TISSUE CULTURE OF SELECTED INDIGENOUS MONOCOTYLEDONS

By

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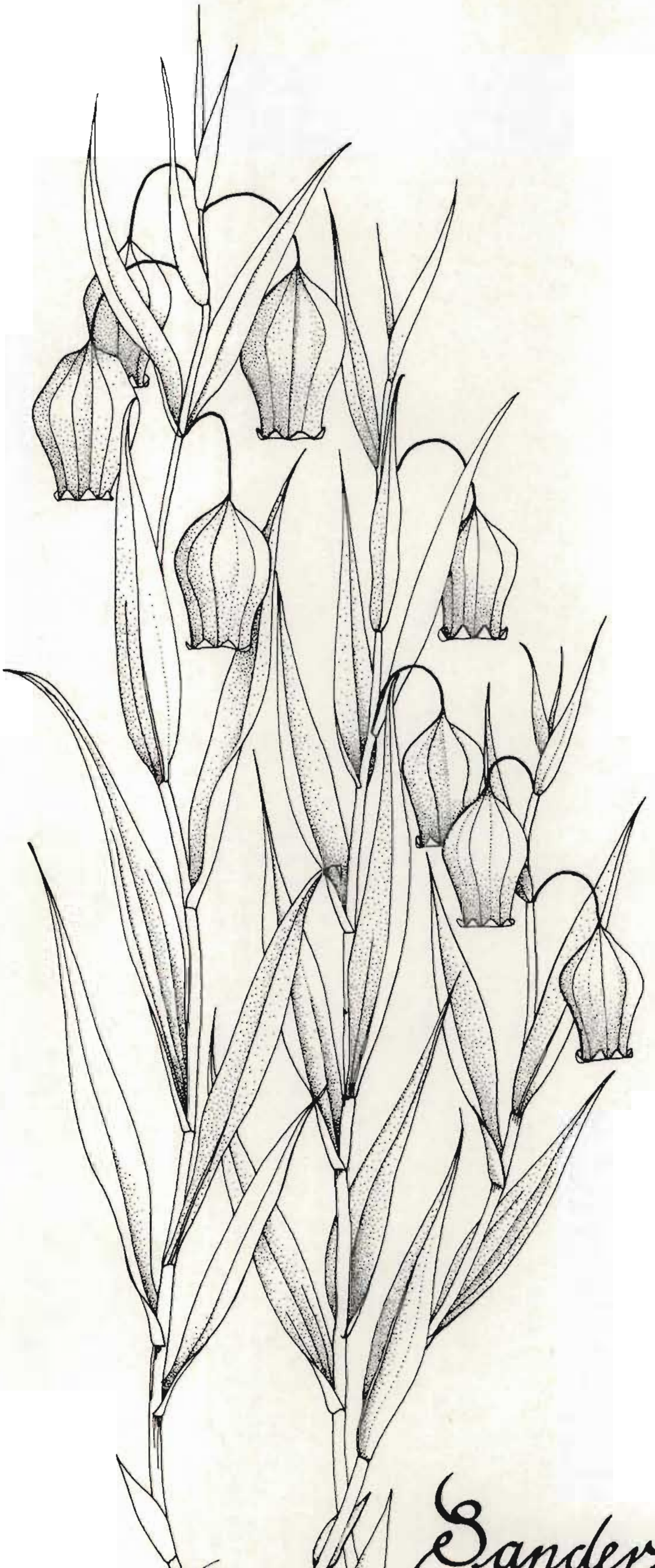
TO MY PARENTS

Clivia miniata

Gloriosa superba



Sandersonia aurantiaca

"One can thus construct the following two hypotheses concerning the origin of organic phenomena such as growth: either this origin is a function of the organism as a whole, or growth does not take place by means of any force residing in the entire organism but each elementary part possesses an individual force, a separate life. ..."

"If therefore, we find that some of these elementary parts which do not differ from others, are capable of being separated from the organism and of continuing to grow independently, we can conclude that each of the other elementary parts, each cell, must possess the capacity to gather new molecules to itself and to grow, that therefore each cell possesses a particular force, an independent life, as a result of which it too would be capable of developing independently if only there be provided the external conditions under which it exists in the organism."

Schwann 1839 (extracts taken from WHITE, 1943)

PREFACE

The experimental work described in this thesis was carried out in the Botany Department, University of Natal, Pietermaritzburg, from January 1985 to July 1988. The work was supervised by Professor J. Van Staden.

These studies have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, are the result of my own investigation.

Finnie

Jeffrey Franklin Finnie
December 1988

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ABSTRACT

Components of the South African indigenous flora are disappearing at an alarming rate, due to pressures on land use. The flora is protected by proclamation of reserves and conservation legislation, however these measures can never be wholly successful. For these reasons, methods for propagting *Clivia miniata*, *Gloriosa superba* and *Sandersonia aurantiaca* using *in vitro* techniques were investigated.

The highly sought after *Clivia miniata* var *citrina* can be successfully cultured using fruit and floral explants. Use of these explants may limit the number of plants produced in culture due to the seasonal nature of flowering. *Gloriosa superba* and *Sandersonia aurantiaca* can be propagated using corm explants, with subsequent *in vitro* stimulation of cormlet formation. To establish a successful tissue culture procedure an integrated approach to all aspects of the culture is necessary. Sterilization techniques should be empirical and specific for each species and explant. The most critical factor in establishing a culture technique is the choice of a suitable explant. Without a suitable explant the success of the culture procedure may be severely limited. Nutritional and environmental variation may modify the explant response in culture, but initial culture response can be directly related to the origin of the explant, particularly, size, time of the year, age and physiological status.

Since the discovery of colchicine in *Gloriosa* by CLEWER, GREEN and TUTIN (1915) a number of researchers have put forward the idea that *Gloriosa* would serve as a source of colchicine. The present trend in biochemical production is via artificial synthesis, however many desirable compounds still have to be extracted from plant material for biochemical production. The utilization of plant cells

that are cultured in vitro provides a viable alternative to the problems involved in the production of chemical compounds.

Levels of colchicine in Gloriosa and Sandersonia are very similar, in the range of ± 0,9%. From evidence presented by BELLET and GAIGNAULT (1985), levels of colchicine in the two study species is much higher than the recorded level (0,62%) of Colchicum. This higher level of the alkaloid makes these two plants a viable source for commercial colchicine production.

Levels of colchicine recovered from in vitro grown roots and callus was 10 - 20 times lower than that found in in vivo tissue. Levels of colchicine extracted from plantlets grown in vitro was the same as that normally recorded for parent tissue. Higher levels of colchicine in malformed roots adds to the evidence that differentiation increases colchicine production in Gloriosa tissue in vitro.

It has been shown that Gloriosa and Sandersonia tissue can synthesize colchicine in vitro. The extent to which the cells' synthetic capacity can be enhanced has yet to be determined. However, research into speedier and more wide ranging methods for metabolite production in culture is receiving attention throughout the world.

CONTENTS

Page

CHAPTER 1

INTRODUCTION

South Africa's wild flowers have a scientific as well as an aesthetic appeal. While many wild plants are grown as ornamentals, some have been hybridised, selected and improved, others are also of scientific interest. Unfortunately, components of the South African indigenous flora is disappearing at an alarming rate, due to farming, urban development and other pressures on land use. The indigenous flora is protected by the proclamation of reserves and strict conservation legislation. However, these measures can never be wholly successful. For these reasons it is necessary for research to be undertaken on Southern African endangered wild flowers rather than the imported exotic flora.

Habitats necessary for many wild species are diminishing rapidly. Of the Amazon Basin's four million kilometers squared of natural forest as much as 100 000 kilometers squared is cleared each year (MYERS, 1976). By the early 1970s, 40% of the tropical moist forests had been lost and their area was shrinking by about eleven million hectares a year (HALL, DE WINTER, DE WINTER and VAN OOSTERHOUT, 1980). The destruction of habitats on this scale endangers many plants. RAVEN (1976) estimated that about 50 000 species or 30% of the world's tropical flowering plants, will have reached endangered or extinct status by the end of the century. MYERS (1976) reports that 20 000 flowering plants are thought to be at risk.

Not all nations are in a position to demarcate large tracts of land as conservation areas, and thus a strategy to conserve the world's wealth of species must be made economically acceptable and politically practical, if it is to withstand competitive pressures from other forms of land use (MYER, 1976).

The survival of threatened species has immediate value, with genetic reservoirs playing an important role in agricultural development, as well as in the fields of medicine, pharmaceutical and industrial processes in the advanced world which has the capacity to utilize these varied genetic resources. A group of important drugs, the alkaloids, are derived from nitrogenous sources found in certain plants. To date only two percent of the planet's estimated 200 000 flowering plant species have been screened for alkaloids, these plants producing more than 1 000 different forms (MYER, 1976; ROBINSON, 1974).

Until recently the growth rate in South Africa was one of the highest for its population size in the world, with a monthly increase of around 60 000 people (TALBOT, 1978). Together with an increase in population goes an increase for greater food production and other essentials, making agriculture one of the major threats to rare plants in the region (HALL and RYCROFT, 1979). An intensive survey has been mounted in the greater part of the Cape Province to estimate the conservation status of the region. In this area there are 38 species presumed recently extinct, 68 endangered, 84 vulnerable, 278 rare, 218 indeterminate and 773 species uncertain but suspected to be in hazard (HALL, 1978).

Numerous cases have been reported where the habitats and the plants contained within them have been partially or completely destroyed. Gladiolus aureus is now down to 35 plants in the wild, and its habitat is under pressure from numerous sources. Moraea loubseri, discovered as a single population in 1973, has had its habitat destroyed by quarrying and is now probably extinct in the wild (HALL and RYCROFT, 1979).

> "Fascination with South African plants is currently undergoing a renaissance; a renewed interest that has been stimulated by a sudden awareness

> of the endangered status of the plants in the wild and by an appreciation of their undoubted economic value." (STIRTON, 1980).

Commercial exploitation and breeding of South African flora has until recently been dominated by Europe and America, with well known examples being: Agapanthus Freesia, Gladiolus, Gerbera, Kniphofia and Strelitzia. However, there has recently been a change towards the breeding of indigenous plants for exploitation on a large scale. This change has been brought about to a large extent by a concern for conservation of rare and endangered plants and a realization of the economic potential of these plants from both a horticultural and natural product approach to utilization.

STIRTON (1980), lists a number of petaloid monocotyledons that show scope for selection as cut flowers and ornamentals, these include: Aristea, Babiana, Brunsvigia, Bulbinella, Clivia, Crinum, Dietes, Eucomis, Geissorhiza, Hypoxis, Ixia, Moraea, Rhodohypoxis, Sandersonia, Scilla, Tritonia, Watsonia and Xerophyta, and continues that there is room for improvement in the more widely known genera such as Agapanthus, Amaryllis, Cyrtanthus, Gladiolus, Nerine and Strelitzia.

The petaloid monocotyledons are "showy plants" (STIRTON, 1980), and are vulnerable due to over collection and exploitation. This problem is compounded by competition from alien plant invader species, land use and abuse and their restricted distribution. Of particular importance are the specialized genetic characteristics of many localized species with attributes associated with restricted range, which is the factor that makes them vulnerable for extinction (MYER, 1976).

In this investigation three petaloid monocotyledon genera were selected for investigation, Clivia miniata Regel., Gloriosa superba L. and Sandersonia aurantiaca Hook. Each genus has its own cultural and ecological problems, with both Clivia and Sandersonia being classified as threatened species.

CHAPTER 2

LITERATURE REVIEW

2.1 The Plants and their Uses

Clivia Lindley., belongs to the family Amaryllidaceae which occurs all over the world, and is comparatively well developed in South Africa where at least 250 species are found. Clivia is a small genus consisting of four species, all of which are endemic to South Africa. The species are: Clivia caulescens Dyer, Clivia gardenii Hook., Clivia miniata Regel and Clivia nobilis Lindl. Two English botanists Lindley and Hooker separately published on the same day in October 1828, a new genus based on the same plant. Lindley called the new genus Clivia while Hooker named it Imantophyllum. In 1830 Roemer and Schultes (Syst. Veg. 7:892) chose the name Clivia and reduced Imantophyllum to synonymy (OBERMEYER, 1972). Lindley established the genus Clivia, named in honour of Lady Clive, Duchess of Northumberland, saying that such a compliment had been long overdue to the noble family of Clive. The plant Lindley described was Clivia nobilis, from plant material collected by James Bowie in the Eastern Cape and grown in the hot houses at Kew and at Syon House, the residence of the Duchess of Northumberland (DUNCAN, 1985).

When Lindley encountered the second species in 1854 (Clivia miniata) he named it Vallota (?) miniata after the French botanist Pierre Vallot (BATTEN, 1986) and miniata from the Latin meaning 'coloured with red lead or cinnabar'. DUNCAN (1985) reports that this species was initially called Imantophyllum (?) miniata by Hooker who published in Curtis' Botanical Magazine in 1854. The plants described by Lindley were received from Backhouse's nursery in York, which were said to have been sent by Andrew Steedman from Natal. In February 1854 Backhouse exhibited a live specimen at a meeting of the Horticultural Society in London (POLE EVANS, 1921; BATTEN, 1986). The plant aroused much interest, and this led to it becoming a popular pot plant. In 1864, E.A. von Regel recognised that this plant belonged to the genus *Clivia* and renamed the plant *Clivia miniata* in Gartenflora. *Clivia gardenii* was described by Hooker in 1856 and *Clivia caulescens* was only relatively recently described by Dyer in 1943 (DYER, 1943) (Figure 2.1).

Clivia miniata Regel bears an inflorescence of red to orange flowers, many hybrids have been cultivated, but the most valuable form of *Clivia* is the yellow flowering *Clivia miniata* var *citrina* Watson. The first published record of a naturally occurring yellow form of *Clivia miniata* was made in 1899 by W. Watson in volume 25 of "The Gardeners Chronicle" (DUNCAN, 1985). The plant described was donated to Kew by Rev. W.H. Bowden, having been found wild in Zululand. Mention was also made of a yellow *Clivia* which flowered in a Mrs Rogers' garden in Cornwall in 1897. POLE EVANS (1931) described this plant as *Clivia miniata* Regel var. *flava* Phillips var nov., a forma typica floribus flavis differt. The plant was collected by a Mr C.R. Saunders in the Eshowe forest, Zululand, who reported that one or two plants were collected in 1888, and a number of plants have been propagated from these originals, all turned out yellow indicating a pure strain!

> "The fact that all plants raised from seed have yellow flowers indicates that we are dealing with a pure strain, but except for the colour of the flowers we have not been able to detect any tangible characters which would separate it from *Clivia miniata* and have therefore kept it as a yellow variety." (POLE EVANS, 1931).

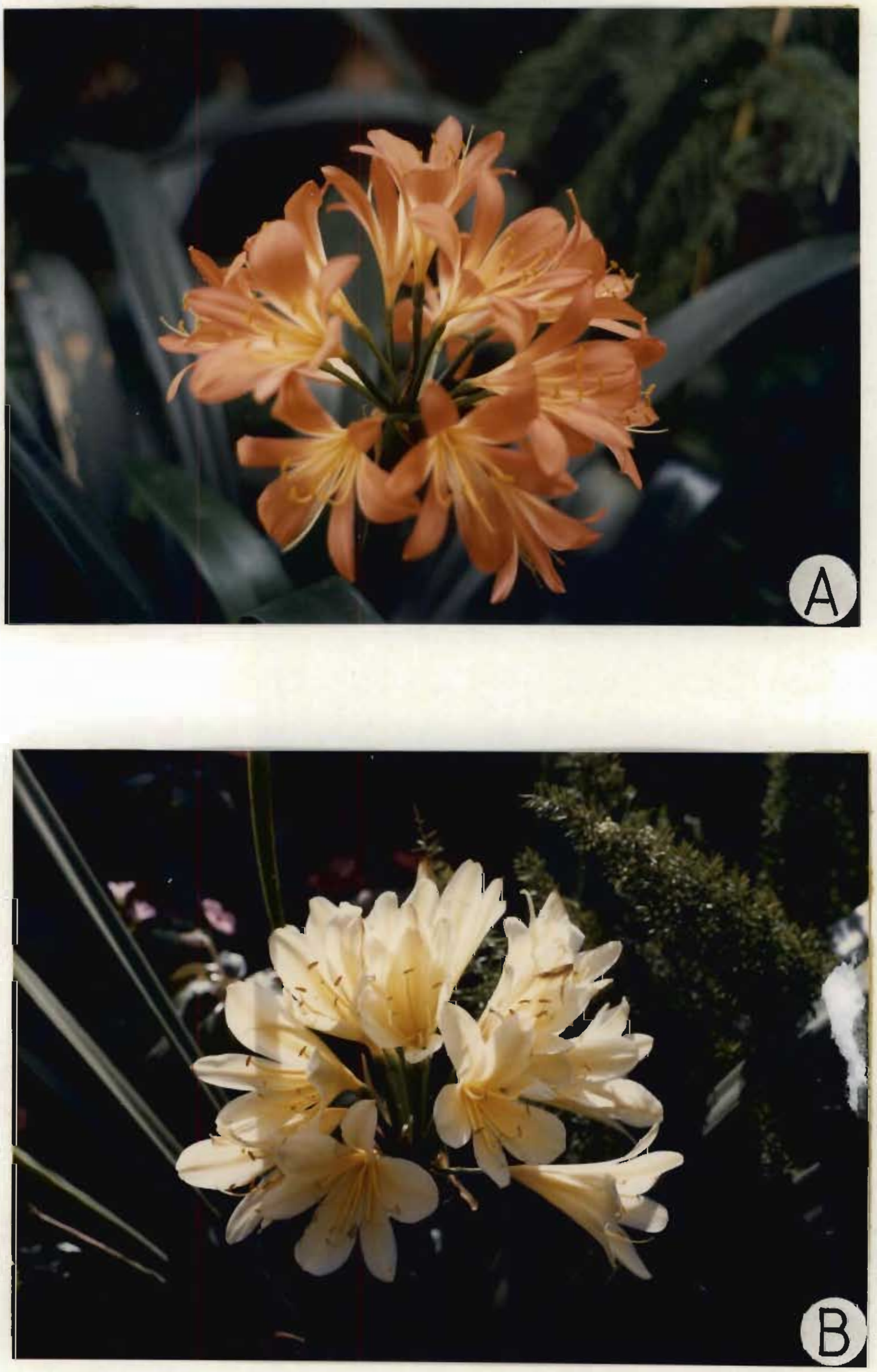


Figure 2.1 Clivia miniata

a) Clivia miniata

b) Clivia miniata var citrina

Contrary to reports that var *citrina* is true breeding, progeny from seeds have been found to be heterogenous with only a small percentage bearing yellow flowers. Breeding is further complicated by a long maturation period of five to seven years, poor flower quality and poor vegetative growth from selfed plants (TARR, pers. com.). "There are a number of reports of the forms of var. *citrina* which do come true to type from seed, while there are as many to the contrary." (DUNCAN, 1985).

The name var. *flava* Phillips is rejected in preference to the earlier published var. *citrina* [other variety names such as var. *sulphurea* and var. *aurea* have no standing (DUNCAN, 1985)]. The Kirstenbosch collection has two distinct forms of var. *citrina* the cultivar "Kirstenbosch Yellow" a slow growing form with broad leaves, light yellow reflexed petals and a fragrance, and "Natal Yellow" with narrower leaves, darker yellow tubular petals and no scent.

Many hybrids have been produced in Europe and America. The best known of these is *Clivia* x *cyrtanthiflora*, which is a hybrid cross of *Clivia miniata* and *Clivia nobilis* (ELIOVSON, 1984).

Clivia miniata is an evergreen herb with a fleshy rhizome, the roots are contractile and have vessels with scalariform perforation plates. It has many leaves which are strap shaped, acute and slightly narrowed at the base, with the leaf bases forming a distinct swelling just above the rhizome, glabrous and bright green. The peduncles are shorter than the leaves, compressed and sharply two edged. Inflorescence is a 12 - 20 flowered umbel, with spathe valves broad, ovate-oblong and membranous, floral bracts are linear with an erect flower. The perianth is straight or slightly curved, with a short tube, lobes are connivent or somewhat spreading, the three outer are narrower than the three inner. Stamens arise from the throat of the perianth tube, with filiform filament and versatile anthers. The ovary is globose with 5 - 6 ovules in each loculus and the style elongate, slender and tricuspidate at a stigmatose apex. Fruit is a bright red berry with the number of seeds ranging from one to few, these seeds do not enter a dehydration stage but remain water rich (POLE EVANS, 1931; DYER, 1976; DAHLGREN, CLIFFORD and YEO, 1985).

Clivia miniata, also known as Boslelie, Benediction lily, Flame lily, Kaffir lily, St Johns lily or September lily (SMITH, 1966; ELIOVSON, 1984) is found distributed from Haga Haga in the Eastern Cape through Transkei into Natal (Figure 2.2) where the plant grows in coastal areas as well as inland along streams, rivers and forests. Flowering time is from August to October, but occasionally out of season inflorescences appear, with flower colour varying from red to orange (or the rare yellow citrina variety Figures 2.1a and b). The plant prefers dappled sunlight as the plant in total shade seldom blooms. The plants multiply from the base forming large groups in the wild. Conventional propagation methods are by division of plant groups, seeds, removal of suckers from the parent plant or stimulation of sucker production by destroying the apical bud. These methods compounded by long maturation periods before flowering, poor flower quality and vegetative growth, makes the conventional plant propagation techniques slow and unprofitable. Thus an in vitro technique would not only increase multiple plant production but also facilitate breeding of the species.

