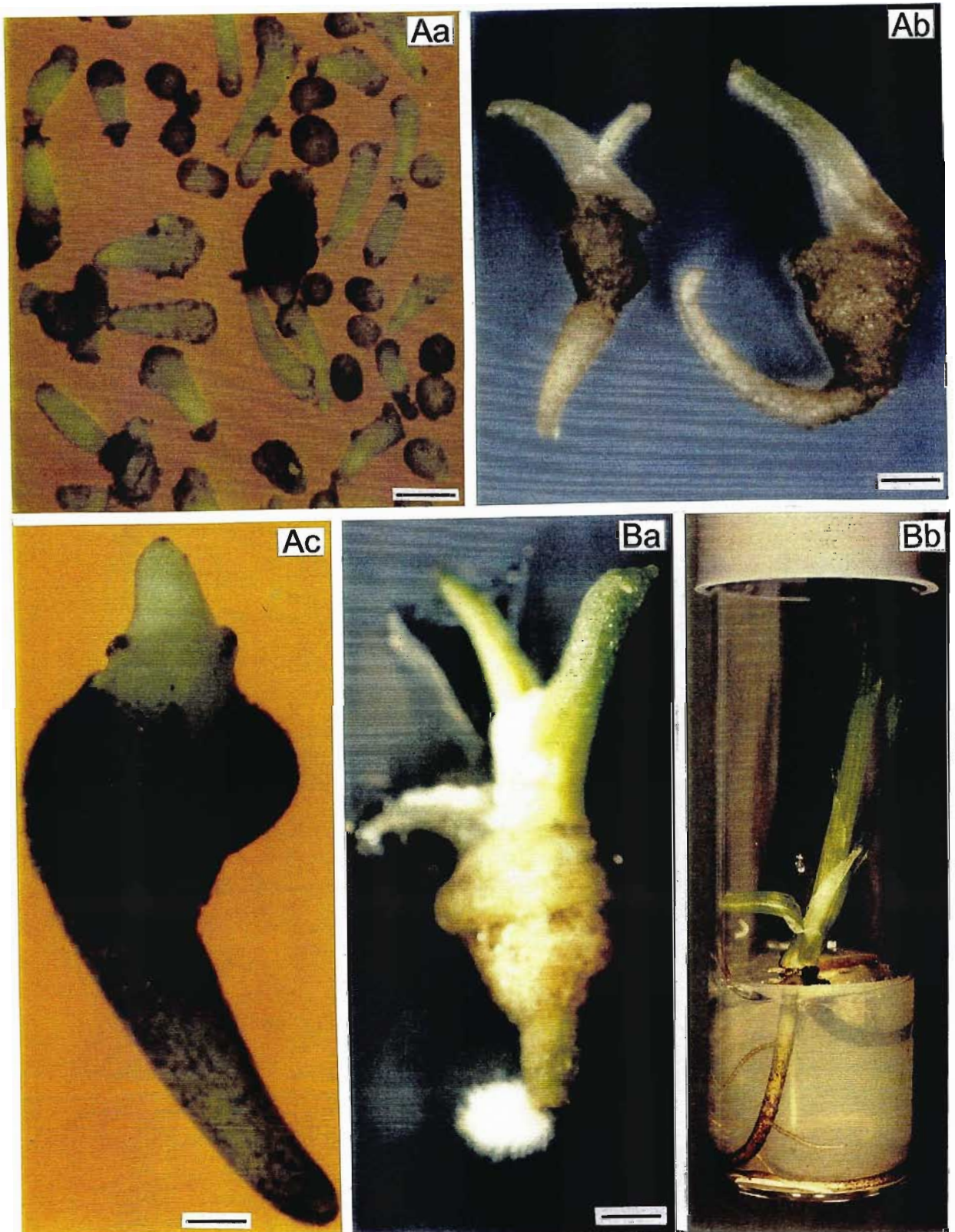
Plate 21

proembryos (Plate 21B a) which had been removed from the 14-week-old “ex nodular callus” liquid culture, produced proembryonic nodular callus (Plate 21B b). Then, as the clusters of nodular callus enlarged, globular proembryos (Plate 21B c) and early stage cotyledonary somatic embryos (Plate 21B d) developed. The proembryos also divided into several round daughter structures beneath the mucilage / crusty covering (Plate 21B d) ensuring continuity of the culture. Further development and germination of the bipolar somatic embryos was identical to that already demonstrated for the first somatic embryos produced from excised zygotic embryos as discussed in Chapter 5. These results clearly demonstrated, however, that an embryogenic callus culture could be re-initiated from the globular proembryos produced in and removed from a 14-week-old “ex nodular callus” liquid culture. The callus had therefore maintained its embryogenic potential for 21 weeks in liquid and for one year after inoculation of the original culture on solid MS medium supplemented with 2,4-D.

#### **6.4.2.2.2      *The Effect of ABA on the Maturation of the Proembryo-like Structures from the “Ex Nodular Callus” Liquid Culture***

Nine weeks after receiving a 2-week ABA pulse followed by a transfer to PGR free MS liquid medium, the composition of the 11-week-old “ABA pulsed” callus culture had altered completely (Plate 22A a) and more closely resembled that of the initial “ex single round” culture (Plate 17). Not only had the original nodular callus totally ceased to proliferate and separated to form many individual entities, but a maturation phase had been initiated. As a result a large number of somatic embryos and related structures were produced in the liquid medium. Measurable differences could not be detected between the components of the cultures pulsed with 0.5 mg or 1 mg l<sup>-1</sup> ABA, however, other than that the 0.5 mg l<sup>-1</sup> ABA pulsed cultures appeared to be slightly more compact and uniform. The following descriptions therefore apply to both of the “ABA pulsed” cultures.

When aliquots of the 11-week-old “ABA pulsed” cultures were viewed the heterogeneous nature of the culture that had developed and proliferated in the PGR free liquid MS medium was very apparent. Components consisted of various globular proembryo-like structures, bipolar somatic embryo-like entities which were either free-floating or in clusters (Plate 22A a), and other malformed and long root-like structures. Even though the somatic embryo-like structures were often morphologically dissimilar, they were clearly bipolar and many began to germinate normally while still in the PGR free liquid medium (Plate 22A b - c). At this stage, however, the germinating somatic embryos became hyperhydric and further germination was inhibited. If expeditiously transferred to PGR free solid MS medium,



**Plate 22.** (A) Components of the heterogeneous 11-week-old "ABA pulsed" culture in PGR free liquid medium which was initiated from the original "ex nodular callus" liquid culture, included (A a) globular proembryos and various bipolar somatic embryo-like entities, free-floating or in clusters (Bar = 2 mm) which began to (A b) and (A c) germinate in liquid medium (Bar = 0.6 mm). (B) On transfer to PGR free solid medium (B a) germination continued normally (Bar = 1 mm) and (B b) rooted plantlets were produced.

however, the somatic embryos continued to germinate normally (Plate 22B a) and produced rooted plantlets without an intermediate callus phase (Plate 22B b), a process which took approximately 12 weeks.

In contrast to the stages of development recorded for the bipolar somatic embryos, however, the malformed long "bipolar" root-like structures did not germinate, but continued to elongate in the PGR free liquid medium. When plated onto PGR free solid MS medium and placed under lights, however, a root developed at the distal end of these structures and small round structures were produced by repeated apical divisions (Plate 23A a - b). The next stages of development were all concentrated in the apical region and included the production of single rooted plantlets from the small round structures and the induction of callus with multiple shoots (Plate 23A c). Repeated subculturing thereafter ensured the establishment of a productive organogenic culture from this callus.

Furthermore large round structures removed from the 11-week-old "ABA pulsed" liquid culture were also observed to develop quite differently from either the somatic embryos or the root-like structures. On transfer to PGR free solid MS medium each round entity produced either a large hairy root or green malformed embryo-like structures (Plate 23B a). The latter were not discrete somatic embryos as they remained attached to the mother structure. Both of these products were identical to those produced from the round structures, induced from the micropylar end of the zygotic embryo explants initially inoculated onto solid MS medium, and which had been transferred to medium with ABA to mature (Plate 13B a). With continued subculturing these large rooted entities developed directly into plantlets while rooted plantlets were regenerated indirectly from the malformed green embryos via callus (Plate 23B b).

It is interesting to note that the morphological stages of development from embryo-like entity to plantlet regeneration recorded for both the malformed root-like structures and the

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**Plate 23.** (A) After transfer to PGR free solid medium (A a) the distal end of the "bipolar" root-like structures elongated and the apex divided repeatedly to produce round structures (Bar = 0.86 mm). (A b) The round structures enlarged (Bar = 1.3 mm) to (A c) produce rooted plantlets and callus. (B) In contrast the large round entities produced (B a) hairy roots and green malformed embryo-like structures (Bar = 0.65 mm) from which (B b) plantlets developed directly or indirectly via an organogenic callus culture respectively.