Clivia miniata is known to the Zulus as uMayime, umMbhezi, ubuhlungu-benamba or ubuhlungu-beyima, and the plant is used as a remedy for numerous complaints (Table 2.1). These cures range from snake bite to use as an aid for conception. The Clivia, a remedy for impotence or barrenness, is included in an infusion to hasten and help delivery. In the infusion 27 plants are used together with the

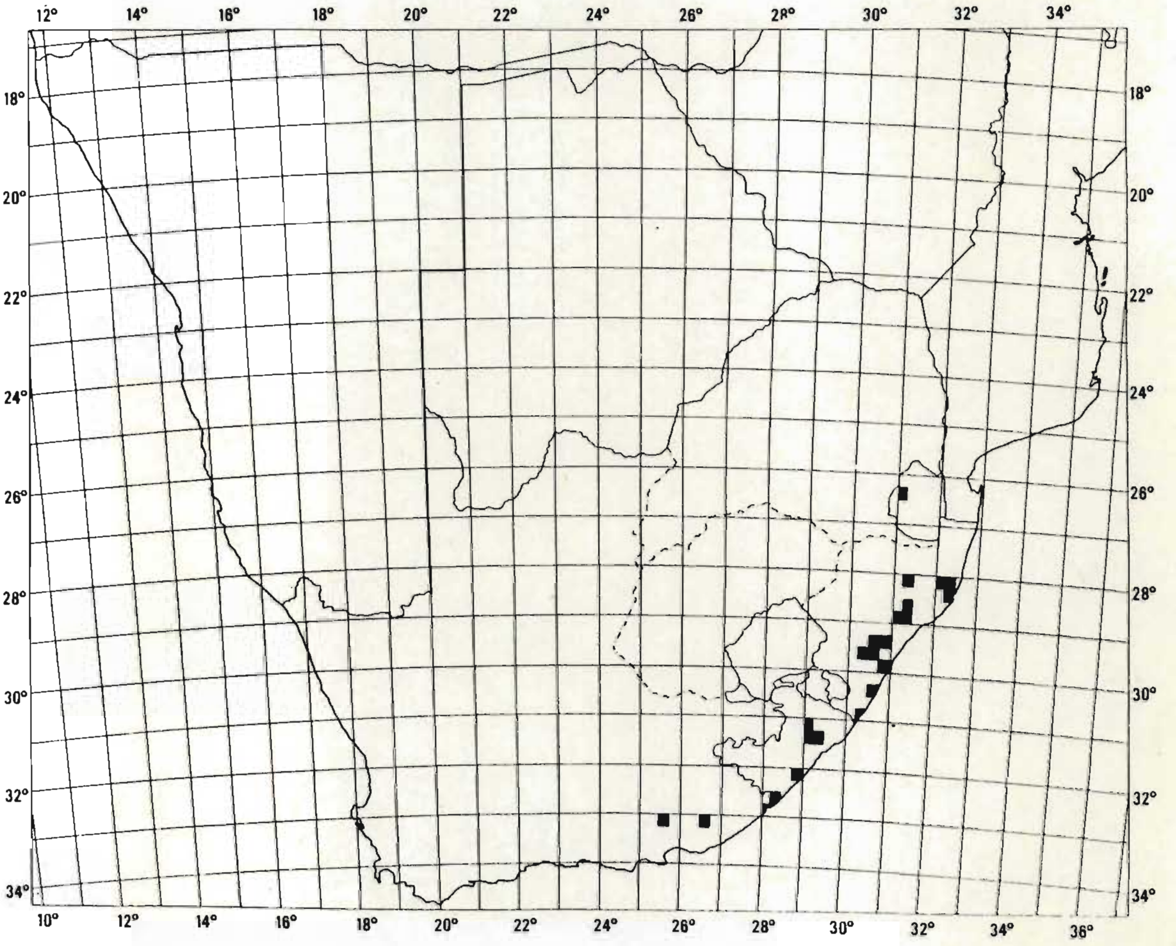


Figure 2.2 Natural distribution of *Clivia miniata* in Southern Africa

placenta of horse and the skin of a crocodile. The infusion is kept in a covered pot and a spoonful is drunk periodically during the last three months of pregnancy. It should not be drunk or looked at by any other person, or the child would take on the appearance of that person (PALMER, 1985).

Table 2.1 Use of *Clivia*, *Gloriosa* and *Sandersonia* by indigenous populations

Species	Tribe/ Area	Plant part	Use	References
Clivia miniata	Zulu	Red berry Root	Febrile complaints - used as an emetic	BRYANT, 1966 WATT and BREYER-BRANDWIJK, 1962.
	Zulu	Whole plant	To facilitate delivery or to procure it when retarded	BRYANT, 1966 PALMER, 1985 WATT and BREYER-BRANDWIJK, 1962
	Zulu Xhosa	Roots	Used to bathe the wound as a snake bite antidote	BRYANT, 1966 WATT and BREYER-BRANDWIJK, 1962
	Zulu		Stomach and intestinal complaints	BRYANT, 1966
	Zulu		A cure for hysteria	BRYANT, 1966
	Zulu	Whole plant	An infusion sprinkled around homes as an armour against evil	PALMER, 1985
Gloriosa simplex L. (*virescens* Lindl.)	Zulu	Powdered root	Treatment for impotency and barrenness	BRYANT, 1966 STEYN, 1934 WATT and BREYER-BRANDWIJK, 1962
iHlamvu	Zulu	Root	To determine the desired sex of a child	BRYANT, 1966 PALMER, 1985 WATT and BREYER-BRANDWIJK, 1962
	East Africa	Root	Homicide	VERDCOURT and TRUMP, 1969 WATT and BREYER-BRANDWIJK, 1962

Table 2.1 (continued)

Species	Tribe/ Area	Plant part	Use	References
	Senegambia	Pounded leaf	Narcotic	WATT and BREYER-BRANDWIJK, 1962
	Tanganyika	Juice	Disinfectant for wounds	WATT and BREYER-BRANDWIJK, 1962
<u>Gloriosa superba</u> L. iHlamvu	India Ceylon	Flower	Religious ceremonies	STEYN, 1934 WATT and BREYER-BRANDWIJK, 1962
nyamahlokane	India Ceylon	Root	Promoting labour pains and procuring abortion	WATT and BREYER-BRANDWIJK, 1962
	Ceylon	Root	Treatment for bruises and sprains	STEYN, 1934 WATT and BREYER-BRANDWIJK, 1962
	India	Root	Tonic, antiperiodic, gonorrhoea remedy, purgative, antihelmintic, haemorrhoids, remedy for snake and scorpion bites, suicide, leprosy and colic.	STEYN, 1934 WATT and BREYER-BRANDWIJK, 1962
	Persia	Root	Bleeding of the nose, impotence, nocturnal seminal emissions	STEYN, 1934 WATT and BREYER-BRANDWIJK, 1962
	South Africa	Root	Antiparasitic and a remedy for ascites	WATT and BREYER-BRANDWIJK, 1962
	Zulu	Root	Used as a charm	PALMER, 1985
	Zulu	Root	Lice killer	BRYANT, 1966
<u>Sandersonia aurantiaca</u> Hook.	Zulu	Root	To determine the desired sex of a child	PALMER, 1985

According to Molle all parts of the <u>Clivia</u> plant contain an alkaloid cliviine (WATT and BREYER-BRANDWIJK, 1962). Other alkaloids have been isolated and named clivonine, lycorine and clivianine (see Table 2.2).

Table 2.2 Alkaloids of Clivia after WATT and BREYER-BRANDWIJK, (1962)

Alkaloid	Species	Chemical formula	Plant part analysed	Percentage
Clivianine	C. nobilis	-	-	-
Cliviine	C. miniata C. nobilis	-	Root	-
Clivonine	C. miniata	-	-	0,07
Lycorine	C. miniata	$C_{16}H_{17}O_4N$	Root	0,3 0,53 dry-weight
Tazettine	C. miniata	$C_{118}H_{12}O_5N$	Root	

ALI, ROSS, EL-MOGHAZY and EL-MOGHAZY (1983) isolated clivonidine, lycorine, clivonine and clivatine from the aerial parts of Clivia miniata, while an alkaloid structurally and stereochemically resembling α-dihydrohippeastrin was isolated by DOPKE and BIENERT (1970).

Extracts of Clivia miniata and Clivia cyrtanthiflora show antiviral activity against viruses pathogenic to man (BERGHE, IEVEN, MERTENS, VLIETINCK and LAMMENS, 1978), while IEVENS, TOTTÉ, BERGHE and VLIETINCK (1978) showed that lycorine and the residual alkaloid fraction in Clivia miniata leaves showed antiviral activity against test viruses pathogenic to man. This theory was revised with lycorine shown to be responsible for the antiviral activity of plant extracts while clivimine, clivonine and cliviamartine were shown to have no antiviral properties (IEVEN, VLIETINCK, BERGHE, TOTTÉ, DOMMISSE, ESMANS and ALDERWEIRELDT, 1982; IEVEN, BERGHE and VLIETINCK, 1983). Lycorine is a powerful inhibitor of growth in higher plants, algae and yeasts, it inhibits leucine incorporation into proteins, uridine incorporation into RNA and brings about a drop in ascorbic acid content. Ascorbic acid biosynthesis is not greatly affected by lycorine in *Clivia miniata*, which raises interesting questions in view of phylogenetic implications concerning ascorbic acid biosynthesis and the presence of lycorine in *Clivia* (ARRIGONI, LISO and CALABRESE, 1975).

Numerous research papers have been published on horticultural techniques used for *Clivia* propagation and growth, especially factors influencing flowering. HIEKE (1971) studied the maturation period of *Clivia* seedlings, MORI and SAKANISHI (1974) the interaction of temperature and flowering and ELDABH, ABOU DAHAB and ELBAGOURY (1978) the effects of gibberellic acid on the growth and flowering of the plant. *Clivia* seed and seedlings have been soaked in colchicine to induce the formation of tetraploids and hexaploids (NIU, XU and LIU, 1986), however, very little has been published on the *in vitro* culture of *Clivia*.

HOLDGATE, AYNSLEY, FENWICK, HILL, KREBS, LYNE, RANGAN, ROTHWELL, SPURR, STOKES, SMITH and THOMAS (1975) claim to have propagated *Clivia in vitro*. This work was done at Twyford Laboratories Ltd., a commercial concern, and details of the culture technique is unpublished. ZUBKOVÁ and SLADKY (1975) used an *in vitro* system to obtain seeds following placental pollination. Normal seed set was achieved but the seed did not germinate even after GA_3 treatment. It is reported that *Clivia* has been cultured using direct adventitious organogenesis, indirect organogenesis and multiple axillary shoot techniques (GEORGE and SHERRINGTON, 1984; VASIL, 1985). However, these reports are also as a result of personal communication with commercial laboratories. CARTER (1984) attempted *in vitro* culture of *Clivia*, no callus nor plantlets were produced. Studies on nucleic acid, protein synthesis and the development of male gametophytes of *Clivia nobilis* cultured *in vitro*, was performed by TANG and ZHU (1985). While cellular ultrastructures during plasmolysis and after protoplast isolation from pollen tubes of *Clivia nobilis* was studied by PARGNEY (1982), this study is not truly an *in vitro* investigation but it does have relevance for culture techniques.

Clivia nobilis was cultured using immature embryos as an explant source. Petals and ovary walls were also used for in vitro experimentation and "different kinds of calli and plantlets appeared" (MIN and JINSHENG, 1984).

Gloriosa superba L. is the well known flame lily whose distribution is widespread in tropical and subtropical areas. The family Liliaceae sensu lato is one of the largest families of flowering plants and is well represented in Southern Africa with approximatley 1 000 species. The family forms a large heterogeneous group with forms ranging from herbs with bulbs, corms and tubers, to trees, shrubs and climbing plants, many of the species have xerophytic features (PERRY, 1985). DAHLGREN and CLIFFORD (1982) have modified the family into smaller homogeneous groups using data from comparative studies as well as using an evolutionary approach. Gloriosa has been classified as family Liliaceae, Tribe Uvularieae (HUTCHINSON, 1959) and Subfamily Wurmbaeoideae Tribe Glorioseae (SANTAVY, 1982), in this thesis the classification of Gloriosa (and Sandersonia) will follow the system proposed by DAHLGREN, CLIFFORD and YEO (1985) (Figure 2.3).

Members of the family Liliaceae sensu lato have been allocated separate families belonging either to the order Liliales or Asparagales. The order Liliales contains the families Alstroemariaceae, Uvulariaceae, Calochortaceae, Liliaceae, Gessiridaceae, Iridaceae, Apostasiaceae, Cypripediaceae, Orchidaceae and Colchicaceae of which Gloriosa is a member.

Members of the family Colchicaceae are geophytes, having either corms or small tubers as their underground organ. The ovary is superior, three chambered with many ovules and three stigmatic branches, the fruit is a capsule. South African genera include; Androcymbium, Baeometra, Gloriosa, Hexacyrtes, Iphigenia, Littonia, Neodregea, Onixotis, Ornithoglossum, Sandersonia and Wurmbea (DAHLGREN, CLIFFORD and YEO, 1985; PERRY, 1985).

Figure 2.3 <u>Gloriosa superba</u>

Gloriosa superba was originally called *Methonica gloriosa* in the 17th century, but Linnaeus renamed *Methonica*, as it could be confused with *Betonica*, to *Gloriosa superba* in his publication Species Plantarum in 1753 (BATTEN, 1986). *Gloriosa* is derived from the Latin *gloriosus* meaning glorious or handsome. Two species of *Gloriosa* are recorded for Southern Africa by POLE EVANS (1931), *Gloriosa superba* L. and *Gloriosa virescens* Lindl., however, DYER (1976) regards *Gloriosa virescens* Lindl. as a subspecies of *Gloriosa superba*, as do a number of popular publications (PLOWES and DRUMMOND, 1976; ELIOVSON, 1984; BATTEN, 1986). Several species come from tropical Africa and Asia, *Gloriosa rothschildiana* O'Brien originates form Uganda while *Gloriosa carsonii* Bak. comes from Central East Africa. (*Gloriosa simplex* is regarded as a synonym for *Gloriosa superba* subsp. *virescens*, and *Gloriosa leopoldii* is the horticultural name for *Gloriosa superba* subsp. *virescens*).

The first published illustration of *Gloriosa superba* appeared in the Botanical Register in 1815. Linnaeus describes the perianth segments as scarlet-red, (in Flora Capensis yellow flowers are included in the same species). The difference between the two South African species is described thus: "in *G. superba* the perianth segments are crisped all along the margins, while in *G. virescens* the segments are crisped only in the upper portion" (POLE EVANS, 1931). *Gloriosa superba* has become regarded as the floral emblem of Zimbabwe. The numbers of the plant have been reduced in the neighbourhood of major growth centres, and is now one of the specially protected indigenous plants scheduled under the Parks and Wild Life Act (PLOWES and DRUMMOND, 1976).

The name of the underground organ varies between references, it is referred to as a tuber by POLE EVANS (1931). CAROW (1975), BATTEN (1986), KRAUSE (1986); a root stock by WATT and BREYER-BRANDWIJK (1962); in this thesis the organ will be described as a corm after the description by DAHLGREN, CLIFFORD and YEO (1985) who refer to the undergound organ as a subterranean, starch rich corm which is stoloniferous.

Gloriosa superba is a geophyte with geniculate corms which are usually deep seated, with supple scandent stems, leaves are cauline, sessile alternate or crowded in places, simple, ovate and attenuate into a terminal recurved tendril which twines around any convenient structure. Solitary flowers are borne on the upper part of the stem on a long pedicel which is sharply bent under the flower. Six free perianth-segments are strongly reflexed, narrowly ovate acuminate and often crisped, segments become horizontal with age. Stamens are radiating long terete and glabrous, with bilocular versatile anthers. The ovary has three locules with numerous axile ovules. The style is sharply bent at the base with three stigmatic arms. The ovoid to cylindrical capsule is three valved, septicidally dehiscent and leathery, it opens out flat to reveal bright red fleshly globose seeds (POLE EVANS, 1931, DYER, 1976; DAHLGREN, CLIFFORD and YEO, 1985).

Gloriosa superba also known as the Flame lily, Superb lily, Gloriosa lily, climbing lily, Turk's Cap, Rooibostelie or Geelboslelie (SMITH, 1966; ELIOVSON, 1984) is found growing in South West Africa, the Bathhurst district in the Eastern Cape, through the eastern parts of South Africa, Zimbabwe, Mozambique into tropical Africa, Madagascar and tropical Asia (India and Sri Lanka) (Figure 2.4). The shoot appears from a bud at the end of the corm after the start of the spring rains and the plant flowers in summer between December and February. Flowers grown in shade do not have as brilliant a colour as those which are grown in full sunlight (BATTEN, 1986). The aerial portions of the plant die back after seed dispersal, the corm remains dormant during winter and daughter corms sprout the following spring. Plant propagation is by division of the corm, and by seed which readily germinates once sown. Generally flower colour is scarlet-red through to yellow.

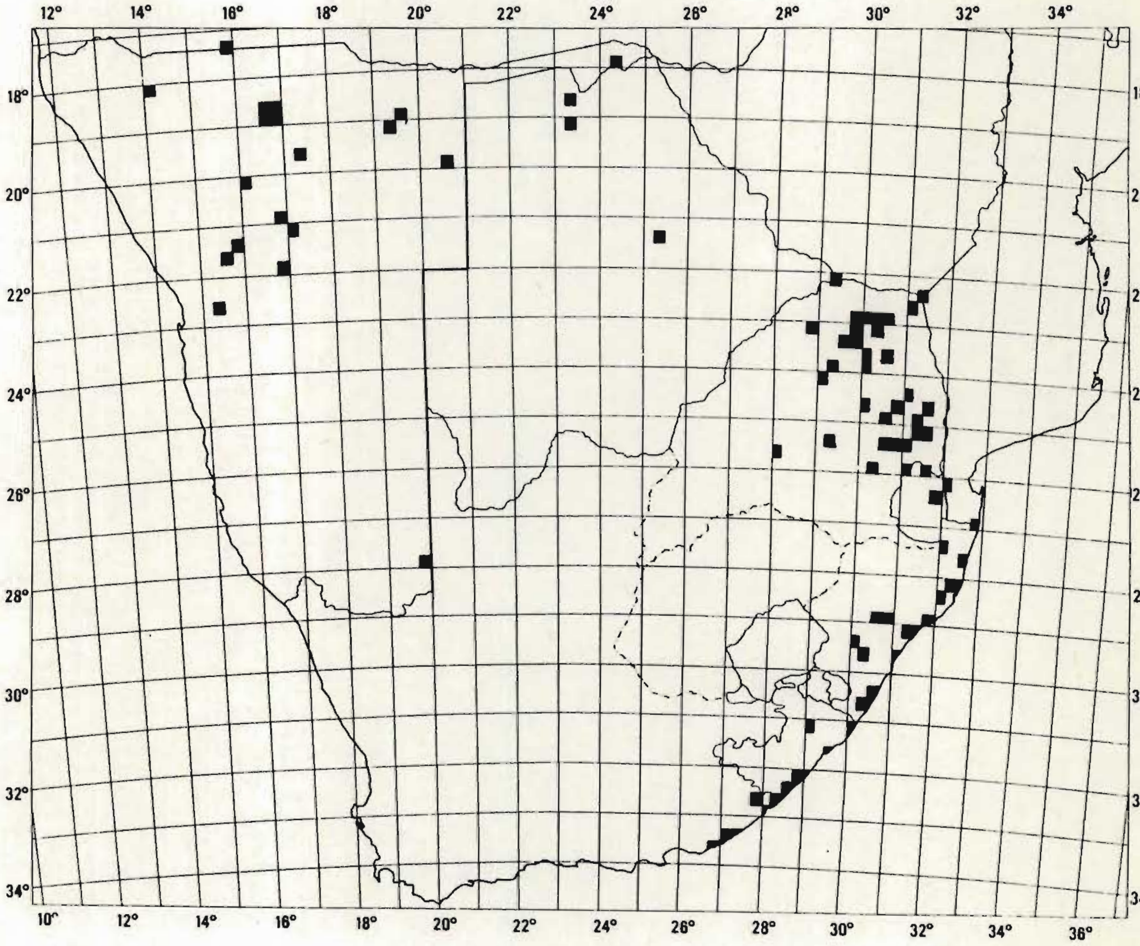


Figure 2.4 Natural distribution of Gloriosa superba in Southern Africa

POLE EVANS (1931) records what may be a colour variety of Gloriosa superba subsp. virescens, which when immature has a yellow colouring below and is reddish brown above, but the yellow colour disappears as the flower ages and the perianth turns a "deep wine red" to purple colour (Burgundy?).

The plant is known to the Zulus as 'iHlamvu' or 'iHlamvu comfana nentombhazana' (BRYANT, 1966), by the Chopi as 'pembekushe' and to the Tonga as 'nyamahlokane' (STEYN, 1934). The plant has numerous uses as remedies and potions to the indigenous populations of both Africa and Asia. The major uses revolve around the use of the plant as a fertility drug, as well as a cure for bites and bruises (Table 2.1). BRYANT (1966) speculated that the root of iHlamvu is "effective" as a cure for barrenness in that as a known lice-killer it may also act as a germicide which could kill microbes responsible for uterine diseases. It is possible to procure the birth of which ever sex of child is desired using iHlamvu, the corm is said to exhibit the shape of the female or the male organ, and all that is necessary is to physic the wife before coition with a decoction of the root which resembles the shape of the organ of the desired sex (BRYANT, 1966). A further use of iHlamvu is when a man courts a girl who does not return his affections, he may phalaza with a piece of the corm after which he will walk slowly past the girl casting his shadow on her. She will then appear to be pregnant, this state can only be rectified by returning the affection, or by using the same 'muti' to break the spell (PALMER, 1985).

Gloriosa superba and Gloriosa superba subsp. virescens are both extremely poisonous and numerous cases have been reported of both human and stock poisoning and death (VERDCOURT and TRUMP, 1969). WARDEN (1881) isolated from the root stocks of plants collected in India a neutral bitter principle superbine, three resins, a fluorescent principle and salicylic acid, the suggested formula for superbine is $C_{52}\ H_{66}\ O_{17}\ N_2$. CLEWER, GREEN and TUTIN (1915) found that Gloriosa superba contained the alkaloid colchicine, it was estimated that the seeds contained 0,6% colchicine while the dried corms contained 0,3%. Numerous reports have been made concerning Gloriosa and its colchicine content (SANTAVY, 1950; WILDMAN and PURSEY, 1968; SARIN, JAMWAL, GUPTA and ATAL, 1974; SRIVASTAVA and CHANDRA, 1977; SANTAVY, 1982; BELLET and GAIGNAULT, 1985; DAHLGREN, CLIFFORD and YEO, 1985), however discussion of colchicine, its biosynthesis and pharmacological actions will be discussed in a following chapter.