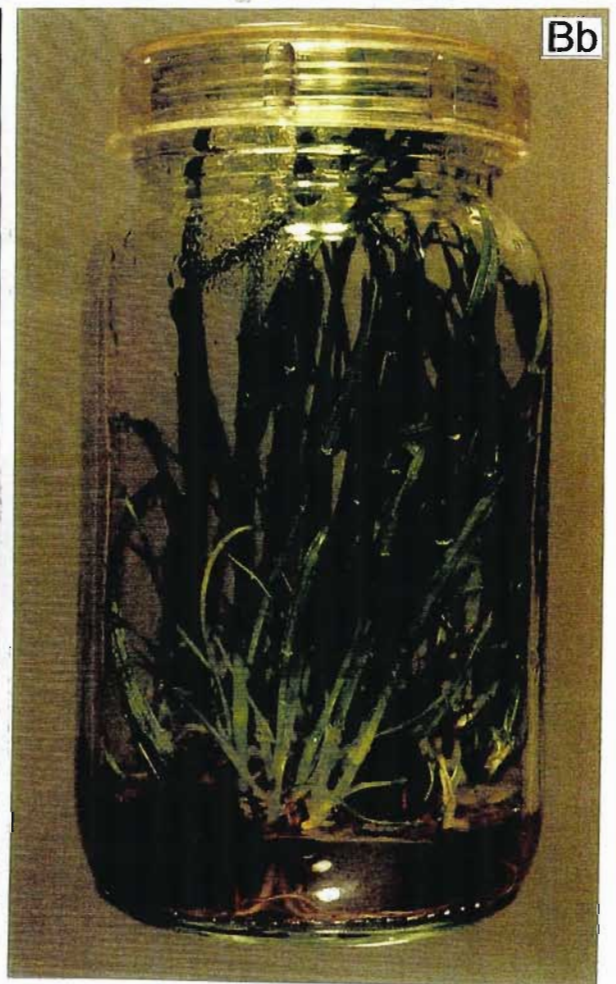
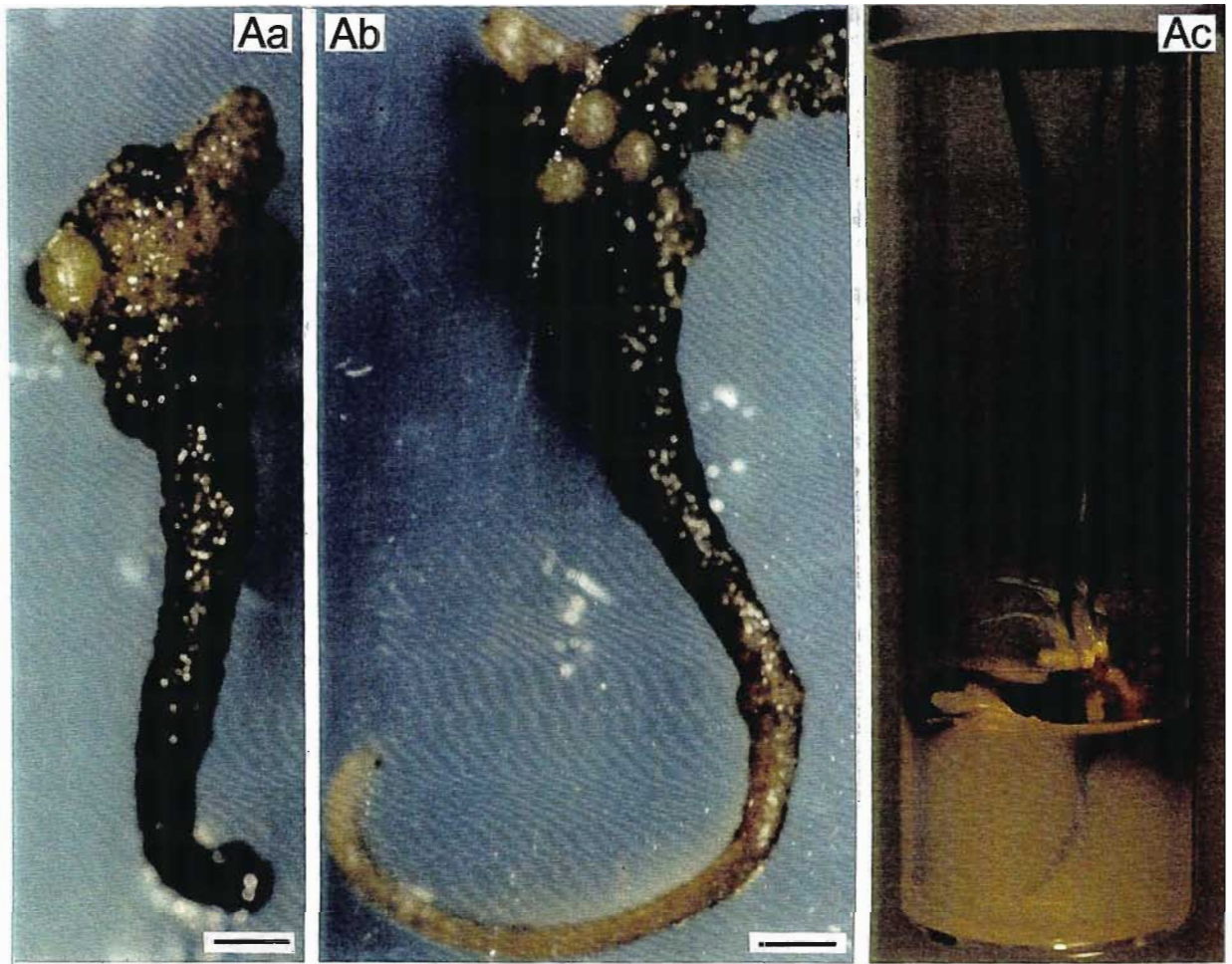


Plate 23

very large round entities were similar to those shown by the clusters of pseudoembryo-like daughter structures obtained from the “ex single round” liquid culture as discussed in Section 6.4.2.1.1 and illustrated in Plates 17 and 18. Development of these latter structures was only induced after their direct transfer from liquid maintenance medium to PGR free solid medium, and from dark to light conditions, without exposure to ABA. It would appear therefore that ABA did not play a role in either their formation or maturation.

In contrast, the authentic bipolar somatic embryos in the “ABA pulsed” liquid culture developed and germinated after exposure to ABA (Plate 22). These were not produced in the “ex single round” liquid culture. Since the small somatic embryos from nodular callus were present in the inoculum used to initiate the “ex nodular callus” liquid culture and not in the inoculum used to initiate the “ex single round” liquid culture, this would suggest that the bipolar somatic embryos in the medium originated from the proembryonic nodular callus and that this culture had therefore retained its embryogenic potential.

#### **6.4.2.2.3      *Changes in Settled Volume of the “ABA Pulsed” Liquid Callus Culture***

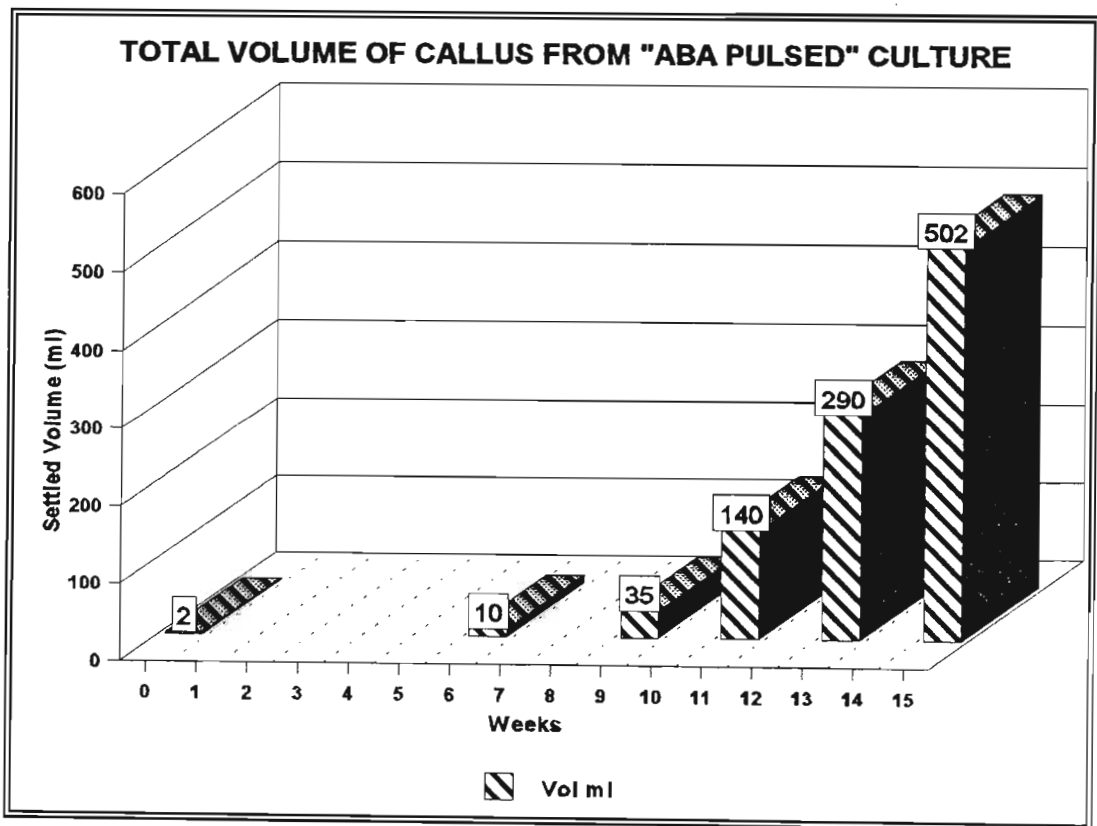
Not only were the morphological changes of the components of the “ABA pulsed” callus culture monitored during its establishment, but also changes in their volume. The 8 replicates of the “ABA pulsed” liquid culture were each initiated from 2 ml of settled “ex nodular callus” culture transferred to 30 ml of liquid MS medium per 100 ml Erlenmeyer flask. After 2 weeks the medium was replaced with PGR free MS medium. Six weeks after inoculation, the average volume of the settled callus in the “ABA pulsed” cultures had increased to 10 ml per flask. At each subculture the components from each flask were washed and subdivided into  $\pm 5$  ml portions, each of which was transferred to a clean culture flask. Initially the increased volume of callus in each flask and subsequent increase in transfers could be monitored for all 8 replicates. After 9 weeks, however, changes in the volume of only one replicate could be monitored satisfactorily not only because of the exponential increase in the volume of callus recorded but also as a result of facility and equipment constraints. The total volume of settled callus recorded over 15 weeks for one replicate increased from 2 ml to 502 ml (Figure 10).