As yet only one report has been found concerning the in vitro culture of Gloriosa, there is however, extensive literature on flowering, corm size, corm formation and plant production. Most of this research has been performed on Gloriosa rothschildiana by European researchers. It is apparent that corm size is directly related to the size and quality of the plant and its flower (ESCHER, STRECH and LADEBUSCH, 1973; CAROW, 1975, 1976b, 1977; APELES DIAZ and CABALLERO RUANO, 1985). The techniques and requirements for dormancy breaking and storage of Gloriosa rothschildiana corms has also been well researched (ZIMMER, 1975; CAROW, 1980a; HENRARD, 1982), as has tuber nutrition and nutrient utilization (CAROW, 1980b; HENRARD, 1982).

KRAUSE (1986) stated that in horticultural practice vegetative propagation of Gloriosa is commonly used, the mother corm usually produces two daughter corms, but there are many reasons why the coefficient of multiplication is considerably lower. "Thus the obtaining of a great number of plants in a short time is possible only from seed" (KRAUSE, 1986). this feature is echoed by CAROW (1979). The fact that multiple plant production is from seed only, together with the statement that Gloriosa superba is a valuable plant for the pharmaceutical industry (BELLET and GAIGNAULT, 1985) makes Gloriosa a "superb" candidate for in vitro studies on both multiple plant production and secondary plant metabolite production.

Sandersonia aurantiaca Hook. is a monotypic species related to *Gloriosa*. *Sandersonia* was classified as Family Liliaceae Tribe Uvularieae (HUTCHINSON, 1959) and Sub Family Wurmbaeoideae Tribe Glorioseae (SANTAVY, 1982). However, as with *Gloriosa*, DAHLGREN, CLIFFORD and YEO (1985) classify *Sandersonia* in the family Colchicaceae Tribus Iphigenieae (Figure 2.5).

Sandersonia was described by W.I. Hooker in Curtis' Botanical Magazine in 1853, the plants that were described having been collected from Fields Hill near Durban and Swartkop near Pietermaritzburg by John Sanderson, a journalist and trader. Sanderson regularly corresponded with Hooker at Kew and Harvey in Dublin, sending them seeds, bulbs, dried material and sketches of specimens. Many of the plants were rare or new and thus Hooker named *Sandersonia* in his honour (BATTEN, 1986). The name *aurantica* is from the Latin meaning yellow and scarlet (orange).

Sandersonia is an erect or scandent geophyte, with a small corm having short spreading lobes and a thin tunic. The stem is simple and supple, with leaves close together alternate clasping below, the leaf is linear to narrowly ovate-acuminate with a cirrhiferous or aristate tip. Flowers cymose on the upper part of the stem and are placed behind a leaf on long arcuate pedicel. The tepals are fused to form a campanulate perigone (Figure 2.5). There are six short stamens included, with bilocular anthers introrse and basifixed. The ovary is tri-locular ovoid, obtusely trigonous with many axile ovules, there are three styles and the stigma is apical. Capsules are globose, verrucose with a large broad funicle (DYER, 1976; DAHLGREN, CLIFFORD and YEO, 1985; BATTEN, 1986).

Sandersonia aurantiaca known also as Christmas Bells and Chinese Lantern lily (SMITH, 1966) occurs from the Eastern Cape through Transkei, Natal, Swaziland to the Transvaal (Figure 2.6). Flowering time is December to January (Christmas),

Figure 2.5 Sandersonia aurantiaca

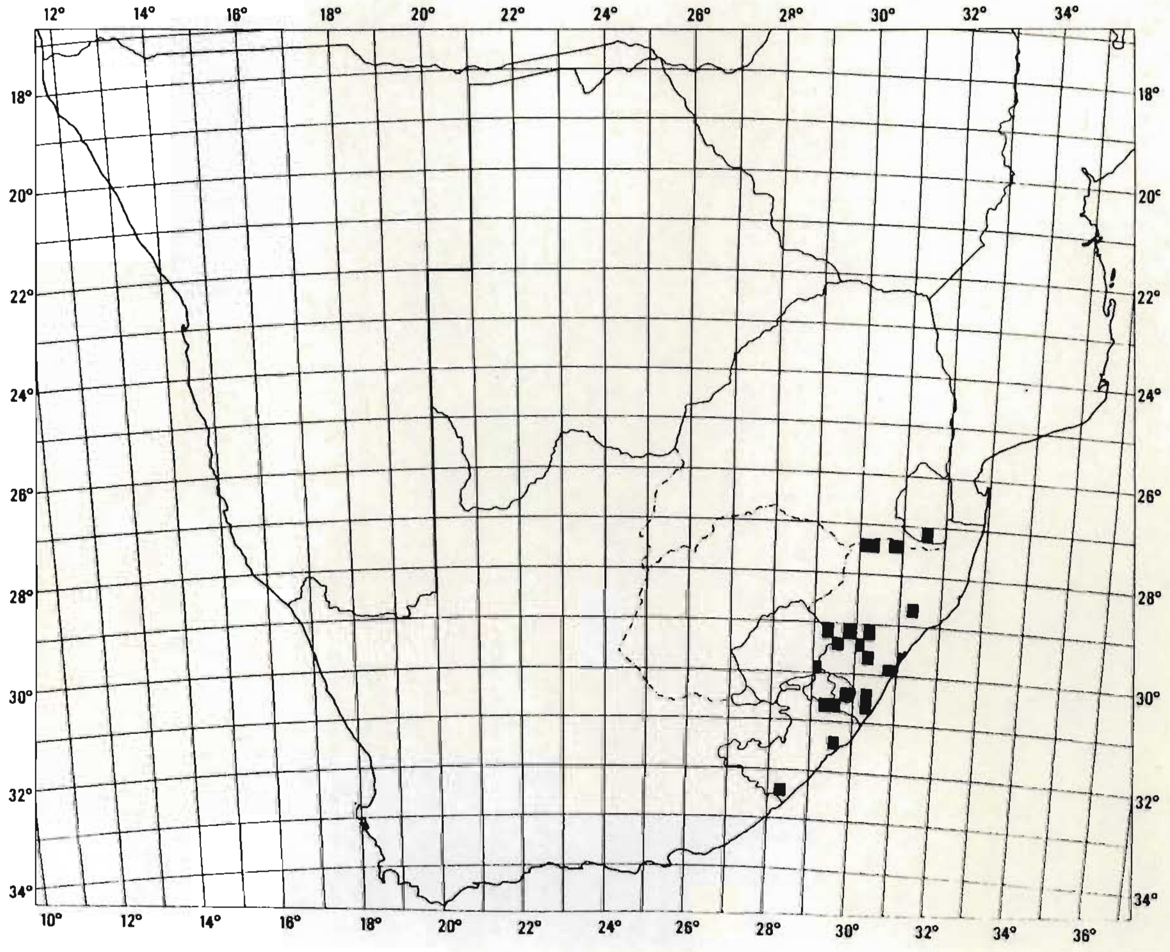


Figure 2.6 Natural distribution of Sandersonia aurantiaca in Southern Africa

the lantern-like flowers are borne on the upper part of the stem and open in succession from the lowest flower, the perianth is persistent and protects the ovary.

Christmas Bells is a popular plant which is now threatened with extinction because of indiscriminate picking and destruction of its habitat for sugarcane fields. The longevity and charm of the flower made Sandersonia a favourite cut flower in Natal before the Second World War. Flower sellers with large bunches for sale were common in the towns before Christmas, and as a result the habitats were slowly eradicated and the plant is now seldom seen except in protected reserves (BATTEN, 1986). Sandersonia is classified as being in danger of extinction in the wild.

PALMER (1985) reports that Sandersonia is used by the Zulu in a similar way to Gloriosa to procure the birth of a son or daughter as desired. Sandersonia has also been shown to contain colchicine (SANTAVY, 1950; WILDMAN and PURSEY, 1968; SANTAVY, 1982). BRUNDELL and REYNGOUD (1985) have studied the response Sandersonia corms to a variety of cultural practices, as well as the course of development from the planted corm to senescence. They report that the original corm dies after producing two joined daughter corms, each with a single growing point, one of which tends to be dominant. From personal experience it has been found that germination of seed is very poor, and thus the major means of plant production is by division of the daughter corms.

2.2 Tissue culture with reference to the Liliaceae and Amaryllidaceae and Iridaceae

Haberlandt, a German botanist, attempted to culture isolated cells in an aseptic environment with a nutrient media containing glucose and peptone, this experiment being considered by many to be the first plant tissue culture experiment. Haberlandt chose to work with single cells and used green cells rationalizing that these cells being photosynthetically active, would not require complex media and thus the technique could be simplified (WHITE, 1943; GAUTHERET, 1985). He unfortunately chose relatively mature and highly differentiated cells for his experiment, palisade cells of *Lamium*, pith cells from *Eichornia*, glandular hairs of *Pulmonaria* and *Urtica*, stamen hairs of *Tradescantia* and stomatal guard cells of *Ornithogalum* (HABERLANDT, 1902; KRIKORIAN and BERQUAM, 1969). The cells survived and enlarged in the media, but because these cells were highly differentiated they were unable to recover their meristematic ability.

SIMON (1908) reported the development of callus, buds and roots on poplar stem segments, but because he did not transfer his cultures and as they were not aseptic his results were largely ignored. These results are possibly the first recorded examples of callus culture and micropropagation (GAUTHERET, 1985). Many researchers recorded unsuccessful cultures in the 1920s, *SCHMUCKER* (1929) reported the successful growth of leaf mesophyll cells of *Bocconia*, however, details of the work were never published nor verified and its correctness is doubted (WHITE, 1943).

Both KOTTE (1922) and ROBBINS (1922) recorded the growth of excised root tips in nutrient media. The major difference between the two sets of results lay in the nutrient media, Kotte using a Knop solution supplemented with Liebig's meat extract, while Robbins supplemented his media with a yeast extract. After subculture the growth rate of the roots gradually declined until the roots died (WHITE, 1943). WHITE (1943) reported the indefinite culture of aseptic tomato roots. Gautheret in 1934 cultured callus aseptically from phloem and cambial explants of Acer, Ulmus, Robina and Salix. Cultures had been inoculated and placed in a cupboard and only inspected two months later (GAUTHERET, 1985). Activity of the cultures ceased after six months. Using the newly "discovered" hormone indoleacetic acid, cultures could be enhanced and subculture was a possibility. The ability to cultivate plant tissues for unlimited periods was published by three scientists in 1939, WHITE (1939), NOBECOURT (1939), GAUTHERET (1939). MOREL and WETMORE (1951) initiated the first callus culture of a monocotyledon.

VAN OVERBEEK, CONKLIN and BLAKESLEE (1941) utilized coconut milk as a supplement for the culture of Datura embyros. MILLER, SKOOG, OKUMURA, VON SALTZA and STRONG (1955) were able to isolate a substance 6-furfuryl aminopurine which they named kinetin. Kinetin and indoleacetic acid were utilized by SKOOG and MILLER (1957) in their classic experiment using tobacco callus to show that shoot initiation is induced by kinetin and that root formation can be induced by auxin. Today it is known that this simple balance of the two hormones in organ formation cannot be applied to all plants, but that organogenesis is controlled by a number of complex interactions involving environmental, nutritional and plant related factors. A substance with kinetin-like properties was isolated from maize endosperm and termed zeatin by LETHAM (1963). The growth factor in coconut milk was shown to be ribosylzeatin by both LETHAM (1974) and VAN STADEN and DREWES (1974) in 1974.

MUIR, HILDERBRANDT and RIKER (1954) were able to place a single cell on a piece of filter paper on top of a callus block, and this cell was shown to multiply and produce cell masses. STEWARD, MAPES and MEARS (1958) and STEWARD, MAPES and SMITH (1958) showed that carrot plants could be cultured from freely suspended cells. VASIL and HILDEBRANDT (1965) showed that plantlets could be produced from cell colonies derived from a single cell. These original works conclusively showing totipotency of somatic cells.

When Haberlandt attempted the first plant cell cultures, he could not have been aware of how valuable plant tissue culture would become in numerous scientific and economically orientated activities. There are four major areas in which plant tissue culture now finds application:

i) the production of pharmaceuticals and natural products;
ii) genetic improvement of crops;
iii) The recovery of disease free clones and preservation of germplasm;
iv) the rapid clonal multiplication of selected varieties.

(MURASHIGE, 1974)

MURASHIGE (1974; 1977) defined three stages for in vitro multiplication of plants, as the production of a plant must follow a sequence of steps, each of these steps must be identified, explored scientifically and optimal conditions must be established if one wishes to apply this technique for commercial purposes. The three stages are as follows:

i) establishment of the aseptic culture;
ii) multiplication of propagula;
iii) preparation for re-establishment of plants in soil.

(MURASHIGE, 1974)

A fourth stage was later added

iv) re-establishing the plants in soil.

(MURASHIGE, 1978)

These stages vary according to the type of culture method required, and in some cases certain stages are not necessary. DEBERGH and MAENE (1981) reason that certain points of the scheme should be elaborated for commercial use. A stage O for stock plant growth under controlled conditions has been included to ensure healthier and more uniform explants.

O the preparation of stock plants under hygienic conditions;

I establishment of aseptic cultures;

II The induction of meristematic centres, their development into buds and their rapid multiplication;

IIIa the elongation of the buds to shoots and the preparation of uniform shoots for stage IIIb;

IIIb the rooting and the initial growth of the in vitro produced shoots under in vivo condition.

The reasons behind the creation of stage IIIb are, in vitro rooting of shoots is the most labour intensive stage as single shoots must be handled, delay in growth after transfer from in vitro to in vivo conditions, rooting is not optimal when done in a constant regime for 2 - 3 weeks, and in vitro formed roots are usually damaged during transplanting increasing the chance of disease entry (DEBERGH and MAENE, 1981).

Stage O preparation of stock plants

The production of healthy uniform stock plants will result in healthier and more uniform explants in vitro. DEBERGH and MAENE (1981) have compared contamination in explants taken from plants having been subjected to 'Stage O' and those not having been pretreated. From this comparison it is evident that sanitary horticultural practices can have a positive effect on the success rate of in vitro cultures (Table 2.3). KNAUSS (1976) reasons that plant diseases are often the economically limiting factor in the production of tropical foliage plants, and recommends the use of an in vitro indexing procedure for the eradication of plant pathogens. Important methods for reducing contamination are, avoiding excessive wetting of the upper portions of the plant (if leaf and stem explants are desirable), low humidity and irrigation from capillary beds or irrigation tubes to reduce potential contamination of aerial organs.

The effect of the proposed Stage O is not solely limited to sanitation but explant survival is higher. Rigorous defoliation of stems is not necessary with 'clean' plants thus allowing the leaves to protect the axillary buds from the sterilant, and the sterilizing agent cannot enter the stem tissue through the scars of the leaf bases. Some researchers prefer to grow stock plants in dry laboratories for a few weeks prior to culture. MURASHIGE (1974) advocates the use of antibiotics as a control measure against contamination; unfortunately these compounds are expensive, may stimulate or retard growth, and no single antibiotic is effective against the entire range of contaminating organisms (KNAUSS, 1976; HOLDGATE and AYNSLEY, 1977; THURSTON, SPENCER and ARDITTI, 1979; DEBERGH and MAENE, 1981; DODDS and ROBERTS, 1982; GEORGE and SHERRINGTON, 1984). Advantages of Stage O include a reduction in cost price per plant and healthier explants that respond uniformly in culture.

Stage I Establishment of the aseptic culture

The aim of Stage I is to obtain an aseptic culture of the required plant material (MURASHIGE, 1974). The culture must be free from contamination, the explant should survive the procedure, and give an appropriate reaction. Although this may seem a simplistic approach each stage is further complicated by the selection of healthy appropriate explants, nutrient media characteristics and the quality of the culture environment.

Table 2.3 Effect of Stage O on the success rate in Stage 1 during the process of tissue culture

Plant species	Type and location of infection using apical or axillary buds as explants		% of non-contaminated cultures	
			Without Stage O %	With Stage O %
Araucaria heterophylla	Bacteria	Vascular tissue	0	50
Bromeliaceae	Bacteria	Bud axil	0	25
Calathea spp	Bacteria	Bud axil, vascular tissue	0	25
Cordyline spp	Bacteria and fungi	Bud axil	Only apical top for 50%	90-100% for all explants
Dieffenbachia spp	Bacteria	Vascular tissue	0	0-50
Dracaena spp	Bacteria and fungi	Bud axil	Only apical top for 50%	90-100% for all explants
Ficus spp	Bacteria	Vascular tissue	2-3	30-40
Marantha spp	Bacteria	Bud axil, vascular tissue	0	25
Medinilla	Bacteria	All over	0	0
Monstera	Bacteria	Bud axil	40	80
Philodendron spp	Bacteria	Vascular tissue	5	25
Spathiphyllum	Bacteria	Bud axil	25	90

Table adapted from DEBERGH and MAENE (1981)

Plants are invariably contaminated with a wide range of micro-organisms. Failure to remove contaminants will result in the carbon rich nutrient medium becoming infected with the resulting rapid proliferation of micro-organisms, which ultimately affects explant performance in culture. It is vital for the explant to be sterilized for the successful isolation of a culture. The sterilant should be easily removeable, as the retention of chemicals will adversely affect the culture. YEOMAN (1973) compares the effectiveness and properties of several sterilizing agents (Table 2.4).

Table 2.4 A comparison of the efficiency of several sterilizing agents commonly used in tissue culture

Sterilant	Concentration used	Ease of removal	Time of sterilization Minutes	Effectiveness
Calcium hypochlorite	9 - 10%	+++	5 - 30	Very good
Sodium hypochlorite	2%*	+++	5 - 30	Very good
Hydrogen peroxide	10 - 12%	+++++	5 - 15	Good
Bromine water	1-2%	+++	2 - 10	Very good
Silver nitrate	1%	+	5 - 30	Good
Mercuric chloride	0,1 - 1%	+	2 - 10	Satisfactory
Antibiotics	4-50 mg/litre	++	30 - 60	Fairly good

* Commercially available NaOCl Trade name Jik

Adapted from YEOMAN (1973)

Hypochlorite solutions have proved to be most effective. Many laboratories use household bleach (Chlorox, Javex and Purex) which contains 5,25% NaOCl as the active ingredient (GEORGE and SHERRINGTON, 1984). In South Africa the available bleach is Jik (Reckitt Household Products) which contains 3,5% active ingredient when packed.

Calcium hypochlorite is cheaper than sodium hypochlorite and is less toxic to plant material (SWEET and BOLTON, 1979), but is not always as effective in sterilization. Because of dissociation hypochlorite has little activity at pH over 8,0 and is more effective when the solution is buffered at pH 6,0 (BEHAGEL, 1971). Sodium hypochlorite breaks down to give chlorine and sodium hydroxide which can easily be removed. Hydrogen peroxide decomposes to give harmless components which evaporate, dilute mercuric chloride is a satisfactory sterilizing agent but is difficult to remove subsequently (YEOMAN, 1973). Explants will vary in their sensitivity to sterilants. To increase the effectiveness of the sterilant a non-toxic wetting agent is often added to the solution (0,05% Teepol or Lissapol, or 0,01% Tween 20 or Tween 80) (STREET, 1973; GEORGE and SHERRINGTON, 1984). The detergent allows for thorough wetting of the surface and allows chemical penetration. Penetration may also be improved by an immersion in alcohol. SHEURINK and WELLES (1979) reported a promoting effect on sprout regeneration when using Tween 20 during leaf sterilization, PIERIK, VAN LEEUWEN and RIGTER (1979) using Anthurium showed an increase in the number of adventitious sprouts when Tween 20 was added at 0,25 - 1,00 $m\ell\ \ell^{-1}$.

The duration of sterilization is an important factor. However, experience shows that sterilization techniques vary from country to country. GEORGE and SHERRINGTON (1984) consider 1,6% NaOCl for 30 minutes a drastic sterilization regime whereas GROENEWALD, KOELEMAN and WESSELS (1975) surface sterilized seeds for 5 minutes in 70% ethanol, 30 minutes in 5% NaOCl and 10 minutes in 0,1% HgCl and 0,1% sodium lauryl sulphate. OSBORNE and VAN STADEN (1987) sterilized roots by sequential immersion in 70% ethanol (4 minutes), 20% Jik (3,5% NaOCl solution) containing 1% Teepol (30 minutes) and 1% $HgCl_2$ containing 1% Teepol (30 minutes), after rinsing and soaking in sterile distilled water (2 hours) the roots were again treated with hypochlorite. The choice of a sterilant and time of treatment depends largely on the nature and sensitivity of the explant. After sterilization the explant is washed several times in sterile water to remove as much as possible of the sterilant.

YEOMAN (1973) emphasizes that a sterilization procedure should be established for each tissue. In order to achieve aseptic culturable material the researcher must be flexible in his approach (DE FOSSARD, 1976).

"The most important determinant of rate of plant increase and quality of regenerated plants is the initial explant. In the absence of a suitable explant, success is at best limited" (MURASHIGE, 1977). Aspects that should be considered when selecting a suitable explant are: explant source, age, size, quality, orientation and the season in which the explant is obtained. The most regenerative organ or tissue may be different for each plant.

Virtually every organ can serve as a source of explants, the explant can range from simple organs to complex reproductive structures. Monocotyledons have been cultured from a wide range of explants: bulbscales (MII, MORI and IWASE, 1974: MURASHIGE, 1974; HUSSEY, 1975a; STEINITZ and YAHEL, 1982; TAKAYAMA and MISAWA, 1982a; JHA, MITRA and SEN, 1984), callus (DAVIES and HESLOP, 1972; PIERIK and STEEGMANS, 1976a), corms (HILDEBRANDT, 1971; HUSSEY, 1975a; PAGE and VAN STADEN, 1985), embryos (ZIMMERMAN and ASCHER, 1982), flowers (KAUL and SABHARWAL, 1972; BAJAJ and PIERIK, 1974; PIERIK and STEEGMANS, 1975b; HEUSER and APPS, 1976; PAGE and VAN STADEN, 1986), fruit walls (MIN and JINSHENG, 1984), gynoecium (KAUL and SABHARWAL, 1972), inflorescence stalks (HUSSEY, 1976a; HOSOKI and ASAHIRA, 1980; RICE, ALDERSON and WRIGHT, 1983; LIU and BURGER, 1986), leaf/bases (SEABROOK, CUMMING and DIONNE, 1976; GABRYSZEWSKA and SANIEWSKI, 1984), ovaries (MAJUMDAR, 1970a; SEABROOK, CUMMING and DIONNE, 1976, HUSSEY, 1975a), seeds (GROENEWALD, KOELEMAN and WESSELS, 1975), shoot tips (KAR and SEN, 1985) and stems (ZIV, HALEVY and SHILO, 1970; KUNISAKI, 1977).