The recorded increase in the volume of the callus was, however, not only as a result of an increase in the number but also in the size of the entities produced. The heterogeneous nature of the callus and size differences between the components at 9 and 15 weeks is illustrated in Plate 24A and 24B. At 9 weeks, with an average embryo size of  $12 \text{ mm}^3$  and a callus volume of 35 ml, the average estimated number of embryos and clusters was

calculated to be 83 per ml or a total of 2905. At 15 weeks, however, the average embryo size had increased to 148 mm<sup>3</sup>, the callus volume from 2 ml to 502 ml and the number of flasks in the replicate to 16. From these figures the average estimated number of embryos and clusters was calculated to have decreased from 83 to 7 per ml, but increased to 3514 in the total volume of 502 ml. It was therefore estimated that 28,112 somatic embryos and embryo clusters of *H. colchicifolia* could have been produced within 15 weeks from the 8 replicates or 16 ml of embryogenic "ABA pulsed" callus culture inoculum.

The large increases in the size of the callus components observed between the 9<sup>th</sup> and 15<sup>th</sup> weeks, however, also resulted in increased malformations, hyperhydricity and a rapid depletion of the liquid medium (Plate 24B). During the same period browning was observed in some of the flasks. This process, probably due to accumulation of phenolics, was irreversible and resulted in black discolouration of the medium and total necrosis of the culture (Plate 24C).

Although a prolific embryogenic culture producing large numbers of somatic embryos and related structures had been established, complete embryo germination and rooted



**Figure 10.** The total settled volume of callus produced from 2 ml of the "ABA pulsed" callus liquid culture measured over a period of 15 weeks.

plantlets, regenerated both directly from somatic embryos and indirectly via an intermediate callus phase, were only induced after transfer to agar solidified medium. For mass production of plantlets via liquid culture to be successful, however, it would be preferable to complete the regeneration cycle in liquid and this aspect was therefore investigated.

#### **6.4.2.2.4 Somatic Embryo Germination and Plantlet Regeneration in Liquid Culture**

Experimentation proved that if (i) the medium of 12-week-old “ABA pulsed” callus liquid cultures was timeously reduced to 2.5 ml just after the somatic embryos and embryo clusters had started to germinate and before they became too large, and (ii) the cultures were transferred to the light, complete germination could be induced *in situ*. Initially the leaves of the uppermost embryos elongated and turned green on transfer to the light (Plate 24D). Thereafter somatic embryos and embryo clusters continued to germinate to produce large numbers of “seedlings” with fully developed leaves and roots, which were transferred to 125 ml Erlenmeyer flasks with minimal liquid medium to dry (Plate 24E). These “seedlings”, subsequently plated on to PGR free MS rooting medium, developed into rooted plantlets (Plate 24F) which were acclimatized as per the procedure outlined in Chapter 4.

As calculated in Section 6.4.2.2.3 it was estimated from the settled volume of callus measured, that a total of approximately 3514 somatic embryos and embryo clusters could be produced in liquid in 16 flasks from an initial 2 ml of inoculum after 15 weeks using the procedures described. This would be an estimated total of 220 embryos and clusters per 100 ml culture flask. However, by timeously reducing the liquid medium of 12-week-old “ABA pulsed” callus cultures and repeated subculturing for 10 weeks, the effects of hyperhydricity were controlled and as a result the average number of fully germinated

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**Plate 24.** (A) The heterogeneous components produced in the “ABA pulsed” culture in PGR free MS medium were fairly uniform after 9 weeks. (B) Enlarged somatic embryos and callus clusters after 15 weeks. (C) Phenolic leachates resulted in black discolouration and total necrosis of the culture. (D) Embryo germination was induced in minimal liquid medium and leaves turned green in the light. (E) Large numbers of “seedlings” with leaves and roots were obtained. (F) Rooted plantlets on PGR free MS medium.

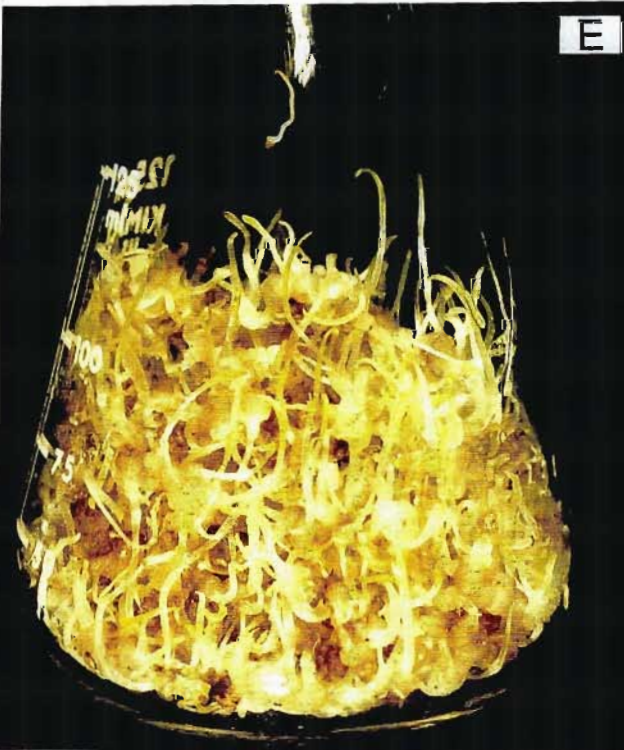
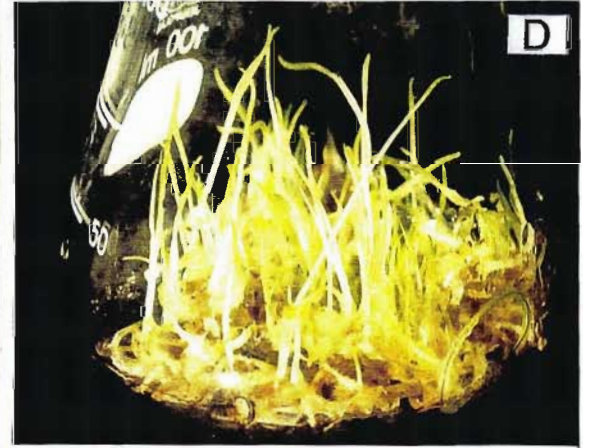
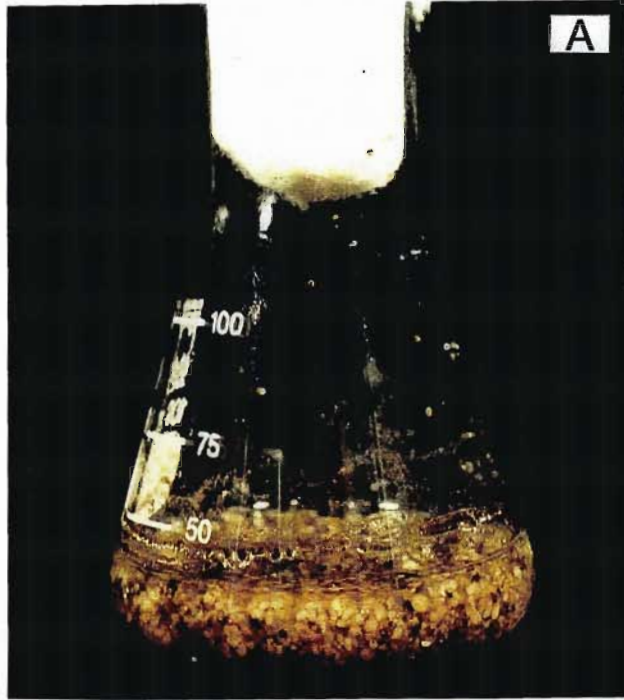


Plate 24

green somatic embryos and embryo clusters actually obtained per 100 ml flask was 235 and 92 respectively. By using this method an estimated 3760 "seedlings" and 1472 embryo clusters could therefore be obtained from 2 ml of embryogenic callus in 25 weeks. Rooted plantlets produced were acclimatized and then cultivated in pots within 6 months.