"Totipotentiality is probably characteristic of all plant cells but its expression may be limited to particular cells" (MURASHIGE, 1977).

It is apparent from a review of the literature that the above quoted examples comprise a minute fraction of explants used successfully in tissue culture. Table 2.5 reviews the *in vitro* culture of the Liliaceae, Iridaceae and Amaryllidaceae sensu lato.

Table 2.5 Members of the Liliaceae, Iridaceae and Amaryllidaceae propagated by means of *in vitro* culture

Genus and explants used for propagation	Reference
	LILIACEAE
Allium	
Base plate, bulb, callus, leaf, meristem, receptacle, root, scales, stem tip.	FRIDBORG, 1971; KEHR and SCHAEFFER, 1976; ABO-EL-NIL, 1977; DUNSTAN and SHORT, 1977; HUSSEY, 1978; HUSSEY and FALVIGNA, 1980; LUTEYN and PHILLIPS, 1982; ZIV, HERTZ and BIRAN, 1983; SHAHIN and KANEKO, 1986; etc.
Aloe	
Callus, leaf, seed	GROENEWALD, KOELEMAN and WESSELS, 1975; GROENEWALD, WESSELS and KOELEMAN, 1976 YAGI, SHOYAMA and NISHIOKA, 1983; HAYASHI, 1987; FINNIE, DINKELMAN and VAN STADEN, 1988;

Table 2.5 (continued)

Genus and explants used for propagation	Reference
Androcymbium	
Bulb.	FINNIE, pers. com.
Asparagus	
Bud, callus, cladode, hypocotyl, meristem, protoplast, shoot tip, stem, suspension culture.	GORTER, 1965; TAKATORI, MURASHIGE and STILLMAN, 1968; WILMAR and HELLENDOORN, 1968; STEWARD and MAPES, 1971; MURASHIGE, SHABDE, HASEGAWA, TAKTORI, and JONES, 1972; STOLZ and CODY, 1974; BUI-DANG-HA, NORREEL and MASSET, 1975; FONNESBECH, FONNESBECH and BREDMOSE, 1977; REUTHER, 1977; 1984; YANG, 1977; KAR and SEN, 1982; 1985; YAKUWA, HARADA and TSUJI, 1982; KHUNACHAK, CHIN, LE and GIANFAGNA, 1987; REUTHER and BECKER, 1987; Etc.
Aspidistra	GEORGE and SHERRINGTON, 1984.
Astroloba	
Floral stalk, leaf, perianth	HAYASHI, 1987.
Bowiea	
Bulb, inflorescence axis	JHA and SEN, 1985a; COOK, CUNNINGHAM and VAN STADEN, 1988.
Chortolirion	
Floral stalk, leaf, perianth	HAYASHI, 1987.
Convallaria	SHERIDAN, 1968.
Eucomis	
Bulb	COOK, pers. comm.
Fritillaria	
Leaf, scale, stem.	HUSSEY, 1976a; 1980.

Table 2.5 (continued)

Genus and explants used for propagation	Reference
Haworthia	
Callus, gynoecium, inflorescence, leaf, ovary, perianth, stem.	MAJUMDAR and SABHARWAL, 1968; KAUL and SABHARWAL, 1970; 1972; 1975; MAJUMDAR, 1970a; 1970b; MAJUMDAR and SCHLOSSER, 1972; WESSELS, GROENEWALD and KOELEMAN, 1976; OGIHARA and TUSNEWAKI, 1978; OGIHARA, 1979; KEMP and STOLTZ, 1981; BEYL and SHARMA, 1983; KOCHAR, 1983; SUN, HEIL, KAHL and KOHLENBACH, 1987.
Gasteria	
Callus, leaf.	BEYL, WANG and SHARMA, 1981; BEYL and SHARMA, 1983; BAYLEY and VAN STADEN, 1987.
Gloriosa	
Inflorescence, leaf, seed, corm.	FINNIE and VAN STADEN, 1987; KLEIN and DELANGE, 1988.
Heloniopis	
Leaf, stem.	KATO and KAWAHARA, 1972; KATO and HONGA, 1974; KATO, 1975; 1978; KATO and OZAWA, 1979.
Hemerocallis	
Callus, flower, petal, protoplast, scape.	CHEN and HOLDEN, 1972; HEUSER and APPS, 1976; MEYER, 1976b; STRODE and OGLESBY, 1977; CHEN and GOEDEN-KALLEMEYN, 1979; FITTER and KRIKORIAN, 1981; KRIKORIAN and KANN, 1981; 1985.
Hosta	
Flower, leaf, nucellus, scape, shoot tip.	HAMMER, 1976; MEYER, 1976a; MEYER, 1980; PAPACHATZI, HAMMER and HASEGAWA, 1980a; 1980b; 1981.

Table 2.5 (continued)

Genus and explants used for propagation	Reference
<u>Hyacinthus</u>	
Bulb, flower, inflorescence, leaf, ovary, scales, stem.	PIERIK and WOETS, 1971; PIERIK and RUIBING, 1973; SANIEWSKI, NOVAK and RUDNICKI, 1974; HUSSEY, 1975a; 1975b; 1980; PIERIK and POST, 1975; PIERIK and STEEGMANS, 1975a; PAEK, 1982; VAN AARTRIJK, BLOM-BARNHOORN and VAN DER LINDE, 1986.
<u>Lachenalia</u>	
Leaf.	KLESSER and NEL, 1976; NEL, 1983; HUSSEY, 1980.
<u>Lilium</u>	
Callus, embryo, filaments, inflorescence, leaf, meristem, ovary, petal, protoplast, scales, seed, stamen.	ROBB, 1957; SHERIDAN, 1968; HACKETT, 1969; ALLEN, 1974; MONTEZUMA-DE-CARVALHO, LUDOVINA and GUIMARAES, 1974; SIMMONDS and CUMMING, 1976; ANDERSON, 1977; HUSSEY, 1976b; 1977a; 1980; KATO and YASUTAKE, 1977; STIMART and ASCHER, 1978a; NIIMI and ONOZAWA, 1979; SIMMONDS, SIMMONDS and CUMMING, 1979; TAKAYAMA and MISAWA, 1979; 1980; 1982a; 1982b; 1982/83; 1983; STIMART, ASCHER and ZAGORSKI, 1980; NOVAK and PETRU, 1981; VAN AARTRIJK and BLOM-BARNHOORN, 1981; 1984; GU and CHENG, 1982; ZIMMERMAN and ASCHER, 1982; PAEK and SHIN, 1983; LIU and BURGER, 1986; Etc.
<u>Liriope</u>	FRETT and DIRR, 1983; CUMMING and PECK, 1984.

Table 2.5 (continued)

Genus and explants used for propagation	Reference
Muscari	
Bulb, inflorescence stem, leaf, ovary wall, stem.	HUSSEY, 1975a; 1980.
Ophiopogon	
Shoot tip	STRODE and OGLESBY, 1977; FRETT and DIR, 1983.
Ornithogalum	
Bulb, inflorescence, leaf, ovary, sepal, stem.	HUSSEY, 1975a; 1976c; 1980; NEL, 1981.
Poelinitzia	
Floral stalk, leaf, perianth.	HAYASHI, 1987.
Ruscus	
Axillary bud, inflorescence, rhizome, stem.	ZIV, 1983; JHA and SEN, 1985; 1985b.
Sandersonia	
Ovary, seed, corm.	FINNIE and VAN STADEN, 1987 KLEIN and DELANGE, 1988.
Scilla	
Bud, inflorescence stem, leaf, ovary, scales.	HUSSEY, 1975a; 1980.
Tulipa	
Bulb, inflorescence stem, scales, shoot.	NISHIUCHI and MYODA, 1976; NISHIUCHI, 1979; 1980a; 1980b; 1986; HUSSEY, 1980; WRIGHT and ALDERSON, 1980; RICE, ALDERSON and WRIGHT, 1983; GABRYSZEWSKA and SANIEWSKI, 1984; ALDERSON, TAEB and RICE, 1986; TAEB and ALDERSON, 1987.

Table 2.5 (continued)

Genus and explants used for propagation	Reference
Urginea	
Bulb, scales.	JHA, MITRA and SEN, 1984; EL-GRARI and BACKHAUS, 1987.
Veltheimia	TENNANT and DELANGE, 1987.
	IRIDACEAE
Crocosmia	GEORGE and SHERRINGTON, 1984.
Crocus	
Ovary, stigma.	SANO and HIMENO, 1987; CHICHIRICCO and GRILLI CAIOLA, 1987; HOMES, LEGROS and JAZIRI, 1987.
Dierama	
Corm.	PAGE and VAN STADEN, 1985.
Freesia	
Anther, callus corm, flower bud, inflorescence stem, leaf, shoot, stem.	BRANTS and VERMEULEN, 1965; BAJAJ and PIERIK, 1974; PIERIK and STEEGMANS, 1975a; HUSSEY, 1975a; 1977a; 1980; STIMART and ASCHER, 1978b; 1982; BACH, 1987.
Gladiolus	
Anther, corm, inflorescence stem, leaf, shoot, stem, stolon tip.	ZIV, HALEVY and SHILO, 1970; SIMONSEN and HILDERBRANDT, 1971; WILFRET, 1971; HUSSEY, 1975a; 1977b; 1980; ZIV, 1979; BAJAJ, SIDHU and GILL, 1983; DICKENS, KELLY, MANNING and VAN STADEN, 1986.
Iris	
Bulb, embryo, inflorescence, meristem, peduncle, scale, seed, stem.	BARUCH and QUAK, 1966; FUJINO, FUJIMURA and HAMADA, 1972; MEYER, FUCHIGAMI and ROBERTS, 1975; HUSSEY, 1976a; 1976b; 1977a; 1980; REUTHER, 1977; RADOJEVIC, SOKIC and TUCIC, 1987.

Table 2.5 (continued)

Genus and explants used for propagation	Reference
Ixia	
Bulb.	MEYER and VAN STADEN, 1987; 1988.
Neomarica	HOSOKI, 1975.
Shizostylis	
Inflorescence stem.	HOLDGATE et al., 1975; HUSSEY, 1975a; 1976b; 1980.
Sparaxis	
Bud, corm, flower, inflorescence stem, stem.	HUSSEY, 1975a; 1976b; 1980.
Tritonia	
Bulb.	DINKELMAN, pers. com.
	AMARYLLIDACEAE
Agapanthus	
Axillary shoot.	HUSSEY, 1980.
Alstroemeria	
Axillary shoot, flower, meristem, pedicel, rhizome tip, stem.	ZIV, KANTEROVITZ and HALEVY, 1973; HUSSEY, 1980; HAKKAART and VERSLUIJS, 1985; LIN and MANETTE, 1987.
Amaryllis	
Bulb, flower, inflorescence, peduncle, scape.	MURASHIGE, 1974; HOLDGATE et al., 1975; BAPAT and NARAYANASWAMY, 1976; PAJERSKI and ASCHER, 1977.
Clivia	
Embryo, ovary wall, petal, placenta.	HOLDGATE et al., 1975; ZUBKOVA and SLADKY, 1975; GEORGE and SHERRINGTON, 1984; MIN and JINSHENG, 1984.
Crinum	YAMADA, 1963.

Table 2.5 (continued)

Genus and explants used for propagation	Reference
<u>Furcraea</u>	
Bulbil, leaf, stem.	LAKSHAMANAN and JANARDHANAN, 1977.
<u>Galanthus</u>	
Leaf, scale, stem.	HUSSEY, 1980.
<u>Hippeastrum</u>	
Adventitious buds, inflorescence, leaf, ovary, scale, stem.	MII, MORI and IWASE, 1974; HUSSEY, 1975a; 1976b; 1980; PAJERSKI and ASCHER, 1977; SEABROOK and CUMMING, 1977.
<u>Ipheion</u>	
Bulb, inflorescence, leaf, ovary, stem.	HUSSEY and WYVILL, 1973; HUSSEY, 1975a; 1980.
<u>Paramongaia</u>	FINNIE pers. com.
<u>Narcissus</u>	
Bulbils, inflorescence stalk, leaf, leaf base, ovary, ovary wall, scales, scape, stem.	HUSSEY and WYVILL, 1973; STONE, 1973; HOLDGATE *et al.*, 1975; HUSSEY, 1975a; 1976b; 1980; 1982; SEABROOK, CUMMING and DIONNE, 1976; HOSOKI and ASAHIRA, 1980; STEINITZ and YAHEL, 1982.
<u>Nerine</u>	
Leaf, meristem, scales, stem.	HAKKAART, MAAT and QUAK, 1975; HOLDGATE *et al.*, 1975; PIERIK and IPPEL, 1977; HUSSEY, 1980; GROOTAARTS, SCEL and PIERIK, 1981.
<u>Vallota/Cyranthus</u>	
Leaf, scales, stem.	HUSSEY, 1980.

Often the physiological age of the explant plays a large role in explant growth and differentiation (MURASHIGE, 1977; NARAYANASWAMY, 1977). GREEN and PHILLIPS (1975) observed that embryo explants taken from mature kernels of Zea produced only callus, whereas explants from young caryopses produced both callus and plants. Bulbscales from young bulbs and the internal bulbscales of old bulbs produced bulbs and roots with little callus production, whereas bulb formation was reduced with an increase in callus production when old bulbscales were used as explants (TAKAYAMA and MISAWA, 1980). Using tobacco stem explants from apical regions, resulted in roots and shoots being more readily produced, than explants taken from the basal region, showing a progressive decline in organogenesis down the stem (MURASHIGE, 1974). PIERIK, STEEGMAN and VAN DER MEYS (1974) reported that juvenile and adult callus tissue of Anthurium behaved identically in culture, they reasoned that differentiated tissue of adult plants rejuvenates when in the callus phase. However, only 30% of adult genotypes were induced to form callus whereas most of the juvenile tissue could be cultured. GEIER (1986) also reported that dedifferentiation was restricted to immature tissue of Anthurium. Juvenile tissue does not always produce the most highly morphogenic explants (DAVIES and DALE, 1979; WICKHAM, RODREGUEZ and ARDITTI, 1980; FINNIE and VAN STADEN, 1986).

Associated with physiological age is the developmental phase of the explant which is often associated with the season in which the explant was taken. Variations in temperature, light and water stress during the year will result in variation in carbohydrate, protein and hormone levels in the plant which ultimately affects the response of the explant in culture according to the time at which the explant was cultured (GEORGE and SHERRINGTON, 1984).

Bulb scale explants of Lilium obtained during spring and autumn regenerated readily while explants taken in summer or winter were not as regenerative (ROBB, 1957), while explants taken in summer and spring were found to be highly regenerative by KUKULCZANKA, KLIMASZEWSKA and PLUTA (1977). This can be explained by the co-operation of endogenous growth regulators in organogenesis, including endogenous indoleacetic acid (HATCHER, 1959; ODANI, 1974), abscisic acid (DAVIDSON and YOUNG, 1974; JENKINS and SHEPARD, 1974; WRIGHT, 1975) and cytokinins (MII, MORI and IWASE, 1974). Seasonal variation is known to influence the behaviour of explants on defined media (FELLENBERG, 1963; CASSELS, LONG and MOUSDALE, 1982). The dormancy requirements of various plants should be met before attempting culture to achieve maximum response, especially in lilies and gladioli (SEABROOK, CUMMING and DIONNE, 1976; NARAYANASWAMY, 1977; NISHUICHI, 1980a; GABRYSZEWSKA and SANIEWSKI, 1984).

Survival and development in culture has been related directly to the initial size of the explant used (MURASHIGE, 1974). The smaller the explant the less the regenerative ability. Meristems are impractical for rapid asexual multiplication, as the explants are small and their development and survival in culture is limited. Isolation of sterile tissue is inversely related to size (MURASHIGE, 1974). DAVIES and DALE (1979) using leaf discs showed that explants smaller than 2 mm had a high mortality rate, but if they survived decontamination, they produced the same number of shoots as a 5 mm explant. PAEK (1982) found that in Hyacinthus the number of bulblets per explant produced increased as the explant size increased but the number of bulblets per unit length of explant decreased. Large explants consisting of parenchyma, vascular tissue and cambium showed a greater capacity for shoot and bud formation than did smaller explants irrespective of auxin or cytokinin concentration (OKAZAWA, KATSURA and TAGAWA, 1967).

TRAN THANH VAN (1973), however, showed that it was possible to obtain mitosis and subsequent bud and root formation in one cellular layer of explant, small groups of homogeneous cytologically differentiated cells were capable of directly forming complex organs such as flowers. TRAN THANH VAN (1973) reasoned that thin layers of tissue have the following advantages over standard explants: rapid reactions, and direct microscopic observation making the determination of the distribution and subsequent development of organogenic centres possible.

Orientation of the explant may also affect explant performance in culture. In Lilium bulblets are always formed on the adaxial side of the bulb scale, even when placed adaxial side down on the medium (ROBB, 1957; LESHEM, LILIEN-KIPNIS and STEINITZ, 1982). It is important in Asparagus, Gladiolus, Narcissus and Tulipa that the scape sections and leaves must be inverted to obtain maximum reaction in culture (TAKATORI, MURASHIGE and STILLMAN, 1968; ZIV, HALEVY and SHILO, 1970; SEABROOK, CUMMING and DIONNE, 1976; GABRYSZEWSKA and SANIEWSKI, 1984). ZIV, HALEVY and SHILO (1970) using Gladiolus stems found that roots always developed on the basal end and buds on the upper surface regardless of initial explant position. Leaf explants always produced shoots at the proximal cut end even if inverted (RAJU and MANN, 1971), this polarity may be related to the polar transport of auxin in the explant. LESHEM, LILIEN-KIPNIS and STEINITZ (1982) found no polarity effects on the regenerative ability between proximal and distal parts of scale sections, nor did bulblets occur in any particular position. BEYL and SHARMA (1983) reported that in Gasteria and Haworthia there was a consistent trend towards greater embryogenesis with explants in the prone orientation over those with an upright orientation. PAEK (1982) discovered that regeneration is inversely related to the natural orientation, however GEORGE and SHERRINGTON (1984) feel the explant position is not important.

The nutrient needs of a culture can be satisfied by a mixture of mineral salts combining the essential macro- and micro-elements together with a source of carbon. For cultures to establish themselves various supplements are necessary in this medium; these include vitamins, amino acids, growth regulators and a chelate. The nutritional needs of the explant depends on the method of propagule multiplication.

A number of media have been used over the years for plant tissue culture with varying concentrations of the nutrient components (Table 2.6). There does seem to be a consistency in the relative amounts of magnesium, calcium and phosphate salts $Mg > Ca > PO_4$ (SCHENK and HILDEBRANDT, 1972). Composition of media is generally based on the elemental molecular weight, it is reasoned that the composition of media based on easily weighable amounts is desirable (GAMBORG, 1966; SCHENK and HILDEBRANDT, 1972).

It is apparent that no single nutrient medium is effective for all tissue cultures, and the basic salt requirements for culture in present day experimentation is largely based on the media devised by MURASHIGE and SKOOG (1962) and WHITE (1963) with modifications to meet special requirements (MURASHIGE, 1974). A standard basal medium generally consists of a mixture of salts of chlorides, nitrates, sulphates, phosphates and iodides of Ca, Mg, K, Na, Fe, Mn, Zn and B, vitamins, a carbon source, organic growth factors, a source of reduced nitrogen and plant hormones (NARAYANASWAMY, 1977). N, P, K, Ca, Mg and S are the elements essential for plant survival, Na and Cl are also necessary for tissue turgor and ionic balance. Nitrogen availability greatly affects survival and growth in culture, nitrate is the most important form of nitrogen, however, a source of reduced nitrogen is also required. Uptake of nitrate requires an acid pH but the medium turns less acid with the loss of nitrate while ammonium ion uptake results in a

Table 2.6 A comparative table of nutrient media used over the years for plant tissue culture (values given as mg ℓ^{-1})

	GAUTHERET (1942)	MURASHIGE and SKOOG (1962)	MILLER (1965)	SCHENK and HILDEBRANDT (1972)	SEABROOK, CUMMING and DIONNE (1976)
NH_4NO_3		1 650	1 000		1 650
KNO_3	125	1 900	1 000	2500	1 900
$CaCl_2$		440		200	440
$MgSO_4$	125	370	71,5	400	370
KH_2PO_4	125	170	300		
NaH_2PO_4					300
$NH_4H_2PO_4$				300	
$Ca(NO_3)_2$	500		500		
NaFeEDTA		37,3?	13,2		37,3
$FeSO_4$		27,85?		15	
Na_2EDTA		37,25?		20	
H_3BO_3	0,05	6,2	1,6	5	6,2
$MnSO_4$	3	22,3	14	10	22,3
$ZnSO_4$	0,18	8,6	3,8	1	8,6
KI	0,5	0,84	0,8	1	0,84
$NaMoO_4$		0,26		0,1	0,25
$CuSO_4$	0,05	0,026		0,2	0,025
$CoCl_2$	0,05	0,026		0,1	0,025
KCl			65		
$NiSO_4$	0,05				
$BeSO_4$	0,15				
H_2SO_4	1				
$Fe_2(SO_4)_3$	50				
$Ti(SO_4)_3$	0,2				
$Cu(NO_3)_2$			0,35		
$NH_4Mo_7O_4$			0,1		
Thiamine HCl	0,1-1	0,1	0,8	5	0,5
Nicotinic acid	0,5	0,5	2	5	5
Pyridoxine HCl	0,1	0,5	0,8	0,5	1
Glycine	3	2			2
Adenine sulphate					160
Myo-inositol		100	100	1 000	100
Cystenic HCl	10				

lowering of the pH until further uptake is inhibited (OHIRA, YAMADA and OJIMA, 1976). PIERIK, VAN LEEUWEN and RIGTER (1979) showed the promoting effect of low levels of NH_4NO_3 on sprout regeneration in callus of Anthurium was caused by the NH_4^+ ion and not the NO_3^- ion. GEIER (1986) also working on Anthurium found that low levels of NH_4NO_3 (200 mg/ℓ) proved beneficial for the induction of regeneration in leaf segments, whereas high NH_4NO_3 levels (720 mg/ℓ) accelerated root formation on callus.