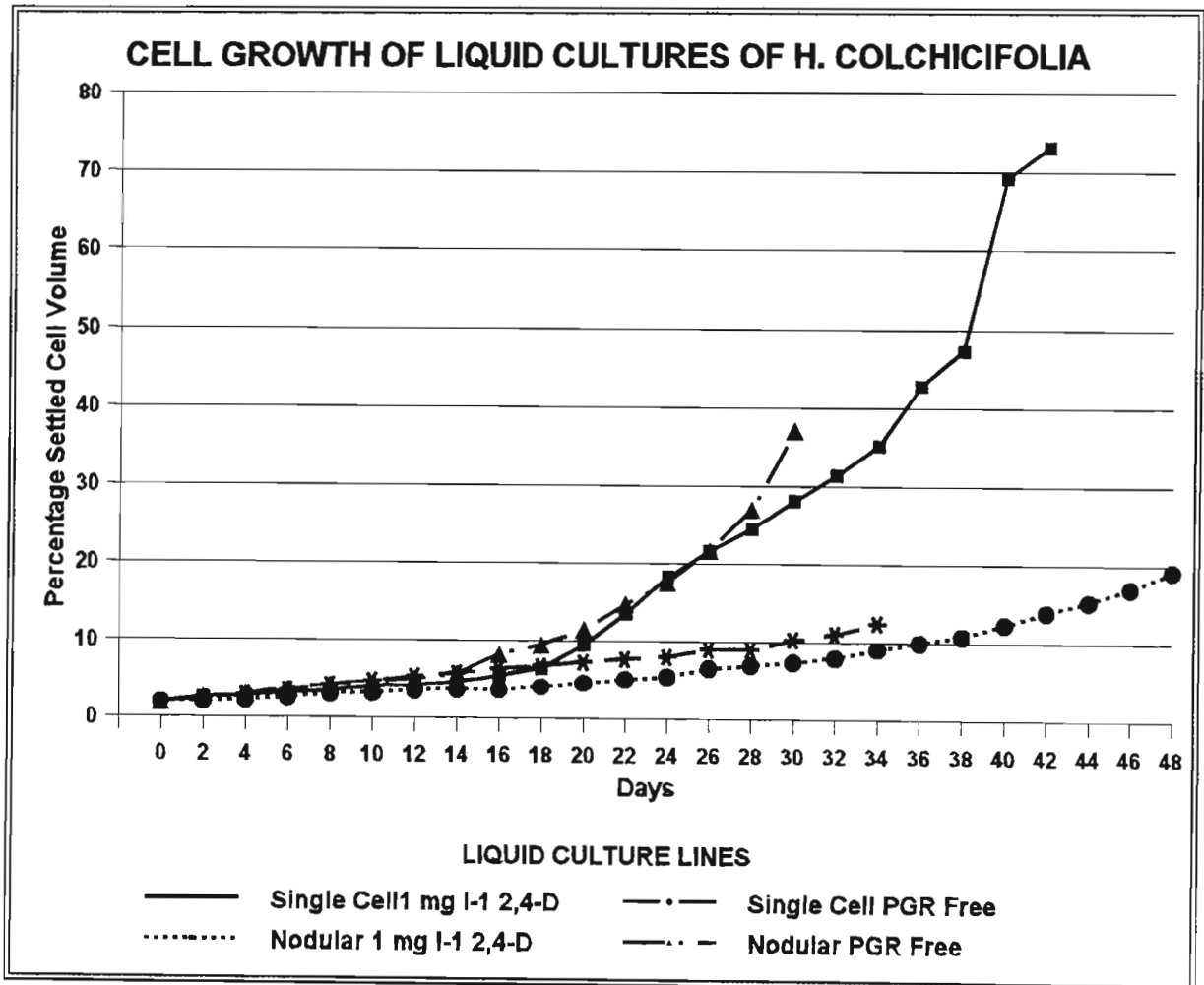
#### 6.4.2.3 Growth Rates of The Principal Liquid Cultures

To quantitatively compare the growth rates of the principal liquid cultures with respect to their induction and maintenance, settled cell or callus volume (%) and therefore cell or callus growth was determined for two "single cell" suspension cultures, one with 1 mg l<sup>-1</sup> 2,4-D and one without PGRs, an "ex nodular callus" liquid culture with 1 mg l<sup>-1</sup> 2,4-D, and an "ABA pulsed" nodular callus liquid culture without PGRs. Furthermore the growth rate was also determined for a fifth more concentrated "single cell" suspension culture with 1 mg l<sup>-1</sup> 2,4-D, using a 250 ml volume side-arm flask. Results are presented graphically in Figures 11 and 12 respectively.

Replicated growth analyses indicated that, in terms of settled cell or callus volume, the cells or callus components of each liquid culture showed a different growth response over time (Figure 11). Initially during the induction phase growth of all of the cultures showed a typical lag phase as recorded every second day for the settled cell volume (%) until the 14<sup>th</sup> day. After 14 days growth of the "ex nodular callus" liquid culture with 2,4-D (maintenance medium) (- - ● - -) and the "single cell" suspension culture without PGRs (- \* -) continued to show only a slight increase. Growth recorded for the "ex nodular callus" culture with 2,4-D (- - ● - -) continued to increase linearly without an exponential increase in volume until the conclusion of the experiment at 48 days, indicating that callus maintenance had been achieved in liquid MS medium with 10 mg l<sup>-1</sup> 2,4-D. Growth of the PGR free "single cell" suspension culture (- \* -) also showed no exponential increase. It increased linearly until, after 34 days, the rapidly dying cell aggregates (Section 6.4.2.1.2) reached a point of saturation and further growth of the culture was totally inhibited.

After 14 days, however, the settled cell volume (%) of the other two cultures increased although not exponentially (Figure 11). The settled cell volume (%) of the "ABA pulsed" nodular callus cultures in PGR free liquid medium (-▲-) showed the most rapid increase, but could no longer be measured after 30 days, however, because the somatic embryos and clusters of pseudoembryo-like structures became too large to be decanted into the side-arms of the flasks. This confirmed the success of the sequential ABA pulse and subsequent transfer to PGR free liquid medium in promoting growth and embryo

maturation in the embryogenic nodular callus cultures. In contrast a sigmoidal growth curve, which reached a point of saturation after 40 days, was obtained for the "single cell" suspension culture with 1 mg l<sup>-1</sup> 2,4-D (—■—). This was typical of a growth curve of a cell suspension culture in which a lag phase is followed by an exponential increase in growth and then a linear phase until a saturation point is reached and growth levels off again. A typical sigmoidal growth curve was also obtained for the more concentrated "single cell" suspension culture with 1 mg l<sup>-1</sup> 2,4-D (Figure 12) but the exponential increase in growth was induced after 7 days instead of the 14 days recorded for the culture with half the concentration. During the exponential and linear growth phases of the single cell suspension cultures of both concentrations, the settled cell volume doubled in 4 to 5 days initially decreasing to 7 days as the culture reached saturation point. Once the single cell cultures were established, the most successful time period between subcultures, and which

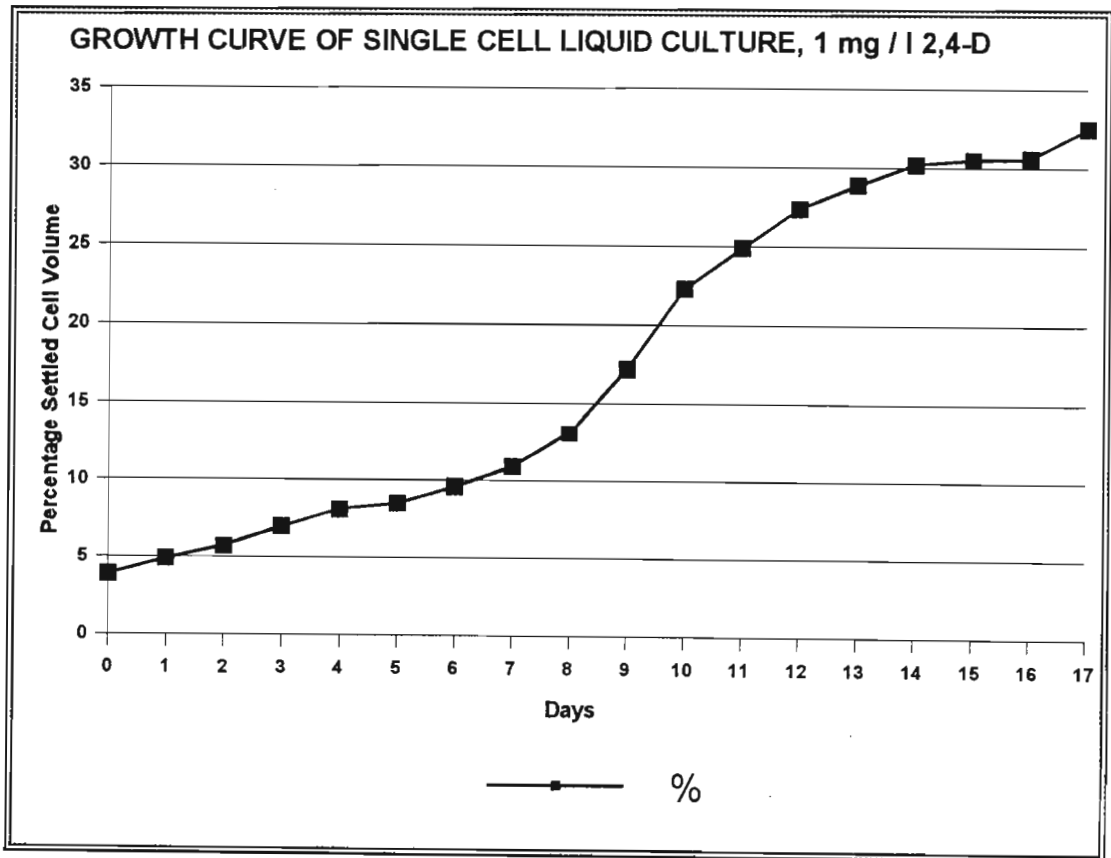


**Figure 11.** Cell growth of two "single cell" suspension cultures, either with 1 mg l<sup>-1</sup> 2,4-D or without PGRs, and two "ex nodular callus" liquid cultures either with 1 mg l<sup>-1</sup> 2,4-D, or from an "ABA pulsed" culture without PGRs.

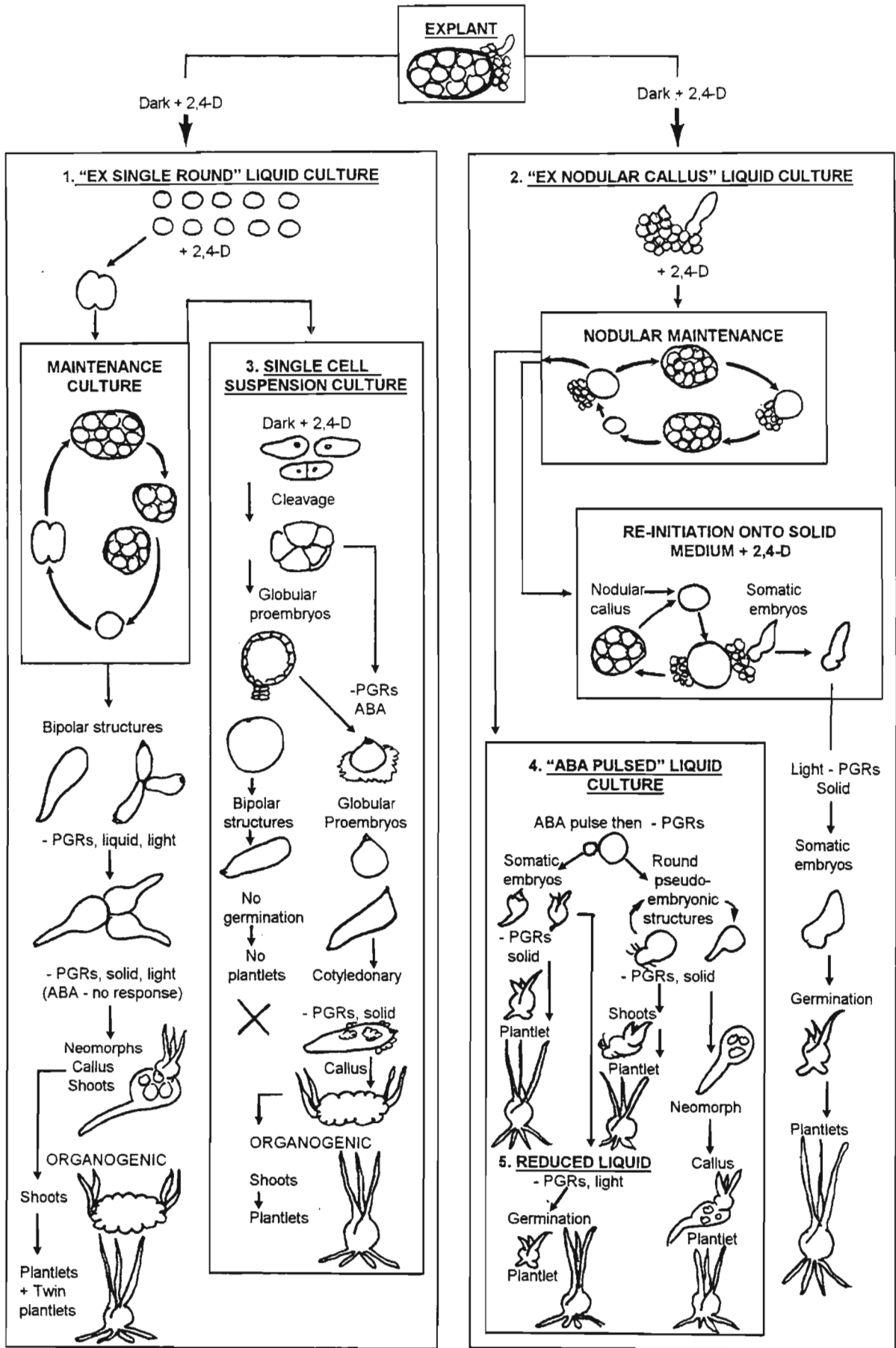
was adhered to throughout this study, was 5 days. Similarly the “ABA pulsed” nodular culture transferred to PGR free liquid medium was subcultured at 5 to 7 day intervals as it also doubled in volume rapidly. The principal maintenance cultures, however, remained constant and therefore only required to be subcultured at 10 to 14 day intervals.

### 6.5 SUMMARY OF THE DEVELOPMENTAL PATHWAYS LEADING TO THE ESTABLISHMENT OF EMBRYOGENICALLY COMPETENT LIQUID CULTURES OF *H. COLCHICIFOLIA*

The results presented in this section clearly demonstrate that embryogenically competent callus liquid and cell suspension cultures of *H. colchicifolia* can be established from secondary and tertiary phase embryogenic explants which had been initiated from excised zygotic embryos and maintained on solid MS nutrient maintenance medium. A diagrammatic overview of the resultant culture lines and micropropagation protocols developed for *H. colchicifolia* showing the various stages of development of the different embryo-like structures, as illustrated in Plates 17 to 24, is presented in Figure 13.



**Figure 12.** Settled cell volume (%) determined daily for the concentrated “single cell” suspension culture in MS medium with 10 mg l<sup>-1</sup> 2,4-D.



**Figure 13.** Diagrammatic pathways showing *H. colchicifolia* plantlet regeneration via somatic embryogenesis in liquid culture.

This shows that the two principal liquid cultures, the “ex single round” and “ex nodular callus” cultures, were established from the large round pseudoembryonic structures and the small proembryonic nodular callus clusters with developing somatic embryos respectively. Both of these cultures were regulated by the addition of 2,4-D to the MS liquid medium and both required a maintenance cycle for continuous proliferation of the large round pseudoembryonic structures in the case of the former culture and the smaller proembryonic nodular structures in the case of the second culture. It was also established that proembryonic nodular callus with somatic embryos did not develop in the “ex single round” liquid cultures, but conversely round pseudoembryonic structures and neomorphs developed in the “ex nodular callus” liquid cultures after a 2-week ABA pulse.

The advantage of solid over liquid medium to monitor the development of isolated structures also became apparent in this instance, as emphasised in the literature, because individual pseudoembryonic structures and neomorphs could not be isolated and immobilized in liquid medium as had been achieved on solid medium (Chapter 5). Critical morphological stages of development of these embryo-like structures could, therefore, not be monitored and their origins in the liquid cultures could not be conclusively traced. Their repeated formation in liquid media as had occurred on solid media (Chapter 5), however, further emphasised their importance in the formation and maintenance of the embryogenic cultures, and that they appear to form an integral part of the somatic embryogenic pathway for the regeneration of *H. colchicifolia* plantlets.