The micronutrients Fe, Mn, B, Zn, Cu, Co and Mo are components of many cell proteins and enzymes, and are necessary for chloroplast functioning and chlorophyll synthesis. Cu and Zn are constitutents of oxidase enzymes and superoxide dismutase, some of which play a role in oxidation, hydroxylation and transformation of superoxide radicles. Fe and Mo are part of the nitrate reductase and nitrogenase enzymes, with other micro-elements being involved in the functioning of the genetic apparatus and the activity of growth substances. Table 2.7, adapted from GEORGE and SHERRINGTON (1984) summarizes the role micro-nutrients play on explant growth and functioning.

Iron is added in the form of a chelate of EDTA (ferric-sodium-etylenediamine-tetra acetate) to ensure its availability over a wider range of pH, and it may have a similar effect as auxin when added at low concentrations. Most explants are able to synthesize their own vitamin requirements, however, initially not all cultured plant cells are able to perform this process. Historically the organic requirements of cultures were met by the inclusion of coconut milk, casein hydrolysate, yeast extracts and fruit juices. These 'factors' were replaced by vitamins when it became necessary to produce defined media (NARAYANASWAMY, 1977).

Table 2.7 The biochemical effects and role of micronutrients in plant tissue culture

Element	Effect
B	Maintenance of meristem activity Low levels associated with depressed cytokinin synthesis.
Co	Components of vitamin B12.
Cu	Occurs in plastocyanin, oxidase enzymes, super oxide dismutase.
Fe	Affects the formation of amino acids, ferredoxin, protoporphyrinogen.
Mn	Maintenance of chloroplast ultrastructure; can replace magnesium in some enzyme systems; is active in photosystem II.
Mo	Decreases chlorophyll content; low levels result in poorly developed chloroplasts; is active in nitrate reductase and nitrogenase.
Zn	Is present in RNA and DNA polymerase enzymes; is a component of the enzymes associated with tryptophan synthesis; is involved with the enzymes, nitrogenase, nitrate reductase, oxidase, super oxide dismutase.

TAKAYAMA and MISAWA (1979) reported that increasing the strength of solid MURASHIGE and SKOOG (1962) media, resulted in the stimulation of bulblet formation while root growth was inhibited. Cations or high salt concentrations were effective in inducing embryogenesis, the ions may be involved in the activation of hormone biogenetic systems (HALPERIN, 1969). Using a liquid medium TAKAYAMA and MISAWA (1982/83) found that bulblet differentiation was not affected by the concentration of Murashige and Skoog media between one-eighth and full strength, while a double strength media inhibited both the growth and numbers of bulblets produced. NARAYANASWAMY (1977) recorded that the media of GAMBORG and EVELEIGH (1968) and SCHENK and HILDEBRANDT (1972) supports the growth of a wide variety of tissues of both the monocotyledons and dicotyledons. SEABROOK, CUMMING and DIONNE (1976) found that for Narcissus the media of Schenk and Hildebrandt and, Gamborg and Eveleigh gave no response while modified MURASHIGE and SKOOG (1962) and ZIV, HALEVY and SHILO (1970) media gave good growth.

> "The carbohydrate requirement of explants can be satisfied by the incorporation of sucrose at a concentration of 2 - 3%, glucose has been superior to sucrose only occasionally. Still other carbohydrates have been used but none has shown superiority over sucrose or glucose" (MURASHIGE, 1974).

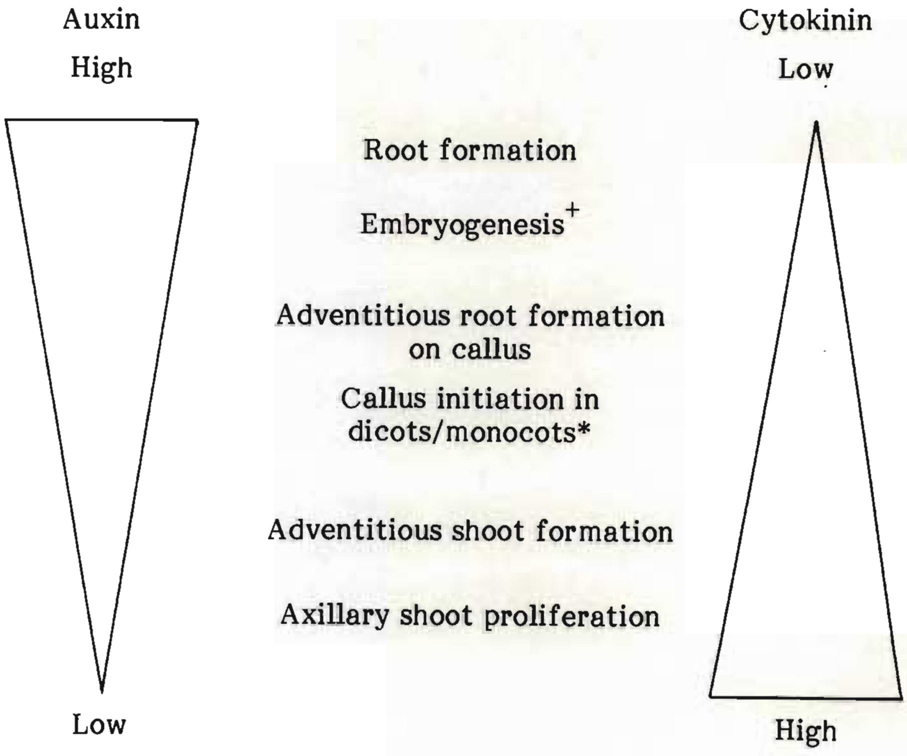
RAGHAVAN (1976) found that embryos grow better when supplied with sucrose than with glucose, fructose or other sugars. BURGHARDTOVA and TUPY (1980) showed that maize embryos prefer invert sugars to glucose and fructose alone and fructose to glucose. The effectiveness of sucrose in promoting root growth may be related to greater entry into vascular tissue and a rapid transport to the meristem (BUTCHER and STREET, 1960; STREET, 1969). Optimum levels of sucrose for bulblet formation are lower than those required for root formation (STEHSEL and CAPLIN, 1969; NAVARRO, ROISTACHER and MURASHIGE, 1975; TAKAYAMA and MISAWA, 1979). TAKAYAMA and MISAWA (1980) found that inhibition of scale leaf formation in Lilium by 90 g ℓ^{-1} sucrose is easily reversed on transfer to 30 g ℓ^{-1} sucrose, indicating the sucrose regulation of scale leaf formation, but that it does not have a direct effect on the induction of dormancy. However, internal changes related to sucrose concentration are involved in the regulation of dormancy. Sucrose levels which result in good callus growth may not be optimal for morphogenesis, the effect of cytokinins and the efficiency of nitrogen ions may be related to sucrose concentration (GAMBORG, CONSTABEL and SHYLUK, 1974).

"The most critical organic components of plant propagation media are auxins and cytokinins" (MURASHIGE, 1974). SKOOG and MILLER (1957) using tobacco pith cultures found that the balance between auxins and cytokinins plays a fundamental role in the course of morphogenesis. A relatively high concentration of auxin favours root initiation, while suppressing shoot formation. Cytokinins induce shoot and suppress root formation, with bud and root formation intimately related to auxin/cytokinin ratios. It must be realized that the same growth regulator may cause differing reactions in culture in differing species and cultivars, possibly due to the explant's ability to produce its own endogenous growth regulators, or the levels of the growth regulators within the plant at the time of isolation (HEMPEL, 1979), or different rates of uptake and metabolism as shown for cytokinins (VAN STADEN and MALLET, 1988).

Despite the voluminous literature available no single combination of hormones can entirely satisfy the needs of the various stages of tissue culture. It is apparent that in most cases a higher ratio of cytokinin to auxin, or cytokinin alone promotes the formation of adventitious shoots on a variety of explants. Callus normally grows best on media containing both auxin and cytokinin normally with more auxin than cytokinin, but media containing low levels of both hormones appears best as a general growth medium.

A review of the literature on the in vitro propagation of the Liliaceae, Amaryllidaceae and Iridaceae reveals that the hormone requirements for successful culture vary from plant to plant and researcher to researcher.

Since the isolation of 6-furfuryl aminopurine (kinetin) by MILLER, SKOOG, OKUMURA, VON SALTZA and STRONG (1955), a large volume of literature has been accumulated on the effect of cytokinins on in vitro culture. Although cytokinins occur naturally in plants (zeatin and its derivatives), because of the small size of the explant used for cultures the explant is not usually able to synthesize its own hormonal requirements, nor does it contain sufficient amounts of endogenous hormones to support growth. Callus growth is often dependent on cytokinins for growth and callus bioassays have been developed to test the biological effects of cytokinins (LINSMAIER and SKOOG, 1965; MURASHIGE and SKOOG, 1962; MILLER, 1963; 1965). KEMP and STOLTZ (1979) have shown that monocotyledon callus contained zeatin and zeatin riboside even when grown in the absence of hormones, indicating the tissues' ability to synthesize its own growth regulators in culture.



\+ Organogenesis in monocotyledons is often promoted in a medium without auxin or reducing the auxin concentration.

* In monocotyledons callus induction may require high levels of auxin, cytokinin may be unnecessary to unimportant.

Figure 2.7 Graphical summary of the various promotory effects of cytokinins and auxins on <u>in vitro</u> system (adapted from GEORGE and SHERRINGTON (1984).

Species belonging to the Liliaceae and Amaryllidaceae generally require higher levels of cytokinin in culture than do those of the Iridaceae (HUSSEY, 1976b), with species that have a slow rate of natural propagation, requiring higher amounts of cytokinin (BAP) (HUSSEY, 1980). The addition of cytokinin to many species results in the complete release from inhibition of apical dominance and the formation of multiple branched plantlets (MURASHIGE, SERPA and JONES, 1974; BOXUS, 1974; EARLE and LANGHANS, 1975; ABBOTT and WHITELEY, 1976; HUSSEY, 1976b; 1977a; DICKENS, KELLY, MANNING and VAN STADEN, 1986). Cytokinins can overcome and prevent dormancy in many of the bulbous species (HUSSEY, 1976b, 1977b; ZIV, HERTZ and BIRAN, 1983; DICKENS, KELLY, MANNING and VAN STADEN, 1986). Conflicting reports arise concerning the effects of cytokinin on callus formation, in certain cases callus may or may not be induced, at very high levels of cytokinin callus often occurs (HUSSEY, 1976b; 1978). GRESSHOFF (1978), stated that cytokinins are not required to initiate shoot development in monocotyledons, while most cytokinin reports concern the effect the hormone has on morphology (TAKAYAMA and MISAWA, 1982a). The hormone's known effect on morphology include bud formation and promotion, corm formation, axillary branching, bulblet and bulbscale formation and plantlet development (ZIV, HALEVY and SHILO, 1970; HUSSEY, 1976b; 1980; TAKAYAMA and MISAWA; 1979; 1982a; 1983; DICKENS, KELLY, MANNING and VAN STADEN, 1986). The presence of cytokinin also ensures continued growth on transplanting in Stage III (ZIV, 1979).

SHERIDAN (1968), reported that auxin (IAA) stimulated callus formation but was not essential while ZIV, HALEVY and SHILO (1970) and REUTHER (1977) maintain that auxins are essential for callus formation, alternatively JHA, MITRA and SEN (1984) found that auxin alone was unable to induce callus. The auxins have been shown to play an important role in morphogenesis of cultures, with "organogenesis being governed by the concentration of auxin" (MII, MORI and IWASE, 1974), and high levels promote organ formation without intermediate callus production. Other effects include inhibition of corm formation, but stimulation of root growth, promotion of roots, buds, shoots, bulbils and bulbs (with additions of cytokinins having little or no effect on the number and quality of organs formed) (HUSSEY, 1976b; PIERIK and IPPEL, 1977; TAKAYAMA and MISAWA, 1979; PAGE and VAN STADEN, 1985; DICKENS, KELLY, MANNING and VAN STADEN, 1986). Callus cultures supplemented with auxins undergo rhizogenesis (KAUL and SABHARWAL, 1972; BUI-DANG-HA, NORREEL and MASSET, 1975; STIMART, ASCHER and ZAGORSKI, 1980), and reduction in the amount of auxin from induction levels also induces rhizogenesis (GROENEWALD, WESSELS and KOELEMAN, 1977; BAJAJ, SIDHU and GILL, 1983).

As mentioned before many aspects of differentiation and organogenesis are controlled by an interaction between auxin and cytokinin concentrations. HOSOKI and ASAHIRA (1980) and BAJAJ, SIDHU and GILL (1983), both found that low auxin and cytokinin levels did not induce callus, but that high auxin concentrations with cytokinin resulted in callus. Root production occurs when the cytokinin : auxin ration is 1 : 1 or $<1:1$ while shooting is induced when cytokinin levels are higher than the auxin level (BAJAJ and PIERIK, 1974; PIERIK and STEGMANS, 1975; MORI, YASEGAWA and KANO, 1975; SEABROOK, CUMMING and DIONNE, 1976). GRESSHOFF (1978), found that cytokinin is not necessary to initiate shoot development, while auxin reduction or removal could result in organogenesis (HUSSEY, 1976a; HEUSER and APPS, 1976). These phenomena show the possibility that monocotyledons have a cytokinin autonomy (SHERIDAN, 1968; GRESSHOFF, 1978). Callus cultures induced using both hormones will shoot on reduction in the concentration of auxin or increases in the cytokinin levels (ABO-EL-NIL, 1977; WESSELS, GROENEWALD and KOELEMAN, 1976), because auxin may inhibit or partially stimulate synergistically the cytokinin effect or organogenesis (TAKAYAMA and MISAWA, 1979).

On the other extreme SHERIDAN (1968), SIMMONDS and CUMMING (1976) and HEMPEL (1979) have shown that many species do not require hormones for callus induction and organogenesis.

It is apparent that no simple formula can be devised to satisfy the hormonal needs of explants, and it is now known that a simple balance of two hormones for plantlet formation cannot be applied to all plants, but rather that organogenesis is controlled by a number of complex interactions involving environmental (light and temperature), nutritional (media, hormones, charcoal, morphactins, and as yet undefined factors) and plant related factors.

The pH of a medium is a critical factor, which can affect: cell membranes, the buffering capacity of the cytoplasm, whether the nutrients remain in soluble form, the uptake of nutrients and the gelling efficiency of agar. Despite these factors, studies on pH has been neglected for tissue culture. The usual pH range is between 5 and 6, however pH drift does occur during culture (MURASHIGE, 1974). Uptake of nitrate ions results in a shift towards neutrality while uptake of ammonium ions results in the acidification of the media (STREET, 1969). MURASHIGE and SKOOG (1962), add agar, preheat the medium and then adjust the pH to 5,7 - 5,8 which allows all the salts to remain in a soluble form. GAMBORG, MILLER and OJIMA (1968) found a greater increase in growth at levels below pH 5,5. The pH of the media may influence the regeneration of plant tissue cultures. TAKAYAMA and MISAWA (1979) using Lilium explants induced root formation within the range 4 - 7 with an optimum at 6, while bulb formation occurred between pH 4 - 8 with an optimum between 5 - 7. Iris bud formation is inhibited below 5,0 and above 5,7 (FUJINO, FUJIMURA and HAMADA, 1972).

The use of a liquid or solid media could ensure the success or failure of a culture, the choice is often based on the available facilities and the personal preferences of the investigator. Bromeliad cultures can only be started in liquid culture, whereas Asparagus requires initiation on a solid medium (MURASHIGE, SHABDE, HASEGAWA, TAKATORI and JONES, 1972; MURASHIGE, 1974).

Agar is the most common form of gelling agent used, advantages include: stability of the gel at all incubation temperatures, the gel is not hydrolysed by plant enzymes nor does it react with media components (GEORGE and SHERRINGTON, 1984). DEBERGH (1982) reported that the properties of the gel depend on the brand. Certain agars contain Ca, Mg, K and Na which may affect nutrient levels. Highly purified agar is too expensive for commercial concerns. Purified Difco bacteriological agars should be used for studies on nutrition and metabolism. ICHI, KODA, ASAI, HATANAKA and SEKIYA (1986) recorded the presence of growth inhibitory substances in agar and recommend the use of gellan gum (Gelrite, PS-60) as gelrite gelled media enhances callus and shoot growth to a greater extent than does agar. Concentrations of agar between 0,7 - 1% are used in experimental cases, but many commercial laboratories use 0,5%, soft gels may lead to 'sinking' of the explant in the medium and the possibility of vitrification, while excessive high concentrations of agar inhibit growth of tissue. The concentration will depend on the plant being cultured (MURASHIGE, 1974; DAVIS, BAKER and HANAN, 1977; SINGHA, 1982). The advantages of solid media include; explants are easily recoverable, aeration of the media is not necessary, the media is stationary allowing for normal growth without gravitational complications, and disintegration of cells and organs does not occur.

Despite the above advantages a number of researchers advocate the use of liquid media (HOSOKI, 1975; EARLE and LANGHANS, 1975; MIKKELSEN and SINK, 1978; LIU and STABA, 1979; TAKAYAMA and MISAWA, 1982/83; 1983), these researchers prefer 'shake cultures' as the number of vessels necessary for culture is reduced. In a solid media the explant surface contact area is limited while in liquid media there is large surface contact area, resulting in more efficient nutrient uptake and an increase in growth rates. In addition, toxic compounds accumulating around the explant are easier to disperse in a liquid medium. Using a shake culture scheme TAKAYAMA and MISAWA (1982/83) estimate that they can produce between $1,2 \times 10^{10} - 3,2 \times 10^{12}$ bulbs per year using one medium sized bulb as an explant.

The lighting requirements of cultures is not the same as those for autotrophic whole plants, as in culture photosynthesis is unnecessary or reduced because of the carbon source in the media. Light is however, necessary to regulate certain morphogenetic responses, and should be considered in terms of intensity, photoperiod and quality (MURASHIGE, 1974; 1977). Illumination in the range of 300 - 3000 lux is normally used during Stages I and II, higher illuminations of 3000 - 10000 lux is recommended for Stage III to improve the explant's chance of survival on transplanting. WARD and VANCE (1968) and WEIS and JAFFE (1969) indicated that the criticial portion of the light spectrum is the blue region, LETOUZI and BEAUCHESNE (1969) showed that the blue region promotes shoot formation and red light favours roots. SIEBERT, WETHERBEE and JOB (1975) using tobacco showed that both flux density and wavelength are important. (Tissue would grow in the dark, but shoot formation was stimulated by near UV light (371 nm) at low irradiances (90 lux) but inhibited when flux density was more than 540 lux, whereas blue light (420 nm) caused stimulation at 1080 and 2160 lux). TRAN THANH VAN (1977) summarizes the effect of light regimes on morphogenesis of tobacco explant in Table 2.8, bud and callus formation was indifferent to the actions of monochromatic radiation. Plants needing specific photoperiods, are likely to manifest this need in <u>in vitro</u> culture (MURASHIGE, 1978).

Table 2.8 Light and morphogenesis, the influence of light at 24° ± 2°C on morphogenesis of <u>Nictotiana</u>

Nature of light	Organ formation % Flower	Root	Bud	Callus
Continuous light	91	16	100	100
Darkness	33	100	91	100
Far red/darkness	50	91	100	100
Far red/darkness interrupted by red light every 6 hours	91	63	91	100

Light has been found to be inhibitory to callus growth in certain species (KLEIN, 1964; YEOMAN and DAVIDSON, 1971) or stimulatory in others (DE CAPITE, 1955; SEIBERT, WETHERBEE and JOB, 1975). HAMMERSCHLAG (1978) discovered that light of differing intensities was both stimulatory and inhibitory, with a cultivar specificity in response to light intensity. In the case of organ formation STIMART and ASCHER (1978a) showed light suppressed bulb formation, while NIIMI and ONOZAWA (1979) showed light favours bulblet regeneration. TAKAYAMA and MISAWA . (1979) found no light effect on bulblet and root formation in <u>Lilium</u>, LESHEM, LILIEN-KIPNIS and STEINITZ (1982) ascribed the variation in light response to the use of different explant organs.

WAREING and PATRICK (1975) point out that the light effect may be due to sink strength as well as hormone directed transport of assimilates or regulation of ethylene formation. Spectrophotometric assays show the presence of high levels of phytochrome in actively growing regions of a number of monocotyledons (KOUKKARI and HILLMAN, 1966) supporting MURASHIGE's (1974) belief that key organogenic processes in tissue culture are photomorphogenic phenomena, probably regulated by phytochrome.

Temperature has also been shown to be an important factor in morphogenesis and development; it appears that the general practice is to maintain cultures at a constant temperature of ± 25°c. The use of daily and seasonally fluctuating temperatures is more desirable, but such facilities are not always available. Optimum temperature for tissue culture varies from species to species and may affect morphogenetic capacity (APPELGREN and HEIDE, 1972; SUTTER and LANGHANS 1978; KATO and OZAWA, 1979; STIMART, ASCHER and WILKEN, 1982; DURMISHIDZE, GOGOBERIDZE and MAMALADZE, 1983). An important factor to consider when culturing bulbous monocotyledons, is the dormancy requirements of the bulbs. Table 2.9 lists species of Liliaceae, Iridaceae and Amaryllidaceae requiring cold treatments in response to dormancy in culture. The variation in temperature requirements exemplifies the significance of exploring and satisfying seasonal temperature requirements of some plants (MURASHIGE, 1974).

Table 2.9 Species requiring cold treatments to break dormancy

Plants		Reference
Liliaceae	Heloniopsis	KATO and OZAWA, 1979.
	Hyacinthus	HUSSEY, 1977a; PAEK, 1982.
	Lilium	MURASHIGE, 1974; HUSSEY, 1977a; VAN AARTRIJK and BLOM-BARNHOORN, 1978; TAKAYAMA and MISAWA, 1980; 1982a.
	Tulipa	RIVIERE and MULLER, 1979; NISHIUCHI, 1979.
Amaryllidaceae	Narcissus	HUSSEY, 1977a.
Iridaceae	Gladiolus	HILDEBRANDT, 1971; HUSSEY, 1977a.
	Iris	HUSSEY, 1977a

Figure 2.8, adapted from GEORGE and SHERRINGTON (1984) graphically summarizes the various methods available for in vitro propagation of plants, these methods may incorporate the use of axillary bud or adventitious shoots or embryos

Figure 2.8 The principal methods of micropropagation using either a direct or indirect technique. Additional methods not shown in the figure include the techniques of protoplast and anther culture which may fall in both the direct or indirect category.

In vitro propagation from axillary shoots is the most reliable technique for plant regeneration, this may be accomplished by using shoot tips (apical or axillary meristems) or by using the single node method. The first successful shoot apex culture was performed by LOO (1945) using Asparagus. The widespread application of shoot tip cultures in commercial practices, has led to misuse of the term meristem culture. Meristem cultures, mericlones or meristemming has been applied to relatively large stem explants (± 10 mm long), true meristem culture makes use of an explant less than 0,1 mm in height (normally only the terminal dome). Culture of this explant is difficult and survival and multiplication rate in culture is very poor. Normally only a single plant can be derived from this explant, but true apical meristem culture is important in the development of pathogen free plants (MURASHIGE, 1974; QUAK, 1977).

The use of shoot apex culture for clonal propagation was first applied by MOREL (1960). The method of multiplication relies on the induction of bud primordia (normally by cytokinins) each of which can produce a shoot. Explants used to start shoot apex cultures may be either the lateral or the main shoot meristem, the cultures after induction can be subdivided and the process repeated (DODDS and ROBERTS, 1985). (Not all shoots arising from shoot tip culture originate from axillary buds, adventitious shoots may arise from explant tissue, or from callus produced at the cut surface).

The techniques of axillary meristem culture allows for rapid multiplication of the plant due to the exponential nature of subculturing. The most important factor regulating growth in this type of culture is the presence or absence of cytokinins

(HUSSEY, 1976b, 1981; SAWHNEY and NAYLOR, 1982). For long term multiplication and storage of selected genotypes, culture techniques are required that include genetic stability and minimum loss of totipotency. "Techniques that exploit the natural mechanism of axillary shoot formation seem most likely to achieve this" (HUSSEY, 1976b).

In vitro layering involves the use of Stage II shoot tips with separate nodes, axillary shoot growth is induced without growth regulators by either; placing shoots horizontally on new media or use of single nodal pieces. Advantages include the ease of culture, and reduction in the chance of callus formation (e.g. *Asparagus* and *Alstroemeria*) (HUSSEY, 1980).

In certain plants multiplication via adventitious shoots may be a result of meristem formation directly on explant tissue, but not from previously formed callus. Direct adventitious meristem formation is dependent on explant origin, regeneration may occur on a wide range of organs or only one or two tissue types. Induction of the meristem, (in probably the epidermal tissue) is due to hormonal influences (HUSSEY, 1982a), the process is usually achieved by triggering or enhancing cell division in explants by auxin or auxin plus cytokinin, with high concentration of auxin and/or longer exposure to auxin (HUSSEY, 1977a). Adventitious plantlet formation allows for genetically uniform plants to be produced provided they are formed from non-polysomatic tissue with the minimum of callus formation. As there are limited numbers of axillary shoots on a plant, adventitious shoot induction provides an ideal starting point for axillary shoot culture (HUSSEY, 1977a; 1982b). Direct embryogenesis may occur in culture. "Genes regulating embryogenesis sometimes can remain activated in newly formed seedling tissues, which retain an embryogenic competence enabling new adventive embryos to be initiated readily from superficial cells" (GEORGE and SHERRINGTON, 1984).

Propagation by indirect organogenesis involves the induction and manipulation of callus (cell aggregates which arise from disorganised growth of small plant organs or detached plant tissue, of previously cultured cells).

Callus cultures are useful in analysing the effect various factors have on organogenesis and embryogenesis, the technique allows for rapid reproduction of plants in certain cases. The regenerative capacity of callus cultures depends on:

i) the period between initiation and onset of morphogenesis;
ii) the frequency of plantlet initiation;
iii) if after sub-culture regeneration can be re-induced;
iv) the number of subcultures without loss or decrease in the frequence of morphogenesis;
v) the production of entire plantlets from the induced callus;
vi) the frequence of genetic abnormalities.

Genetic changes in cultured cells occur frequently (KAO, MILLER, GAMBORG and HARVEY, 1970), with the production of polyploid and aneuploid cells. The cells may become polyploids due to endoreduplication, selective stimulation of polyploids already produced, chromosomal aberrations and nuclear fragmentation (MURASHIGE and NAKANO, 1965; D'AMATO, 1978; KASHA, KOTT and SEGUIN-SWARTZ, 1982; THORPE, 1982). Cells from the initial explant may have genetic variations (KASHA, KOTT, SEGUIN and SWARTZ, 1982) and these abnormalities increase during passage in culture. Ploidy differences may occur in callus obtained from different explants taken from the same mother plant (YAMADA, NISHI, YASUDA and TAKAHASHI, 1967). Variations may arise as a result of numerous factors, while many cultures remain genetically stable (MURASHIGE, 1974).

The variety of explants used for the induction of callus in monocotyledons has a narrower range than that for dicotyledons (GEORGE and SHERRINGTON, 1984), with embryos, young leaves, stem nodes and inflorescences being the most frequently used sources of explant. Most callus cultures are derived from either the vascular cambium or parenchyma cells (YEOMAN and STREET, 1973). Yeoman stated that the initiation of callus is dependent on the culture environment and not the origin of the explant, whereas EVANS, SHARP and FLICK (1981), maintain that the successful initiation of callus is explant dependent. Figure 2.9 summarizes the growth response of a typical cell culture, cell division does not occur throughout the callus mass, but rather at meristematic areas near the surface. There is great variability in the type of differentiation found in callus cultures, a culture consisting entirely of parenchyma cells is rarely found (NARAYANASWAMY, 1977), with cytodifferentiation occurring to form tracheary elements, sieve elements, suberized cells, secretory cells and trichomes. The nutritional requirements of callus cultures of the Liliaceae, Iridaceae and Amaryllidaceae have been discussed above, subculture of callus is necessary due to accumulation of toxins and depletion of the media.

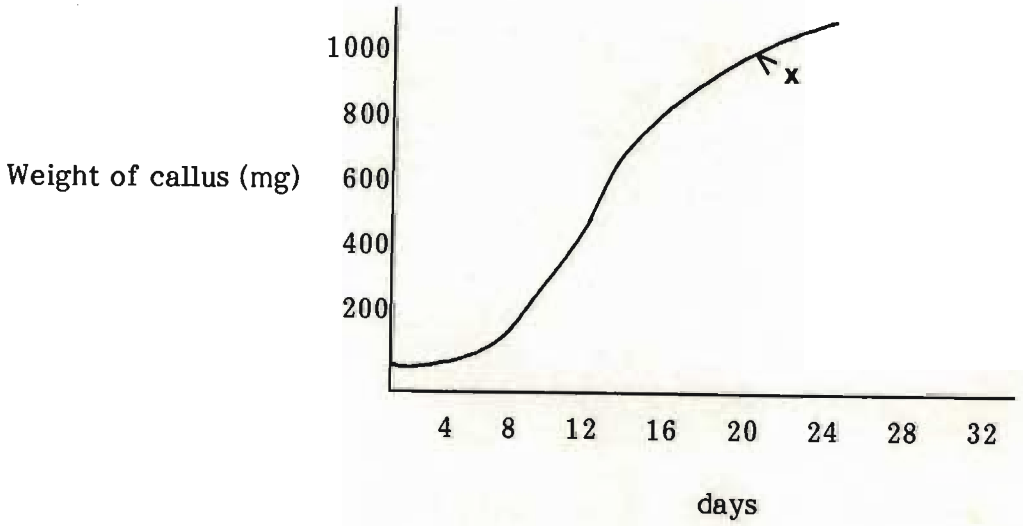


Figure 2.9 Growth response of a typical callus culture, x = time for approximate subculture in this callus (DODDS and ROBERTS, 1985)

After prolonged subculture changes often occur in callus cultures, hormone habituation, the loss of organogenic potential and change in the external characteristics of the callus (THORPE, 1980).

When callus is grown in an agitated liquid medium it forms a suspension composed of free cells and cell aggregates ranging from a few to several hundred cells, depending on the friability of the callus (NARAYANASWAMY, 1977). Increase in cellular material reaches a saturation point beyond which no further increase occurs; at this stage subculture by dilution is necessary. With most suspension cultures maximum density is reached within 6 - 25 days (STREET, 1977). The initiation of a suspension is largely determined by trial and error (KING and STREET, 1979), normally a large amount of callus is required to serve as innoculum (2 - 3 g for 100 ml of medium) (HELGESON, 1979), cells will not grow below a critical cell density (8 - 15 x 10^{-3} cells/ml) (STREET, 1977). A major advantage of the cell suspension technique, is the much faster rate of cell multiplication in a suspension as initial growth of morphogenic callus can be slow initially (the system is advantageous provided the suspension retains its capacity to reform callus with organogenic capacity). The technique has been used by KRIKORIAN and KANN (1981) using Hemerocallis.

COCKING (1960) first succeeded in producing protoplasts, however, no further progress was achieved for the following ten years. Division by regenerated cells was reported by NAGATA and TAKEBE (1970) and KAO, KELLER and MILLER (1970), plants were obtained the following year (NAGATA and TAKEBE, 1971). POWER, CUMMINS and COCKING (1970), POWER and COCKING (1971), KAO and MICHAYLUK (1974) and KAO, CONSTABEL, MICHAYLUK and GAMBORG, (1974) showed the possibility of the fusion of somatic protoplasts and the combining of genetic material from normally incompatible plants. SIMMONDS, SIMMONDS and CUMMING (1979) felt that Lilium species are suitable for protoplast studies, as chromosomal and meiotic irregularities resulting from in vitro techniques could be more readily tolerated by vegetatively reproducing species, than by those relying on sexual processes.

Protoplast isolation can be achieved from a number of explants, but to obtain consistently high yields of viable protoplasts, often proves difficult. This variability may be as a result of the physiological condition of the explant, the age and conditions under which isolation occurs (UCHIMIYA and MURASHIGE, 1974; WATTS, MOTOYOSHI and KING, 1974; VASIL and VASIL, 1980). Protoplast isolation may be achieved by mechanical means or by the use of enzymes (primarily cellulase and pectinase) (POWER and COCKING, 1969; 1977). Purification of the protoplasts can be by means of filtration or by floatation in sucrose solutions (EVANS, KEATES and COCKING, 1972). The protoplast technique for the in vitro culture of the Liliaceae, Iridaceae and Amaryllidaceae has not been widely utilized with relatively few examples. Asparagus (BUI-DANG-HA, NORREEL and MASSET, 1975), Hemerocallis (FITTER and KRIKORIAN, 1981) and Lilium (SIMMONDS, SIMMONDS and CUMMINGS, 1979).

To use the protoplast technique for successful in vitro culture and genetic manipulation, one must be able to readily isolate in large numbers and culture the protoplasts so as they are able to regenerate cell walls, colonies and organs!

The purpose of anther and pollen culture is the production of haploid plants by the induction of embryogenesis from divisions of monoploid spores, either microspores or immature pollen grains (REINERT and BAJAJ, 1977). TULECKE (1953) first observed that mature pollen grains of Ginkgo could be induced to form haploid callus, while GUHA and MAKESHWARI (1964; 1966) reported the direct development of embryos from microspores of Datura. The chromosome complement of haploids can be doubled by the use of colchicine or regenerative techniques, to yield fertile homozygous diploids (VASIL and NITSCH, 1975). Unfortunately, the number of successful pollen cultures is relatively small (SUNDERLAND, 1977), with few examples in the Liliaceae, Iridaceae and Amaryllidaceae, Asparagus, Crocus, Fressia, Gladiolus, Lilium (SHARP, RASKIN and SOMMER, 1971; HONDELMANN and WILBERG, 1973; RAQUIN, 1973; BAJAJ and PIERIK, 1974; BAJAJ, SIDHU and GILL, 1983; CHICHIRICCO and GRILLE CAIOLA, 1987).

The most important factor in anther culture is the relative development of the anther (SUNDERLAND and WICKS, 1969). Anthers of plants fall into three categories, premitotic, mitotic or postmitotic, and selection of the correct 'anther age' is dependent on to which category the plant belongs (SUNDERLAND, 1979). For Nicotiana the best stage for explant utilization is when floral buds are just visible beyond the calyx (referred to as stage 2 by NITSCH (1969) or stage 4 by SUNDERLAND and DUNWELL (1977)). Other factors affecting anther culture include plant age, physiological status, temperature, light, media and growth regulators (REINERT and BAJAJ, 1977). The advantages of pollen culture include the study of somatic cell genetics. Figure 2.10 represents advantages for plant breeders and tissue culturers, when utilizing the anther / pollen culture technique.

Stage II Clonal increase of the plant

Stage II involves propagule multiplication, with a rapid increase of organs and structures which ultimately gives rise to plants (MURASHIGE, 1974; 1977). The methods used have been described previously in this chapter. "The bulk of culture activity and time is usually spent in this stage" (MURASHIGE, 1977). Methods using axillary bud propagation are recommended, although adventitious organogenesis may be faster, a high occurrence of genetically aberrant plants are often the result.

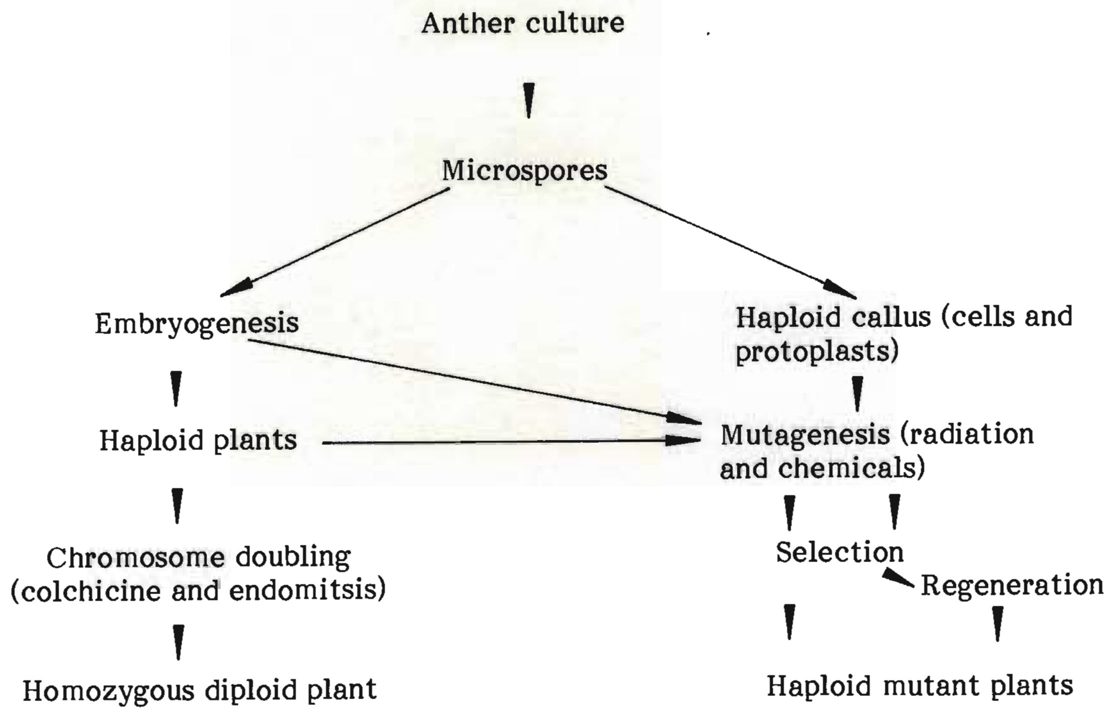


Figure 2.10 The induction of homozygous diploids and mutants, obtained through anther culture (REINERT AND BAJAJ, 1977)

Stage III and IV Re-establishment of plants in soil

Difficulties occur when propagules from Stage II are transplated directly into soil (HASEGAWA, MURASHIGE and TAKATORI, 1973). Stage III is intended to prepare propagules for transfer to soil as a successful tissue culture method must result in the re-establishment in the soil of a high frequency of the tissue culture derived plants (MURASHIGE, 1974). DEBERGH and MAENE (1981) added Stage IIIa and IIIb (the reasons having been discussed earlier). Stage IV involves the transfer into the natural environment, this transfer can result in the loss of plants for two main reasons:

i) plantlets produced in culture have poorly developed cuticles, and on transfer, plantlets suffer rapid water loss (SUTTER and LANGHANS, 1979);

ii) plantlets in culture are provided with nutrients, on transfer the plantlets must become autotrophic, which appears to take a few days (this adversely affects plantlet survival).

Subculturing of in vitro plantlets to a pretransplanting medium with a lower level of nutrients under higher light intensities, provides a hardening stage which prevents desiccation after transplanting (ZIV, 1979), with mist spray techniques or plastic covers to assist acclimatization. It appears that in most cases members of the Liliaceae, Iridaceae and Amaryllidaceae can be directly transferred into soil even when they have leaves and stems (NOVAK and PETRU, 1981), but in spite of the ease of transplanting the plantlets show a strong tendency to become dormant (HUSSEY, 1977a; 1977b; TAKAYAMA, MISAWA, TAKASHIGE, TSUMORI and OHKAWA, 1981). A period of low temperature (2 - 5°C) for between 3 - 8 weeks breaks dormancy when planting out (HILDEBRANDT, 1971; HUSSEY, 1977a; TAKAYAMA, MISAWA, TAKASHIGE, TSUMORI and OHKAWA, 1982) while cytokinins can also release in vitro corms from dormancy (HUSSEY, 1977b). STEINITZ and YAHEL (1982) recorded that for successful establishment in the field the plantlets must be sufficiently developed prior to transplanting (i.e. a bulb ± 1 cm in diameter, two leaves and an abundant root system) without these organs survival rate is poor.

In concluding this mini review it is pertinent to heed the words of warning from BOXUS and DRUART (1980). In vitro cultures have largely surpassed the mere stage of laboratory research, with a large number of commercial laboratories utilizing these techniques.

> "The extreme plant cell totipotency and the equally extreme plasticity of vegetal tissue and organs can lead to a very high reproduction rate of plantlets. This incentive of the in vitro cultures has fortunately not escaped the attention of certain avant garde practicians. Unfortunately

> the apparent facility of the <u>in vitro</u> multiplication techniques and the attraction of the novelty have quite often also led to anarchial developments of techniques still in the experimental stage" (BOXUS and DRUART, 1980).

Despite warnings certain dangers have not been avoided:

i) many believe all culture techniques ensure pathogen free material, and

ii) genetic conformity is guaranteed irrespective of explant origin or culture technique.

CHAPTER 3

REGENERATION OF *Clivia miniata*, *Gloriosa superba* AND *Sandersonia aurantiaca* *IN VITRO*

3.1 Introduction

Clivia miniata, *Gloriosa superba* and *Sandersonia aurantiaca* are the three species under investigation, each genus has its own cultural and ecological problems, with both *Clivia* and *Sandersonia* being classified as threatened species, while *Gloriosa* has potential in both the horticultural and pharmaceutical industry. The popularity of the three species has resulted in an increasing demand for the plants in Southern Africa as well as Europe and Asia, however, no successful technique has been established for the rapid clonal propagation of the plants, with the resulting exploitation of the natural population.

The following study was undertaken to devise a method for rapid propagation of the three indigenous monocotyledonous species. Many *in vitro* techniques were applied to the study plants, ranging from the relatively simple callus culture to the more involved use of protoplasts. It should be noted from the outset that statistical replication of certain of the experiments was impossible, due to the limited number of plants available for experimentation especially *Clivia miniata* var *citrina* and *Sandersonia aurantiaca*. In the case of *Clivia miniata* var *citrina* the readily available var *miniata* was utilized as explant material for the development of a technique which in turn can be applied to var *citrina* which in theory should respond similarly in culture. The similarity of *Gloriosa* and *Sandersonia* in both their growth habit and biochemistry allows for a comparative study to be performed between the two species, in terms of explant origin as well as cultural requirements.

3.2 Materials and Methods

3.2.1 Plant material

The natural distribution of the study plants was discussed in the previous chapter. For experimental purposes plants of C. miniata var miniata were grown in the Botany Department botanic gardens in a well shaded area. C. miniata var citrina explants were kindly donated by Mr and Mrs L. English. Plants of Gloriosa were collected from the coastal forests in Northern Natal and from a commerical nursery (Hawkfalls Nursery) in the Natal midlands. These plants were grown in pots in a 60% shade house in the Botany Department's botanic gardens. Sandersonia plants were collected from the farm 'Samvula' of Mr and Mrs L. English near Table Mountain in the Pietermaritzburg district. The plants were found growing in sugar cane fields, identification was confirmed only after flowering. The Sandersonia plants were transplanted into pots and grown in a 60% shade house. Explants were cultured within six hours (where possible) after collection unless potted out. In order to establish the most suitable source of explants all portions of the plants were utilized, except in the case of C. miniata var citrina.

3.2.2 Pretreatment of mother plants

Preliminary results from explants taken directly from their natural habitat indicated the need for a pretreatment step or stage 0 as recommended by DEBERGH and MAENE (1981). All plants were transplanted into sterile potting media, fed regularly (during the growth season of Gloriosa and Sandersonia) with Kelpak (a seaweed concentrate) and Nitrosol (an inorganic plant supplement) together with a spraying regime of a pesticide (Malathion) and a systemic fungicide (Benomyl) to reduce possible contaminants. Where possible, the plants were watered without excessive wetting of the aerial portions of the plant. In order to increase the number of Gloriosa plants available for experimentation, Gloriosa seeds were germinated in speedling trays, the plantlets were harvested at one, two and three years respectively.