Nodular callus with authentic bipolar somatic embryos was, however, not produced in the “ex single round” liquid culture as had occurred on solid medium. The discrete bipolar embryo-like structures formed in the “ex single round” liquid culture did not germinate but developed into neomorphs from which plantlets were regenerated. Authentic bipolar somatic embryos were not produced at any stage. However, a “single cell” suspension culture was obtained from cells and cell aggregates which developed spontaneously in the MS liquid maintenance medium. All the stages of development from a single cell to globular stage proembryos through to late stage cotyledonary bipolar embryos were identified lending support to the statement that cell suspension cultures are in fact not morphologically homogeneous but heterogeneous (PIERIK, 1987). In contrast to the somatic embryos produced in dicotyledonous cultures, particularly carrot cultures (STEWART et al., 1958 b; THORPE, 1995; RAI and McCOMB, 2002) these embryos did not germinate either spontaneously or by the addition of PGRs to the liquid medium. Later stage embryos were, however, induced after the addition of ABA and transfer to PGR free medium but again did not germinate and plantlets were only regenerated via the induction

of an intermediate callus phase on the cotyledonary stage embryos. Plantlets were therefore only obtained from the “ex single round” liquid culture via the induction of organogenic cultures indicating that the embryos produced were perhaps not true somatic embryos.

In contrast authentic bipolar somatic embryos were obtained from the embryogenic “ex nodular callus” liquid culture either after re-initiation of the globular entities on to solid MS medium or via an ABA pulsed liquid culture transferred to a PGR free liquid medium. These somatic embryos germinated either in MS liquid or after transfer to a solid medium. A highly prolific embryogenic culture in which the somatic embryos and embryo clusters germinated spontaneously was then obtained by reducing the volume of liquid to a minimum. This had the effect of promoting desiccation, a requirement in seed germination, and therefore increasing the rate of survival of the germinating somatic embryos and embryo aggregates. Plantlet regeneration was prolific by following this pathway. It is therefore suggested that by following the route of inducing somatic embryogenesis and nodular callus on solid media and then utilising liquid media to scale-up somatic embryo production, this micropropagation protocol could be implemented for the mass propagation of *H. colchicifolia* plants.

Furthermore the construction of these pathways, not only by monitoring the developmental stages of single entities on solid medium but also cultures as a whole in liquid medium, has shown that detailed fate maps illustrating the origins and morphological stages of development of somatic embryos and related structures can be constructed. The suggestion by VON ARNOLD *et al.* (2002) that genetic markers can then be used to identify the controlling factors in somatic embryogenesis within these fate maps needs to be implemented. The developmental pathways or “fate maps” developed in this study for *H. colchicifolia* could be used as a model system to test this hypothesis.

## **6.6 OVERALL DISCUSSION AND CONCLUSION**

The main objective of this section of the present study to determine whether the production of somatic embryos and plantlets could be increased in a liquid culture was met. Results showed that an estimated 28 112 somatic embryos and embryo clusters of *H. colchicifolia* could be obtained from germinated somatic embryos and embryo clusters in 16 ml of embryogenic “ABA pulsed” liquid culture within 15 weeks.

By comparing the different pathways leading to plantlet regeneration and the morphological stages of development of the various entities produced both on solid and in liquid media, photographically, quantitatively and schematically, similarities and differences between the entities and the media on which they were produced were highlighted. Results also showed that for *H. colchicifolia*, the induction of somatic embryos *in vitro* from zygotic embryo explants was controlled by the addition of plant growth regulators, specifically 2,4-D. Further stages of somatic embryogenesis were also manipulated by the addition and subtraction of plant growth regulators. The repeated formation of pseudoembryonic structures and neomorphs not only on solid but also in liquid media confirmed their importance in the process and that they form an integral component in the *in vitro* somatic embryogenic pathway developed for the regeneration of *H. colchicifolia* plantlets. Furthermore these results have conclusively shown that the pseudoembryonic structures and neomorphs regularly produced in these *H. colchicifolia* embryogenic cultures are not mutants as "non somatic embryo structures" are now commonly referred to (VON ARNOLD *et al.*, 2002).

A statement re-iterated throughout the literature reviewed for this study, however, was that there is a lack of evidence supplied or published to support the many claims that the embryos produced via *in vitro* culture were in fact bipolar somatic embryos or that the plantlets obtained were in fact regenerated via a somatic embryogenic pathway. Cells, somatic embryos, embryo-like structures and neomorphs similar to those produced in this study have been shown to occur in embryogenic cultures of both dicotyledons (BUTTON and BORNMAN, 1971; PHILIP and SCHRAUDOLF, 1986; BHANSALI, 1990) and monocotyledons (VASIL and VASIL, 1981; BLAKE, 1983; KRİKORIAN *et al.*, 1990; SMITH and KRİKORIAN, 1991; TEIXEIRA *at al.*, 1993) but the origins of these structures was not shown.

Evidence to support such claims is difficult to obtain since, as discovered during the course of the present study, the embryogenic cultures established were inclined to be very heterogeneous. This heterogeneity was rapidly re-established in both single cell and callus cultures and also when discrete entities were isolated and cultured separately. In this study, although results have been supported quantitatively, the most compelling evidence showing the origins and further morphological stages of development of the somatic embryos and related structures produced was found to be visual, particularly detailed photographic records. Once collated schematically the developmental pathways could be easily followed for every isolated entity. Photographic evidence was therefore largely relied on and presented as proof that somatic embryos were produced, plantlets regenerated and

*in vitro* somatic embryogenesis induced for *H. colchicifolia* in this study. Furthermore phenotypically normal plantlets were recovered from these embryogenic cultures. It is envisaged that by using the protocols developed in this study, the automation and large-scale micropropagation of *H. colchicifolia* plants in bioreactors as demonstrated by PAEK *et al.* (2001) for the production of several cultivars of *Lilium* for the horticultural industry, is a possibility in the future.

## *Chapter 7*

# **GROWTH AND DEVELOPMENT OF *H. COLCHICIFOLIA* PLANTS REGENERATED *IN VITRO***

## **7.1 INTRODUCTION**

In their review on somatic embryogenesis in woody plants DUNSTAN *et al.* (1995) emphasised that facilities in research laboratories were often inadequate to cope with the transplantation of *in vitro* regenerated plantlets from laboratory to greenhouse conditions in a horticulturally acceptable way. Reported methods on the acclimatization and subsequent establishment of plantlets were therefore generally found to be appropriate for use in limited scale, but not large scale woody plant production. During the 1980s commercial growers had also expressed a similar viewpoint with respect to the application of micropropagation to other horticultural crops (ZIMMERMAN *et al.*, 1986). Mature field grown trees of commercially important woody monocotyledons, notably the Palmae, have since been established from *in vitro* regenerated plantlets, however (RIVAL *et al.*, 1997). By focussing on the development of a model *in vitro* system specifically for the daylily (KRIKORIAN and KANN, 1981), regenerated plantlets have also been successfully transplanted from the laboratory, acclimatized under greenhouse conditions, planted out in field trials and their field performances evaluated (KRIKORIAN *et al.*, 1986). Subsequently many herbaceous monocotyledons have been produced on a large scale for the horticultural industry utilizing protocols developed in research laboratories, including *H. hemerocallidea* (KYTE and KLEYN, 2001).

The development of micropropagation protocols for the domestication and mass production of wild medicinal plants requires dedicated research, however, as little is known about their requirements and adaptation to horticultural or agricultural conditions. Developmental research on a new plant species is not an easy task, however, as was emphasised by KRIKORIAN *et al.* (1986) during their long-term investigation into the *in vitro* propagation of the daylily.

By carefully following and adapting the initial methods outlined for the *in vitro* regeneration of *H. rooperi* (= *H. hemerocallidea*) (PAGE, 1984), protocols suitable for other *Hypoxis*

species were developed and regenerated plantlets were cultivated to maturity (UPFOLD *et al.*, 1992; APPLETON and VAN STADEN, 1995 a, b). Horticultural trials in which it was established that the application of the liquid fertilizer, Multifeed, was beneficial during the acclimatization of regenerated *Hypoxis* plantlets, were also conducted (DIVES, 1991) and implemented. *In vitro* regenerated plants of *H. hemerocallidea* were also successfully established in initial field trials to determine the effect of stress, soil type, fertilizers and herbicides on field grown plants (McALISTER and VAN STADEN, 1995). Results from the present study have shown, however, that *H. colchicifolia* plants respond differently to other *Hypoxis* species not only *in vitro* but also under natural conditions (Chapter 3). *H. colchicifolia* plants, regenerated *in vitro* and acclimatized under greenhouse conditions as outlined in Chapter 4, were therefore planted out under both horticultural and field conditions to determine whether they were able to adapt to cultivation. Since little is known about the growth habit of this species, morphological changes of the corm, leaves and flowers of the young experimental plants were monitored.

## **7.2 MATERIALS AND METHODS**

### **7.2.1 PLANT MATERIAL**

Plant material was sourced from *H. colchicifolia* plants of various ages which had been regenerated *in vitro* via organogenesis. All the plantlets had developed functional roots *in vitro* after which they had been transplanted to 75 mm diameter plastic pots and acclimatized under plastic in a greenhouse as described in Chapter 4. The acclimatized plants had then been transferred initially to a shade-house with 50% shade. Six months after transplantation the older plants had been transplanted to 125 mm diameter plastic pots and placed in an area providing 10% shade.