3.2.3 Media and bench preparation

Unless otherwise stated, the culture medium devised by MURASHIGE and SKOOG (1962), (Table 3.1) was utilized. This media can be bought as a premix with obvious advantages for the commercial industry. The media was made up of nine stock solutions (as outlined in Table 3.1), which comprised of amounts of analytical grade chemicals, dissolved in distilled water. The solutions were stored in a fridge at 10°C and light sensitive components were kept in the dark. Additional supplements (sucrose, myo-inositol and growth regulators) were added during final preparation of the media, which was made up to volume using distilled water, and the pH was adjusted to 5,8 using sodium hydroxide (NaOH) or hydrochloric acid (HCl). Agar (Difco-bacto, or Oxoid no. 1) was added to the media at a concentration of 0,8%, the agar was dissolved by steaming in an autoclave (or microwave) for five minutes. Ten millilitres of the dissolved media was dispensed into 25 x 100 mm glass tubes and capped with aluminium Cap-O-Test caps. The media together with transfer instruments, glassware and distilled water were sterilized in an autoclave at one bar pressure at a temperature of 121°C for 20 minutes. All transfer procedures were performed on a laminar flow bench, which had been swabbed down and sprayed with 70% ethanol prior to use. During transfer of plant material, culture vessel openings and instruments were flamed before and after each transfer, with the bench and the culturer's hands and arms being regularly swabbed with 70% ethanol.

Table 3.1 MS medium, adapted from MURASHIGE and SKOOG (1962)

Nutrient	Concentration mg 1^{-1}	Mass/500 ml stock (g)	Volume stock ml/litre final medium	Stock solution
NH_4NO_3	1650	82,5	10	i
KNO_3	1900	47,5	20	ii
$CaCl_2.2H_2O$	440	22,0	10	iii
$MgSO_4.7H_2O$	370	18,5	10	iv
NaFe EDTA	37,3	2,0	10	v
KH_2PO_4	170	8,5	10	vi
H_3BO_3	6,2	0,31	10	vii
$ZnSO_4.4H_2O$	8,6	0,43		
$MnSO_4.4H_2O$	22,3	1,115		
KI	0,83	0,042		
$Na_2MoO_4.2H_2O$	0,25	0,013	10	viii
$CoCl_2.6H_2O$	0,025	0,0013		
$CuSO_4.5H_2O$	0,025	0,0013		
glycine	2	0,1	10	ix
nicotinic acid	0,5	0,025		
pyridoxin HCl	0,5	0,025		
thiamine HCl	0,1	0,005		
myo-inositol	0,1 $gℓ^{-1}$			
sucrose	30 g 1^{-1}			
agar	8 g^{-1}	pH5,8		

3.2.4 Sterilization

The first objecive of in vitro culture is to attain development of excised tissue free from contaminants. Plants are inevitably contaminated with a wide range of micro-organisms, and failure to remove contaminants will result in the carbon rich medium becoming infected, with rapid proliferation of micro-organisms.

Presterilization: The explants were processed immediately after harvest (where possible), washed for five minutes in water plus a detergent (Tween 20) to remove all traces of soil and debris, all non-viable portions were removed and the tissue was subjected to a 30 minute soak in 1% benzimidazol (Benomyl), a systemic fungicide, followed by a brief wash in water and a rinse in 80% ethanol plus Tween 20 for ten seconds. The plant was then cut into workable sizes for further treatment. (Using plants subjected to Stage 0, the use of Benomyl in the presterilization regime was found to be unnecessary).

Sterilization: Use was made of a wide range of sterilants, namely, sodium hypochlorite ((NaOCl) (commercial bleach Jik containing 3,5% active ingredient was used), mercuric chloride ($HgCl_2$), hydrogen peroxide (H_2O_2) and 100% ethanol. In all cases the detergent Tween 20 (Polyoxyethylene sorbitan monolaurate) was added at a concentration of two drops per 100 millilitres of sterilant. The explants were sterilized in various concentrations of these sterilants for differing periods of time. The explants were then rinsed three times in sterile distilled water, the first rinse lasting five minutes, the second 15 minutes and the final rinse lasted for 30 minutes. The explants were removed from the final rinse and stored in sterile petri-dishes to prevent desiccation, all plant tissue visibly damaged by the sterilant was removed. The plant material was cut into suitable sized explants and transferred onto the nutrient media. The cultures were then transferred to the growth rooms and monitored for contamination. All cultures unless stated were incubated in a growth room with a culture environment of 25 ± 2°C with a relative humidity of 40% and a 16 hour light : 8 hour dark cycle at an intensity of 27 $\mu E\ m^{-2}s^{-1}$ (Figure 3.1). From these experiments a sterilization regime was adopted for each explant type and used for all future experimentation.

3.2.5 Explant origin

The most important determinant of rate of plant increase and quality of regenerated plants is the initial explant. In the absence of a suitable explant success is at best limited (MURASHIGE, 1974).

Roots: The roots of all three species were used for investigation, but the roots of Gloriosa and Sandersonia are thin and seasonal, making them diffucult to work with, and over-sterilization resulted in the death of a large majority of the explants. The contractile roots of Clivia are abundant and a continuous supply of explants can be assured. Root explants were cut into 1 cm long segments.

Corm/rhizome: Explants derived from corms of Gloriosa and Sandersonia provided the bulk of explants used for tissue culture, one year, two year, and older corms being used for the establishment of cultures. The corms were divided into five distinct zones, segments of the corm between 1,0 - 1,5 cm^3, were inoculated on the media. Although the availability of corms is not seasonal, there is a distinct seasonal response in the in vitro corms with respect to both physiology and growth, a factor which received attention in a future experiment. The rhizome of Clivia was used as a source of explants. However, utilization of this plant part would necessitate the harvesting and "destruction" of the entire plant. Because of the limited number of var citrina plants available use of this source of explants was avoided where possible. Explants between 1 - 1,5 cm^3 were taken at random from all regions of the rhizome.

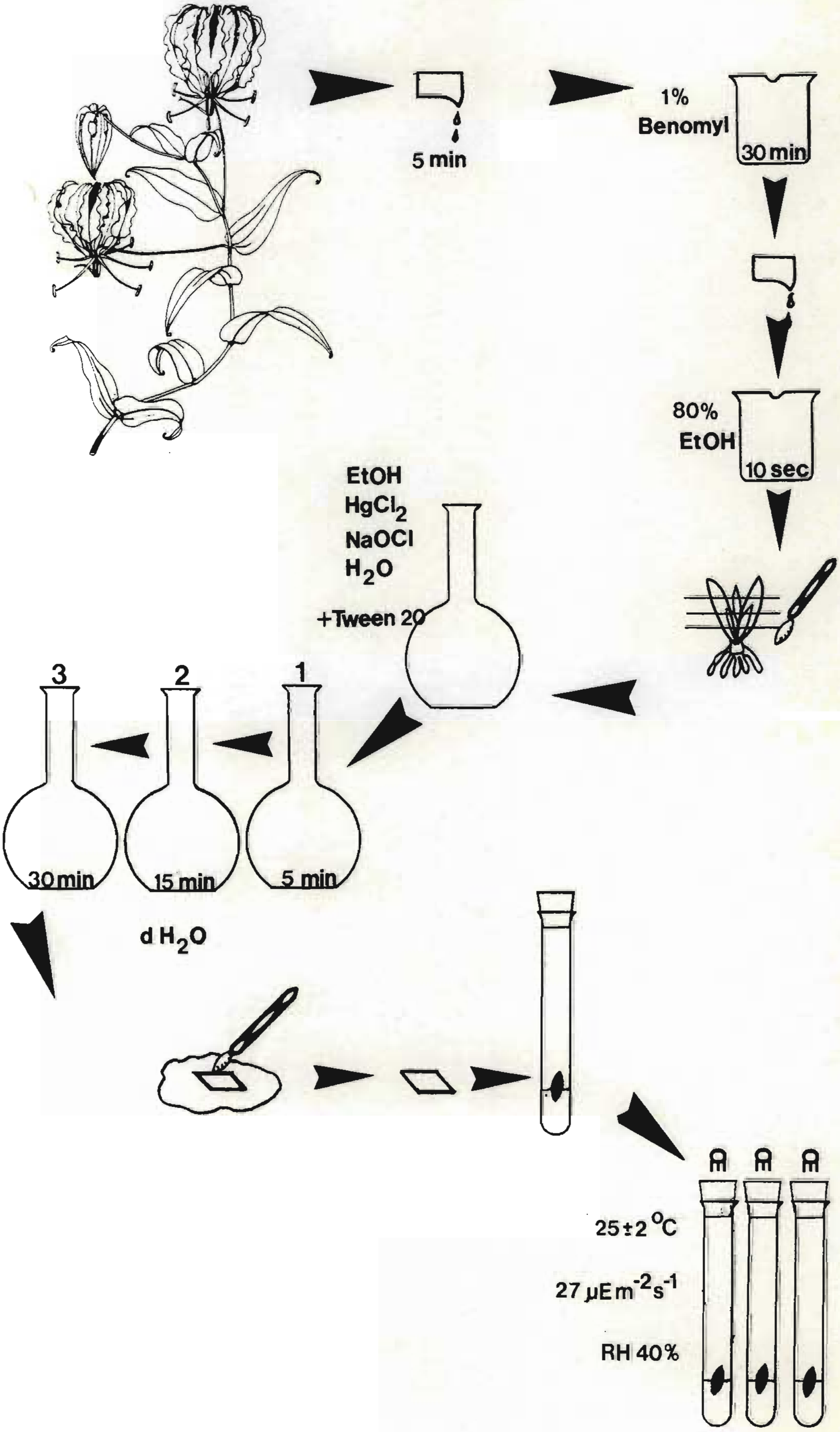


Figure 3.1 Procedure utilized to sterilize and culture plant tissue explants.

Meristem: Meristems were obtained from plants of all three species. meristems excised consisted only of the apical dome, however, once again removal of the meristem may result in the death of the plant. This negative feature is compounded by the fact that there are only a limited number of meristems per plant.

Stem: Mature and immature stems of Gloriosa and Sandersonia were utilized for the establishment of cultures. Due, however, to the seasonal nature of these two plants, explants could only be taken during November, December and January as after this period the plants began to senesce.

Leaves: The suitability of leaves as a source of explants was determined. In the case of Gloriosa and Sandersonia the seasonality problem again was a limiting factor. The abundance of Clivia leaf material provides an ideal source of explant material that does not result in the destruction of the entire plant. Explants 1 - 2 cm^2 were taken from five distinct regions (Figure 3.2) from both mature and juvenile leaves. Leaf bases were also used as explants.

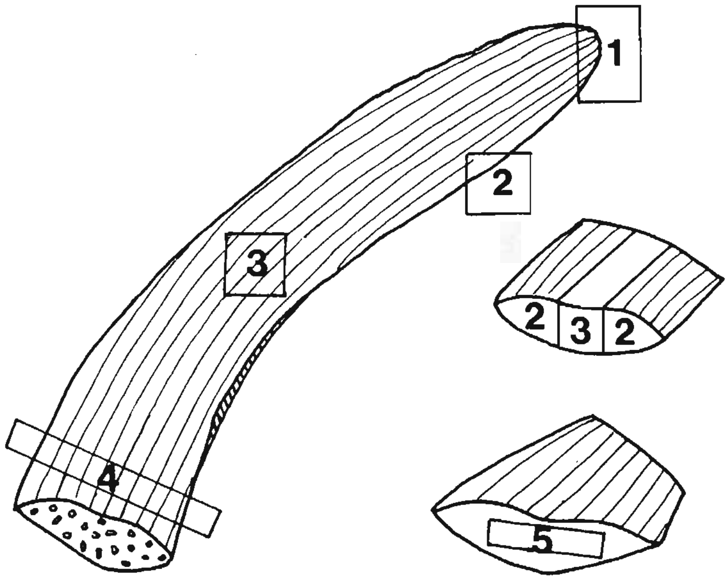


Figure 3.2 Origin of Clivia leaf explants

Inflorescence peduncle: Inflorescence peduncles of Clivia were utilized for culture establishment but for commercial application availability of explant material is limited to the flowering season.

Flowers: Use of flower explants from all three genera included the use of buds of all ages. Later refinement to the procedure involved the use of buds at five distinct ages: (i) just after the bud became visible; (ii), (iii) and (iv) during the development of the buds prior to opening and (v) after the perianth had opened. (These five stages were also used for anther culture). Explants were also taken from the pedicel, isolated petals, immature ovaries, filaments and styles. Explant size varied depending on the age of the flower utilized. Flower explants of Clivia were taken between September and November, while floral explants from Gloriosa and Sandersonia were derived during December and January.

Fruit/capsules: Fruit explants were taken at three distinct ages: (i) just after fertilization; (ii) during development, and (iii) during the ripening process. Although the availability of explants is limited to the flowering season a great deal of success was achieved using these explants, and subsequently the use of Clivia berry explants was extensively pursued.

Seeds and embryos: Probably the easiest way to derive sterile plant material is by aseptic germination of seeds in vitro. Clivia, Gloriosa and Sandersonia seeds were sterilized and germinated on both hormone enriched and hormone free media. Excised embryos were also used as explants. Due to the difficulty involved in obtaining and germinating Sandersonia seeds, excised embryos from this genus were not utilized.

3.2.6 Hormones

"The most critical organic components of plant propagation media are auxins and cytokinins" (MURASHIGE, 1974). During the course of this investigation a large range of available auxins and cytokinins were utilized for experimentation:

Auxins: 1-naphthaleneacetic acid (NAA)
2,4-dichlorophenoxyacetic acid (2,4-D)
indole-3-acetic acid (IAA)
indole butyric acid (IBA)

Cytokinins: kinetin
benzyladenine (BA)
zeatin

After the establishment of successful sterilization techniques, explants were subjected to a number of hormonal treatments. Initial experimentation was performed using either the synthetic auxins 2,4-dichlorophenoxyacetic acid or 1-naphthaleneacetic acid or the synthetic cytokinins benzyladenine and kinetin. These four hormones were chosen because of their stability in culture as well as their relative availability. three concentration grids were set up for experimentation (Figure 3.3a - c).

Where possible combinations of the various hormones were utilized (using one of the above three grids) to establish the hormonal response in culture of leaves, rhizomes, corms, stems, flowers and roots. Unfortunately, because of the lack of explant material, explant response could not be tested in all cases, thus resulting in the modification of these grids or, alternatively, the selection of a hormone combination (after referral to the literature) which would most likely give a positive response. In future experiments using callus derived from the initial cultures, these grids could be utilized for statistically significant experimentation. From the results obtained it was established that most explants are capable of

		BA/kinetin concentrations mg ℓ^{-1}			
		0	0,1	1	5
2,4-D/NAA concentration mg ℓ^{-1}	0				
	0,1				
	1				
	5				

a.

		BA/kinetin concentrations mg ℓ^{-1}			
		0	0,25	2.5	5
2,4-D/NAA concentration mg ℓ^{-1}	0				
	0,25				
	0.5				
	5				

b.

		Zeatin concentrations mg ℓ^{-1}		
		0	0,1	1
IAA concentration mg ℓ^{-1}	0			
	0,1			
	1			

c.

Figure 3.3 (a-c) Concentration grids used for hormonal experimentation producing callus, which could be subjected to further hormonal manipulation. It was also evident that only specific explants were capable of producing multiple axillary/adventitious plants (ensuring genetic stability).

3.2.7 Subculturing and callus manipulation

Large amounts of callus were derived from the explant and hormone experiments. The type of callus was classified into four groups:

- (i) Clivia callus
  - (a) fine white crystalline callus, which showed no further morphogenetic response on the mother explant.
- (ii) Gloriosa and Sandersonia callus
  - (b) when maintained on a medium supplemented with 2,4-dichlorophenoxyacetic acid, the callus remained undifferentiated with the production of an exudate on the interface between the plant material and the medium;
  - (c) when maintained on a medium free from 2,4-dichlorophenoxyacetic acid the callus became rhizogenic;
  - (d) crystalline vitrified callus.

In order to build up sufficient stocks of callus, Gloriosa and Sandersonia callus was routinely subcultured on a media containing 1 mg ℓ^{-1} 2,4-dichlorophenoxyacetic acid and 1 mg ℓ^{-1} kinetin every seven weeks, until sufficient stocks were established for further experimentation. The major objectives for utilization of callus were the development of a system for continuous shoot production, as well as utilization for suspension culture. The hormone grids described in the previous section (3.2.6) were utilized to test the shoot producing ability of the callus. Three types of callus were used for experimentation:

(i) callus maintained on 2,4-dichlorophenoxyacetic acid for longer than 12 months;

(ii) "new" callus excised from the original explant;

(iii) rhizogenic callus.

The bulking up of Gloriosa and Sandersonia callus on the 2,4-dichlorophenoxyacetic acid / kinetin medium was relatively easy to achieve. However, Clivia callus growth was slow and in most cases the callus senesced after one or two subcultures.

A minimum of ten tubes / flasks were used for each of the above treatments, the morphogenetic response was recorded and the callus was massed at the end of each experiment.

3.2.8 Nutrients

Nutrient experimentation involved the use of four types of media:

(i) MURASHIGE and SKOOG (1962) (Table 3.1);

(ii) a modified Murashige and Skoog medium utilized for Anthurium culture by FINNIE and VAN STADEN (1986);

(iii) MILLER'S (1963; 1965) nutrient medium; and

(iv) SCHENK and HILDEBRANDT (1972) medium.

The constituents of media (ii), (iii) and (iv) are summarized in Table 3.2.

The amount of sucrose supplied to MURASHIGE and SKOOG's (1962) medium was varied, namely 0, 10, 20, 40 and 80 $g\ell^{-1}$. Finally the amount of available nitrogen in the MURASHIGE and SKOOG (1962) medium supplemented with 1 mg ℓ^{-1} NAA and 0,1 mg ℓ^{-1} kinetin was varied. Available nitrogen was varied using a full strength Murashige and Skoog as the control and the levels of both NH_4NO_3 and KNO_3 were reduced by a half, quarter and an eighth strength.

Table 3.2 Nutrient media for tissue culture of Clivia, Gloriosa and Sandersonia values expressed as mg ℓ^{-1}

Nutrient	FINNIE and VAN STADEN (1986)	MILLER (1963; 1965)	SCHENK and HILDEBRANDT (1972)
NH_4NO_3	825	1000	-
KNO_3	950	1000	2500
$CaCl_2.2H_2O$	220		200
$MgSO_4.7H_2O$	185	71,5	400
Na Fe EDTA	18,65	13,2	
$FeSO_4$			15
Na_2EDTA			20
KH_2PO_4	85	300	
$NH_4H_2PO_4$			300
$Ca(NO_3)_2.4H_2O$		500	
H_3BO_3	6,2	1,6	5
$ZnSO_4.4H_2O$	8,6	3,8	1
$MnSO_4.4H_2O$	22,3	14	10
KI	0,83	0,8	1
$Na_2MoO_4.2H_2O$	0,25		0,1
$CoCl_2.6H_2O$	0,025		0,1
$CuSO_4.5H_2O$	0,025		0,2
KCl		65	
$Cu(NO_3)_2.3H_2O$		0,35	
$NH_4Mo_7O_4.4H_2O$		0,1	
glycine	2		
nicotinic acid	0,5	2	5
pyridoxin HCl	0,5	0,8	0,5
thiamine HCl	0,1	0,8	5
myo-inositol	100	100	1000
sucrose	30 g ℓ^{-1}	pH 5,8	
agar	8 g ℓ^{-1}		

3.2.9 Environmental experiments

Most of the cultures were grown at a temperature of 25 ± 2°C at a light intensity of 27 $\mu Em^{-2} s^{-1}$ with a 16 hour light : 8 hour dark cycle. Temperature variation was tested at 25°C, 20°C and 10°C respectively (during a power failure the temperature in one of the growth rooms rose to 35°C. Although the high temperature did not adversely affect the growth response of the cultures, this abnormally high temperature provided a stimulus for the growth of internal bacterial contamination from supposedly sterile cultures).

Culture response to light was also investigated using:

(i) 16 hour light : 8 hour dark cycle at an intensity of 27 $\mu Em^{-2}s^{-1}$ as a control;

(ii) continuous low light at an intensity of 0,5 $\mu Em^{-2}s^{-1}$;

(iii) continuous darkness;

(iv) continuous light at an intensity of 35 $\mu Em^{-2}s^{-1}$;

(v) continuous darkness for two weeks, then transferred to light (i) and finally

(vi) cultures were grown in a 60% shade house in the garden with a resulting day / night light and temperature fluctuations.

3.2.10 Seasonality

The seasonal response of Gloriosa corms was investigated to observe whether these organs were subject to the same dormancy mechanisms in culture as they are *in vivo*. Corms were harvested in spring, summer, autumn and winter and cultured at 25°C. Dormant and actively grown corms were cultured at 10°C and 25°C. Established cultures (corm explants that had produced a single cormlet and a shoot) were also subjected to variation in the temperature regime. Half the cultures were grown in 25°C while the remainder were subjected to an alternating temperature regime of 10°C and 25°C.

3.2.11 Pollen and anther culture

Pollen and anther cultures were attempted for all three species. The anthers were taken from buds at five distinct ages (described in Section 3.2.5). The technique used for anther culture is summarized in Figure 3.4, and the media constituents are listed in Table 3.3.