### **7.2.2 THE EFFECT OF PLANTING CONDITIONS ON THE GROWTH OF *IN VITRO* REGENERATED *H. COLCHICIFOLIA* PLANTS**

Thirty plants, selected at random from among each of the 5-month-old (Plant group ONE) and 21-month-old (Plant group TWO) plants acclimatized as described, were measured and their morphological characteristics recorded. Fifteen plants, randomly selected from each age group, were then repotted into 300 mm plastic pots containing a potting medium of pre-enriched compost : Umgeni sand in a ratio of 2 : 1; v : v (Treatment 1). The plants in pots were then placed in a shade-house under 10% shade and watered regularly. The

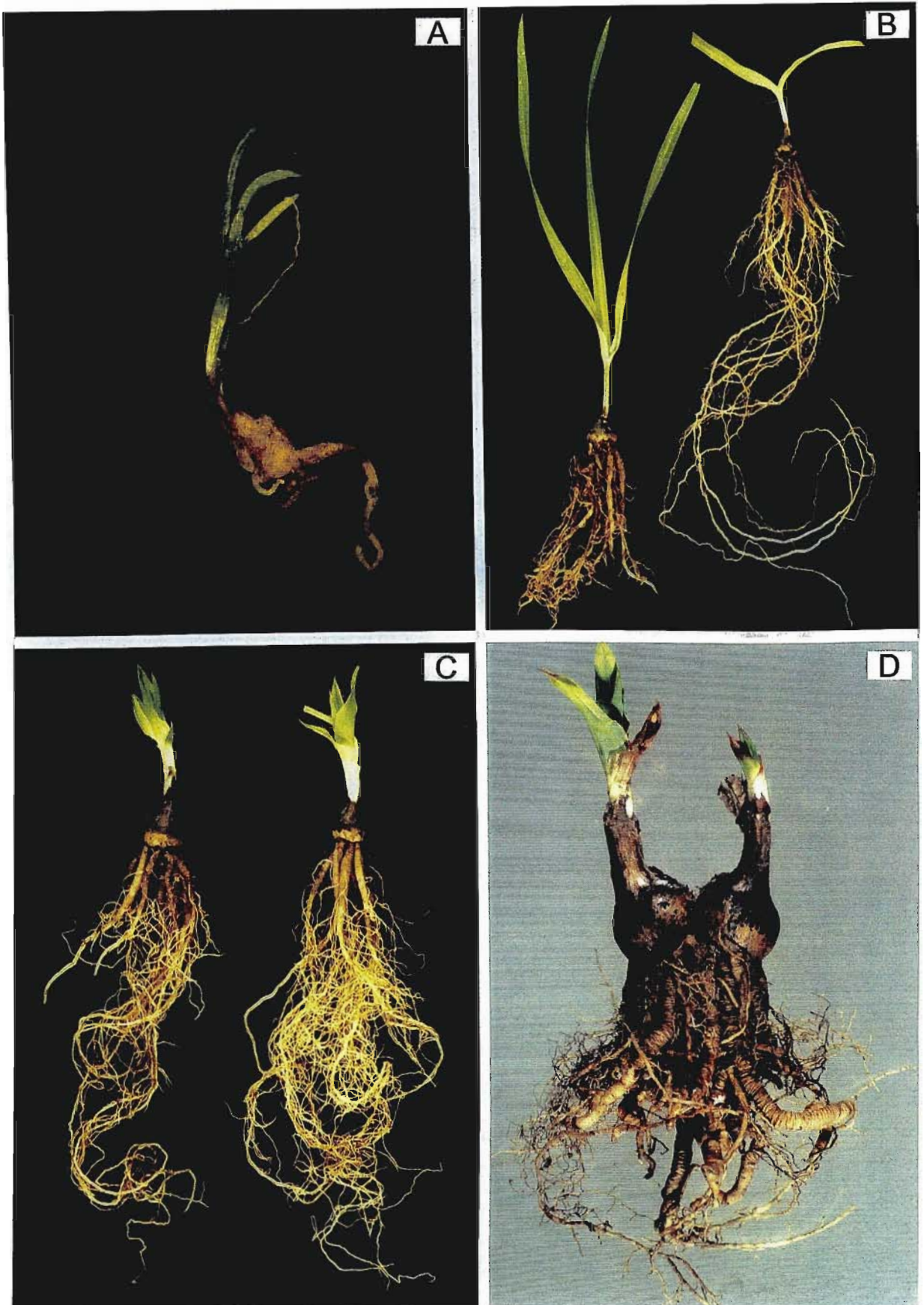
remaining 15 plants from each group were transferred to a garden plot in the Departmental Botanic Garden, University of Natal, Pietermaritzburg (Treatment 2) to simulate growth under natural conditions. The soil in the garden plot was of a shale / clay type which was the same soil type as that in which *H. colchicifolia* grows naturally (Sites 1 and 2, Chapter 3) and the same as that in which *H. hemerocallidea* plants were shown to survive best (McALISTER and VAN STADEN, 1995). In contrast to the pot treatment, the plants in the garden plot were only watered for the first 6 months until established in order to emulate semi-natural conditions thereafter. Flowering and leaf growth were monitored at 4 to 6 month intervals. Corm growth was monitored regularly for the plants grown in pots, but the plants cultivated in the open plot were only harvested after 28 months when final measurements were recorded. At the final harvest, 5 corms were selected at random from the 15 replicates of both treatments for the Group TWO plants only and the mean corm fresh and dry mass determined and percentage water loss calculated for each group of corms.

## 7.3 RESULTS

### 7.3.1 ACCLIMATIZATION OF THE *IN VITRO* REGENERATED PLANTLETS

In comparison to *H. hemerocallidea* (PAGE, 1984), plantlets of *H. colchicifolia* regenerated *in vitro* required a different treatment in order to survive acclimatization. Results presented in Chapter 4 highlighted that plantlets not only required functional roots to be developed *in vitro* prior to transplantation, but also a period under lights to adjust to humidity, light and temperature changes. A higher survival rate was also attained if the period in the mist house was omitted and the plants placed under a plastic humidity tent instead, during which time the leaves were kept as dry as possible. The application of a liquid fertilizer (Multifeed) was sufficient to reverse any signs of chlorosis of the leaves, a problem sometimes found among a small percentage of plantlets produced *in vitro*. Adverse effects of pathogens and fungus gnat were controlled by limiting the application of watering.

Acclimatized *H. colchicifolia* plants produced *in vitro* are illustrated in Plates 25 and 26. Rooted plantlets from germinating somatic embryos (Plate 25A) in liquid culture were fully acclimatized with a corm diameter of 13 mm and a well developed root system when 4 to 5-months-old (Plate 25B). Seven-month-old plants from somatic embryos grown on solid medium are illustrated in Plate 25C. "Twinned" plants produced from neomorphs survived



**Plate 25.** Acclimatized *H. colchicifolia* plants produced *in vitro* from (A) germinating somatic embryos. (B) Four to five-month-old plants with well developed corms and root systems. (C) Seven-month-old plants from somatic embryos grown on solid medium with corms with a diameter of 13 mm. (D) "Twinned" plants produced from neomorphs were also successfully acclimatized.

and were also successfully acclimatized (Plate 25D). All the plants illustrated in Plate 26 were produced via organogenesis from corm explants.

### **7.3.2 THE EFFECT OF PLANTING CONDITIONS ON THE GROWTH OF *IN VITRO* REGENERATED *H. COLCHICIFOLIA* PLANTS**

Data presented in Table 14 shows that growth and development of the corms and leaves was more rapid among the plants in pots (Treatment 1) than those planted in the garden plot (Treatment 2). Similarly the plants in pots began to bloom much earlier at between 25 and 31 months after acclimatization, while only 17% of those planted in the garden plot had bloomed after 49 months. The corm measurements also showed that the corms of the plants in pots had increased in size by approximately 40% more than the corms of the plants in the garden plot after 28 months in cultivation.

At the commencement of the trial the 5-month-old plants had shown no signs of becoming dormant during the first winter season and their leaves remained at an average length of 140 mm and width of 4 to 6 mm throughout the first dormant period (Plate 26A). This juvenile habit persisted until the following season as illustrated by the older 15-month-old plant in Plate 26C. The 21-month-old plants from Group TWO, however, had become dormant during the winter season and their first leaves were just emerging at the time of planting at the commencement of the trial (Plate 26B). The leaves produced annually thereafter increased in length to 727 mm and 78 mm in width after 28 months in pots, but the leaves of the plants in the garden plot only reached a length and width of 380 mm and 64 mm respectively. At the time of harvest 100% of the 49-month-old plants grown in pots in Group TWO had flowered in either the previous or current season. These plants also appeared morphologically more like mature plants (Plate 1) with broader leaves and larger corms, and their fibrous roots were dying back to reveal the contractile roots (Plate 26D).

The effect of horticultural and cultivation practices, compared to simulated natural conditions, on the promotion of corm growth was, however, clearly demonstrated at the final harvest. After 28 months in cultivation, the average fresh and dry corm mass determined for five 49-month-old plants from pots was 78 g and 29 g respectively and for the plants from the garden plot only 29 g and 6 g. The percentage water loss for each group was very similar at 80% and 79% respectively indicating that the differences in corm mass was due to horticultural practices and growth and not to water storage.

**Table 14.** Measurements of the corms and leaves of *in vitro* regenerated *H. colchicifolia* plants planted in pots (Treatment 1, with 15 replicates) and in a garden plot (Treatment 2, with 15 replicates) recorded over a period of 28 months. Each treatment was repeated using 5-month-old plants (Plant group ONE) and 21-month-old (Plant group TWO) plants. Flowering was recorded as the number of plants with a flowering peduncle and expressed as the percentage in flower. Key: A = age in months (M), MTH = month, CD = corm diameter (mm), CL = corm length (mm), LL = leaf length (mm), LW = leaf width (mm), FL = Flowers, F = flowering or inflorescence peduncles present (%), NoM = no measurements taken, Dorm = dormant.

Plant group ONE		Treatment 1 (pots)					Treatment 2 (garden plot)				
		CORM		LEAVES		FL	CORM		LEAVES		FL
A (M)	MTH	CD (mm)	CL (mm)	LL (mm)	LW (mm)	F (%)	CD (mm)	CL (mm)	LL (mm)	LW (mm)	F (%)
5	Aug	13.7 ± 2.1	14.1 ± 1.4	141.4 ± 20.3	4 - 6	0%	13 ± 2.2	14 ± 2.1	137.7 ± 20.6	4 - 6	0%
15	July	NoM	NoM	321 ± 132	NoM	0%	NoM	NoM	136 ± 53	NoM	0%
17	Aug	25.5 ± 7.1	29.5 ± 5.5	Dorm	Dorm	Dorm	NoM	NoM	Dorm	Dorm	Dorm
25	April	NoM	NoM	NoM	NoM	93%	NoM	NoM	NoM	NoM	0%
33	Dec	43.1 ± 4.8	41.5 ± 4.3	595 ± 62	62 ± 16	54%	24.6 ± 5.2	30.1 ± 4.2	356 ± 89	44 ± 15	0%
TWO											
21	Aug	17.3 ± 4.1	15.6 ± 3.8	45.8 ± 27.9	NoM	0%	14.1 ± 4.8	15 ± 4.3	121.5	14.3	0%
31	July	NoM	NoM	489 ± 61	NoM	47%	NoM	NoM	254 ± 69	NoM	0%
33	Aug	37.1 ± 6.8	36.2 ± 5.8	Dorm	Dorm	Dorm	NoM	NoM	Dorm	Dorm	Dorm
41	April	NoM	NoM	NoM	NoM	100%	NoM	NoM	NoM	NoM	0%
49	Dec	53.7 ± 4.3	43.7 ± 5	727 ± 74	78 ± 6.9	93%	34.1 ± 7	35.3 ± 5.1	380 ± 148	64 ± 20	17%

**Plate 26.** Plants used in the cultivation trial. (A) 5-month-old and (B) 21-month-old *H. colchicifolia* plants at the commencement of the trial. (C) A 15-month-old plant showing the juvenile habit common to *H. colchicifolia* plants. (D) 49-month-old plant with first flowers at the time of harvest.

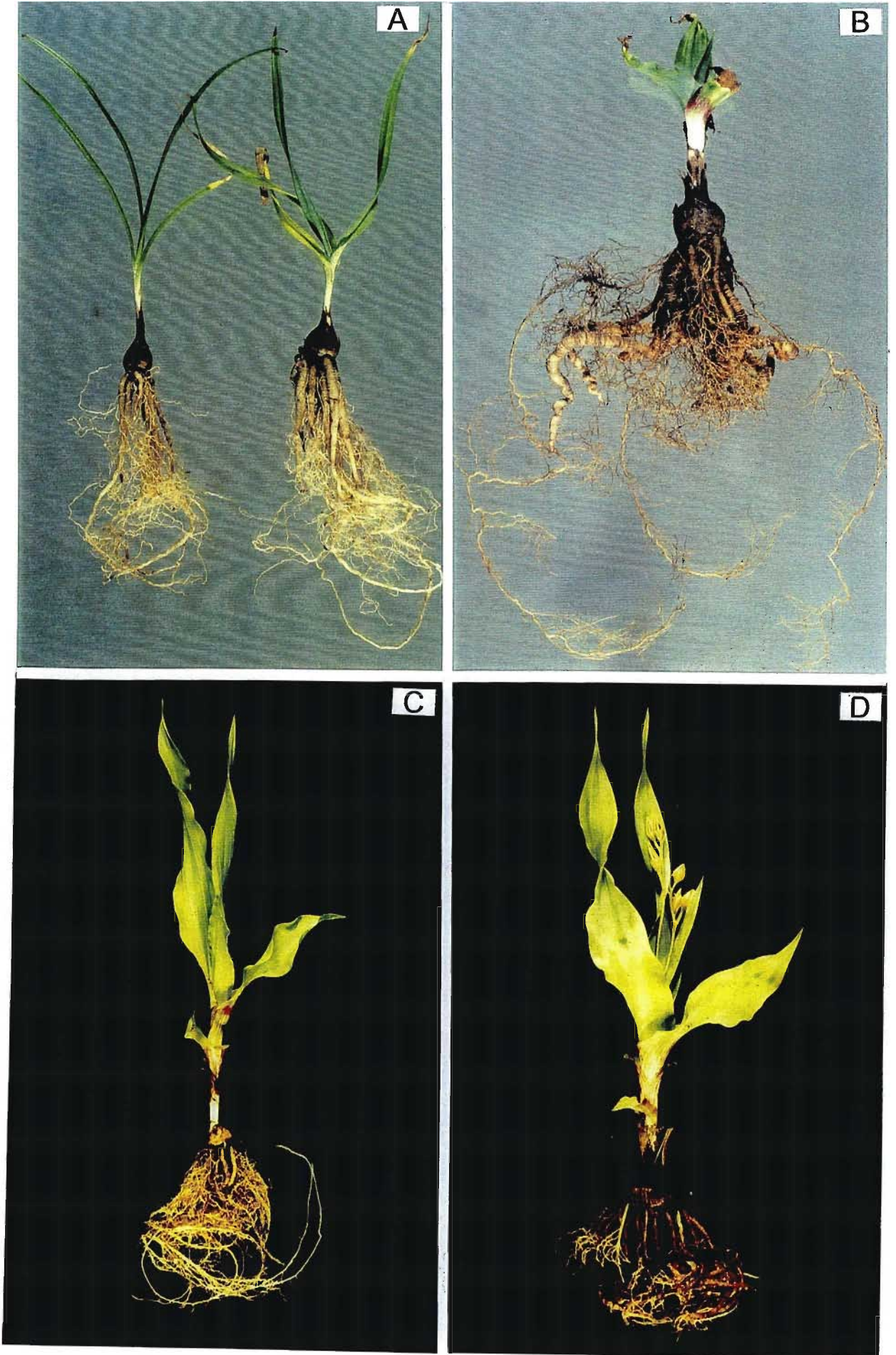


Plate 26.

## 7.4 DISCUSSION

As discussed in Chapter 2, there does not yet appear to be agreement on suitable diagnostic characters for species delimitation within the genus *Hypoxis*. In the cultivation trial great variation was recorded for both the leaf and corm characters measured for the *H. colchicifolia* plants. These results also support the claim by NORDAL *et al.* (1985) that leaf characteristics are totally unsuitable for species delimitation of *Hypoxis*. The *H. colchicifolia* plants also showed a distinct juvenile form for about 3 years prior to the production of the first flowering peduncles and supports the findings in the population surveys presented in Chapter 3. This seasonal variation in the morphology of *H. colchicifolia* plants further emphasises the importance of comparing *Hypoxis* specimens at similar stages of development, either age or seasonal, for accurate identification as found by HERNDON (1988).

Results from the surveys in Chapter 3 showed that *H. colchicifolia* plants did not survive the management practices of mowing and frequent hot fires. Observations in the cultivation trial showed that the young plants planted in the garden plot, and therefore comparable to those in natural areas, only flowered for the first time when 49 months old. This would support the observation that annual grassland management practices are detrimental to these plant populations as the plants are destroyed prior to flowering and seed production. Burns in autumn were not detrimental to survival of *H. colchicifolia* plants. Observations in the cultivation trial confirmed therefore that later burns in autumn would be conducted after flowering and seed production and support the suggestion that all geophytes should not be lumped together as "forbs" but considered individually when grassland management programmes are planned for natural areas.

The regular dormant periods, continuous leaf growth and flowering times recorded for all of the plants in the cultivation trial, both under natural and horticultural conditions, showed conclusively, however, that the *in vitro* regenerated *H. colchicifolia* plants had retained the geophytic growth pattern as described for wild specimens by WOOD (1976). It can therefore be concluded from the field and horticultural performances of the *in vitro* regenerated plants that the micropropagation protocols developed in this study for the mass production of *H. colchicifolia* plants were successful.

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