Table 3.3 Nutrient media used for anther culture, based on the medium of NITSCH and NITSCH (1969)

Nutrient	Concentration mg ℓ^{-1}
KNO_3	950
NH_4NO_3	720
$MgSO_4.7H_2O$	185
$CaCl_2$	166
KH_2PO_4	68
$MnSO_4.4H_2O$	25
H_3BO_3	10
$ZnSO_4.7H_2O$	10
$Na_2Mo\,O_4.2H_2O$	0,25
$CuSO_4.5H_2O$	0,025
NaFe EDTA	18,75
myo-inositol	100
glycine	2
nicotinic acid	5
pyridoxine HCl	0,5
thiamine HCl	0,5
folic acid	0,5
biotin	0,05

sucrose	20 g ℓ^{-1}	
agar	8 g ℓ^{-1}	
IAA	0,1 mg ℓ^{-1}	pH5,5

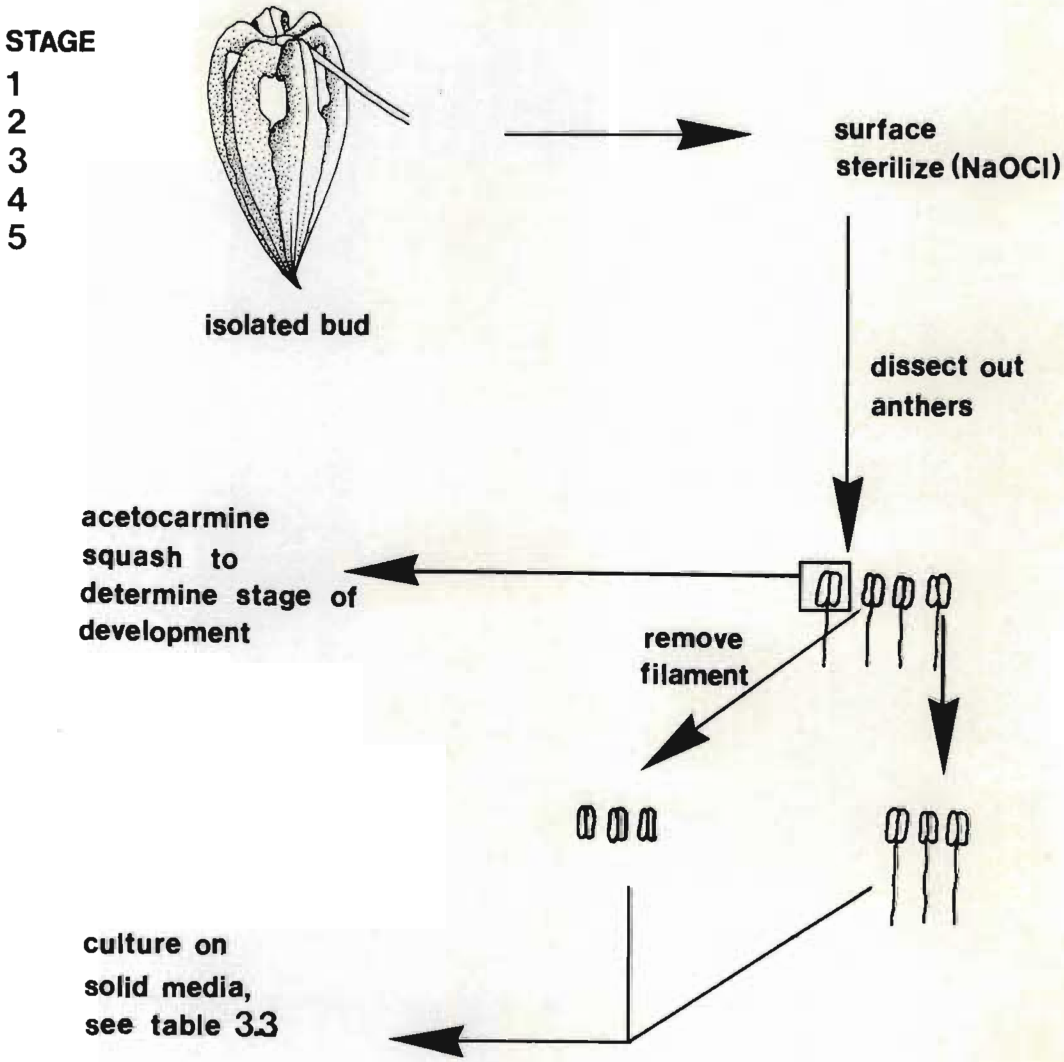


Figure 3.4 Technique used to culture Clivia, Gloriosa and Sandersonia anthers.

3.2.12 Protoplast culture

Protoplasts from both Clivia and Gloriosa leaves were produced using the technique outlined in Figure 3.5. Leaves grown in vivo and in vitro were utilized for protoplast isolation. A half strength MURASHIGE and SKOOG (1962) medium supplemented with Oxoid no. 1 agar was utilized. Cultures were either grown in the dark at 25°C for three weeks and then transferred to a growth room with a light intensity of 0,5 $\mu Em^{-2}s^{-1}$, or at 0,5 $\mu Em^{-2}s^{-1}$. Enzyme and nutrient solutions are listed in Table 3.4.

Table 3.4 Enzyme and nutrient solutions for protoplast culture

Enzyme solution 1	2% cellulase 0,5% pectinase	plus 13% mannitol	pH5,4
Enzyme solution 2	2% cellulase 0,5% pectinase 0,25% macerase	plus 13% mannitol	pH 5,4
Hormone	no hormones 0,1 mg ℓ^{-1}BA; 0,1 mg ℓ^{-1}NAA		
Nutrient medium	½ strength MS medium, 13% mannitol 20 g ℓ^{-1} sucrose		pH 5,4
Agar	8 g ℓ^{-1} or 4 g ℓ^{-1} Oxoid no. 1		

3.2.13 Hardening off

To achieve the hardening off of in vitro grown plantlets, the following technique was utilized:

(i) Rooted plantlets were removed from the culture tubes, and excess agar was washed off;

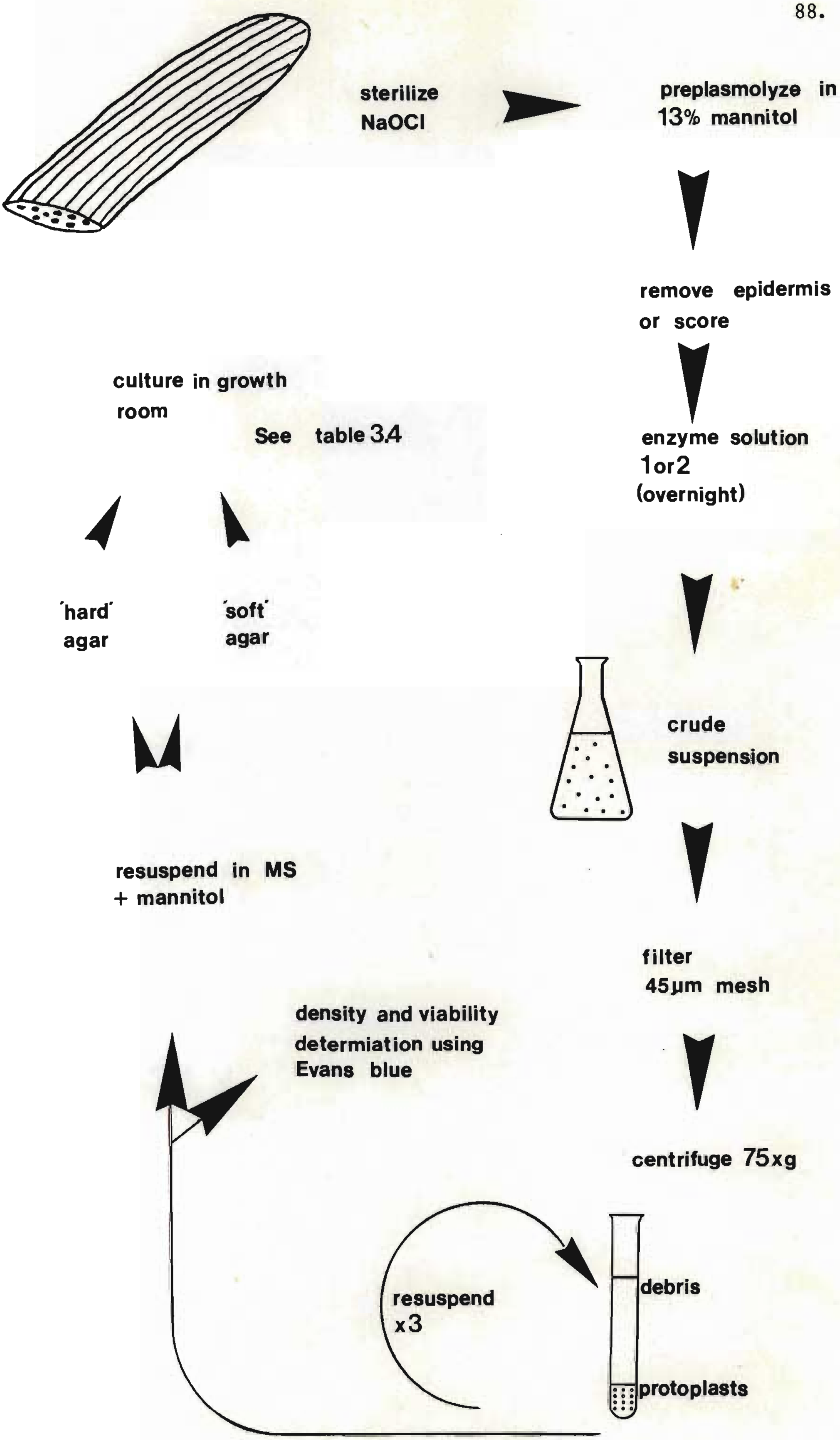


Figure 3.5 Technique used to produce both Clivia and Gloriosa protoplasts.

(ii) plantlets were transplanted into plastic pots containing sterile peat compost;

(iii) the pots were placed in a mist house (five seconds per five minutes) for two weeks;

(iv) the pots were then transferred to a damp greenhouse for a week and then to a 60% shade house for four weeks during which time they were well watered;

(v) plantlets were planted out *in situ*.

Despite the high humidity in the culture tubes, *Clivia* plantlets had a well developed cuticle *Gloriosa* and *Sandersonia* plantlets on the other hand had poorly developed cuticles, and hardening off of these plantlets was difficult to achieve, resulting in the death of the aerial organs. Hardening off of *Gloriosa* and *Sandersonia* cultures with well developed cormlets was relatively simple. The fragile aerial portion of the plantlet was removed and the cormlets were placed directly into a moist potting media, without necessitating a gradual decrease in the relative humidity.

3.2.14 Histological studies

Histological studies were performed on explant tissue and *in vitro* grown tissue. So as to elucidate the results obtained from previous experiments, and to give a visual representation as to the origin of the propagules and the type of cells and tissues involved in the *in vitro* process, use was made of light, scanning and transmission electron microscopy respectively.

Light microscopy: Explant material was fixed in formalin - acetic acid - alcohol (FAA) at room temperature. The plant material was subsequently dehydrated in a tertiary-butanol / ethanol graded series and infiltrated with wax according to the schedule outlined in Table 3.5. Once completely impregnated with wax, the samples were "blocked" in pure wax, mounted on wooden stubs and refrigerated for two hours. Sections were cut on a Jung microtome. These sections were mounted on glass slides pretreated with HAUPT (1930) adhesive. Once dry the slides were stained with safranin and fast green (JENSEN, 1962) as outlined in Table 3.6, and viewed under an Olympus BH_2 photomicroscope.

Table 3.5 Schedule for wax embedding

Fixation:

Fix in FAA for a minimum of 24 hours FAA:

96% ethanol	50 mℓ
Acetic acid	5 mℓ
37% formaldehyde	10 mℓ
distilled water	35 mℓ

Solution	Minimum time (hours)	Temperature °C
Dehydration		
water : ethanol : tertiary butanol		
1) 45 : 45 : 10	1	20°
2) 30 : 50 : 20	12	20°
3) 15 : 50 : 35	1	20°
4) 5 : 40 : 55	1	20°
5) 0 : 25 : 75	1	20°
6) 0 : 0 : 100	2	40°
7) 0 : 0 : 100	18	40°
Infiltration:		
1) Tertiary butanol : liquid paraffin 50 : 50	24	40°
2) Liquid paraffin	12	40°
3) liquid paraffin and a few wax pellets	12	40°
4) liquid paraffin and wax pellets in an open vial	24	60°
5) pure molten wax	48	60°

Table 3.6 Staining with safranin and fast green

Sections dewaxed 2 x 3 minutes in xylene	
Xylene / alcohol	1 minute
95 % alcohol	30 seconds
70 % alcohol	30 seconds
safranin	20 minutes
95 % alcohol	30 seconds
100% alcohol	1 minute
100% alcohol	1 minute
50 % absolute alcohol : 50% xylene	1 minute
fast green	few seconds
50% absolute alcohol : 50% xylene	30 seconds
xylene	1 minute

Electron microscopy:

(i) Resin embedding for transmission electron microscopy was achieved by initially fixing the tissue in glutaraldehyde buffered with sodium cacodylate, followed by three washes in buffer and post fixation with osmium tetroxide. Following subsequent washes the tissue was dehydrated in a graded alcohol series and impregnated with Epon resin as outlined in Table 3.7. Specimens were trimmed and ultrathin sections were cut on a Reichert Jung Ultracut E microtome using glass knives made on an LKB knife maker. Sections were mounted onto copper grids and stained with uranyl acetate and lead citrate according to the technique of REYNOLDS (1963). The grids were given a final rinse in double distilled water, dried and examined using a Jeol 100 CX transmission electron microscope at an accelerating voltage of 80 kV.

ii) Explant tissue for scanning electron microscopy was fixed in gluteraldehyde for 24 hours before being dehydrated in a graded alcohol series range from 10 to 100%. After dehydration the specimens were critically point dried, mounted on specimen stubs, sputter coated with gold-palladium in a Polaron ES-100 vacuum evaporator and viewed in the Hitachi S-570 scanning electron microscope.

Table 3.7 Preparation routine for Epon resin embedding of explant material for transmission electron microscopy

Procedure	Time
Fixation	
Glutaraldehyde buffered with0,05 M sodium cacodylate (pH 6,9 to 7,2)	minimum 24 hours
Wash in 0,05 M sodium-cacodylate buffer	2 x 30 minutes
Post-fixation	
2% Osmium tetroxide buffered with 0,02 M sodium-cacodylate buffer (pH 6,9 - 7,2)	minimum 4 hours
Wash in 0,02 M sodium-cacodylate buffer	2 x 30 minutes
Dehydration	
Dehydration in graded ethanol series (10, 20, 30, 40, 50, 60, 70, 80, 90%)	10 minutes each
100% anhydric ethanol	3 x 10 minutes
Propylene oxide	2 x 30 minutes
Infiltration (Epon/Araldite)	
25% Epon : 75% Propylene oxide plus DMP (1 drop DMP per mℓ)	2 hours caps on
50% Epon : 50% Propylene oxide plus DMP	2 hours caps on
75% Epon : 25% Propylene oxide plus DMP	overnight caps off
100% Epon plus DMP	24 hours caps on
Embedding and polymerization	
Place tissue in 100% Epon plus DMP at 70°C	48 hours

3.3 Results and discussion

3.3.1 *Clivia miniata*

HOLDGATE, AYNSLEY, FENWICK, HILL, KREBS, LYNE, RANGAN, ROTHWELL, SPURR, STOKES, SMITH and THOMAS (1975), GEORGE and SHERRINGTON (1984) and VASIL (1985), reported the culture of *Clivia* using direct adventitious organogenesis, indirect organogenesis and multiple shooting techniques. All these reports are the results of culture experiments carried out in commercial laboratories, and the culture techniques were not published. MIN and JINSHENG (1984) reported the successful culture of callus and plantlets from *Clivia nobilis* using immature embryos, petals and ovary walls.

3.3.2 Sterilization

A prerequisite for successful culture is the establishment of an aseptic technique. As discussed in the previous chapter the sterilizing agent and the treatment time will depend on the sensitivity of the explant material (YEOMAN, 1973). Perhaps it is pertinent to reconsider a table from Yeoman's paper concerning the sterilization of different plant organs (Table 3.8).

Table 3.8 Sterilization procedures for different plant organs adapted from YEOMAN (1973)

Tissue	Presterilization	Sterilization
Seed	10 secs 100% alcohol	20-30 mins 10% CaOCl, 5 mins 1% bromine water
Fruit	10 secs 100% alcohol	10 mins 2% NaOCl
Stem	Scrub in running water, rinse in 100% alcohol	15-30 mins 2% NaOCl
Storage organs	Scrub in water	20-30 mins 2% NaOCl
Leaves	10 secs 100% alcohol	1 min 0,1% $HgCl_2$

It is evident that each plant organ has its own optimal sterilization requirement. Thus, the first experiment in this investigation involved the establishment of suitable sterilization regimes for each explant. The results presented in Table 3.9 a, b, c, d, confirm that each explant requires a specific sterilization regime for optimum explant response in culture.

Table 3.9 Percentage of Clivia explants sterile after 4 weeks in culture

a) Percentage of Clivia explants successfully sterilized using 100% ethanol

Sterilant	Time	Percentage explants sterilized after 4 weeks in culture				
		Leaf	Root	Rhizome	Flower	Fruit/Ovary
100% ethanol	5 mins	5	0	0	-	-
	10 mins	7-10	0	0	-	-

b) Percentage of Clivia explants successfully sterilized using 10% H_2O_2

Sterilant	Time	Percentage explants sterilized after 4 weeks in culture				
		Leaf	Root	Rhizome	Flower	Fruit/Ovary
10% H_2O_2	10 mins	41*	0	0	-	100
	15 mins	52*	0	0	-	100

* large amount of callus production

Table 3.9 (continued)

c) Percentage of Clivia explants successfully sterilized using NaOCl

Sterilant	Time	Percentage explants sterilized after 4 weeks in culture Leaf	Root	Rhizome	Flower	Fruit/Ovary
1% NaOCl	10 mins	10	0	0	-	100
	20 mins	15	0	0	-	100
2% NaOCl	10 mins	53	5	-	100	100
	15 mins	79	10	-	100	-
	30 mins	90*	10	10	-	-
3% NaOCl	10 mins	85*	-	-	-	100
	20 mins	-	27	33	-	-

* decreased viability / dead due to over-sterilization

d) Percentage of Clivia explants successfully sterilized using $HgCl_2$

Sterilant	Time	Percentage explants sterilized after 4 weeks in culture Leaf	Root	Rhizome	Flower	Fruit/Ovary
0,1% $HgCl_2$	1 min	10	0	0	-	-
	10 mins	60	20	20	-	-
	15 mins	100*	66*	33*	-	-

* a number of the explants were dead because of over-sterilization

The roots and rhizomes of Clivia proved to be the most difficult explant to decontaminate, with $HgCl_2$ soaks for long periods being necessary to give sterile explants. Despite the fact that up to 60% of the explants could be decontaminated using

$HgCl_2$, viability was markedly reduced (even though the surface of the explant in contact with the sterilant was cut off prior to culture). Inclusion of Benomyl in the pretreatment for subterranean organs was essential for reducing the percentage contamination of the explants. A large percentage of the contamination was a result of soil borne fungi, with a low percentage of bacterial contamination. In the case of the aerial organs pretreatment with Benomyl had no noticeable effect on the sterilization of the explant. Leaf, flower and ovary explants were easier to decontaminate and percentages of up to 100% could be achieved. The more "violent" sterilization techniques markedly reduced explant vigour with marked shrivelling and necrosis of the explant. The ovary/fruit proved to be the easiest of the explants to decontaminate (provided the fruit was undamaged, and ripening of the berry had not commenced). The optimum sterilization techniques are summarized in Table 3.10 and these values were used for all subsequent experiments.

Table 3.10 Optimum sterilization procedures for Clivia explants

Explant	Sterilant	Time (mins)
Leaf	2% NaOCl	15 - 20
Root	0,1% $HgCl_2$	10 - 15
Rhizome	0,1% $HgCl_2$	10 - 15
Flower	2% NaOCl	10 - 15
Ovary	10% H_2O_2	10 - 15
	2% NaOCl	10 - 15

Ethanol proved effective in the pretreatment stage in reducing surface contamination on explant material. As a sterilant ethanol was ineffective and resulted in partial dehydration of the explant as evidenced in the shrivelling of the plant material. Hydrogen peroxide although not very effective in terms of microbe elimination, did not damage plant tissue as the chemical is easy to remove and is a relatively "soft" sterilant. Those explants that were sterilized in H_2O_2 responded in culture with abundant callus production. Sodium hypochlorite proved to be the best allround sterilant as it is effective not only in decontamination, but is easy to remove resulting in minimal damage to explant tissue. While mercuric chloride is probably the most efficient sterilant, it has major drawbacks in that a large amount of tissue damage may result from over zealous sterilization regimes.

Although not quantified the obvious benefits from the inclusion of a detergent in the sterilizing routine was observed. Omission of Tween 20 resulted in increased percentages of contamination, a feature also recorded by STREET (1973). No beneficial effects on explant's performance using Tween 20 (enhanced shooting of the explant) was recorded as found by PIERIK, VAN LEEUWEN and RIGTER (1979) and SHEURINK and WELLES (1979). A further noticeable trend was the reduction in the amount of contamination during the winter months, but explant response in culture was also reduced. The obvious bonus factor of reduced microbe activity is negated by a reduction in the seasonal growth of the plant.

CARTER (1984) found that her best sterilization procedure resulted in only 57% decontamination of the Clivia explant tested. As shown in Table 3.10 and emphasized by YEOMAN (1973) sterilization procedures must be established for each tissue type, necessitating the flexible approach advocated by DE FOSSARD (1976).

3.3.3 Explant origin

Monocotyledons have been cultured using a wide range of explants. Results obtained from investigation into Clivia explant response in culture are summarized in Table 3.11.

Table 3.11 The response of different Clivia explants in tissue culture

Explant	No response	Callus	Direct organogenesis	Indirect organogenesis
Root	X			
Rhizome		X		
Meristem		X	X	
Leaf		X		
Inflorescence peduncle		X		
Flower		X		X
Fruit		X		X
Seed/Embryo		X	X	X

As evident in Table 3.11 all explants tested apart from the root produced callus, however, in the case of the rhizome, leaf and inflorescence peduncle, this callus appears to be wound callus. These callus cells, despite large nuclei were non-meristematic with minimal amounts of cytoplasm (Figure 3.6). According to MURASHIGE (1974) cultured cells (especially callus), are large highly vacuolated, with little cytoplasm, and only faintly visible nuclei. "These cells are indeed differentiated, but perhaps unorganised" (MURASHIGE, 1974).

Isolation of the meristem resulted in the destruction of the plant, and although the meristem developed into a plantlet, multiple shooting was not obtained. Apical meristem culture for the elimination of viruses in monocotyledons was first achieved in Iris by BARUCH and QUAK (1966). The use of meristem cultures also provides a system for the production of disease free Clivias. However, as discussed by MURASHIGE (1974) meristem explants are difficult to obtain and the poor survival and slow rate of plant multiplication, restricts the use of meristems for the production of pathogen free plants.

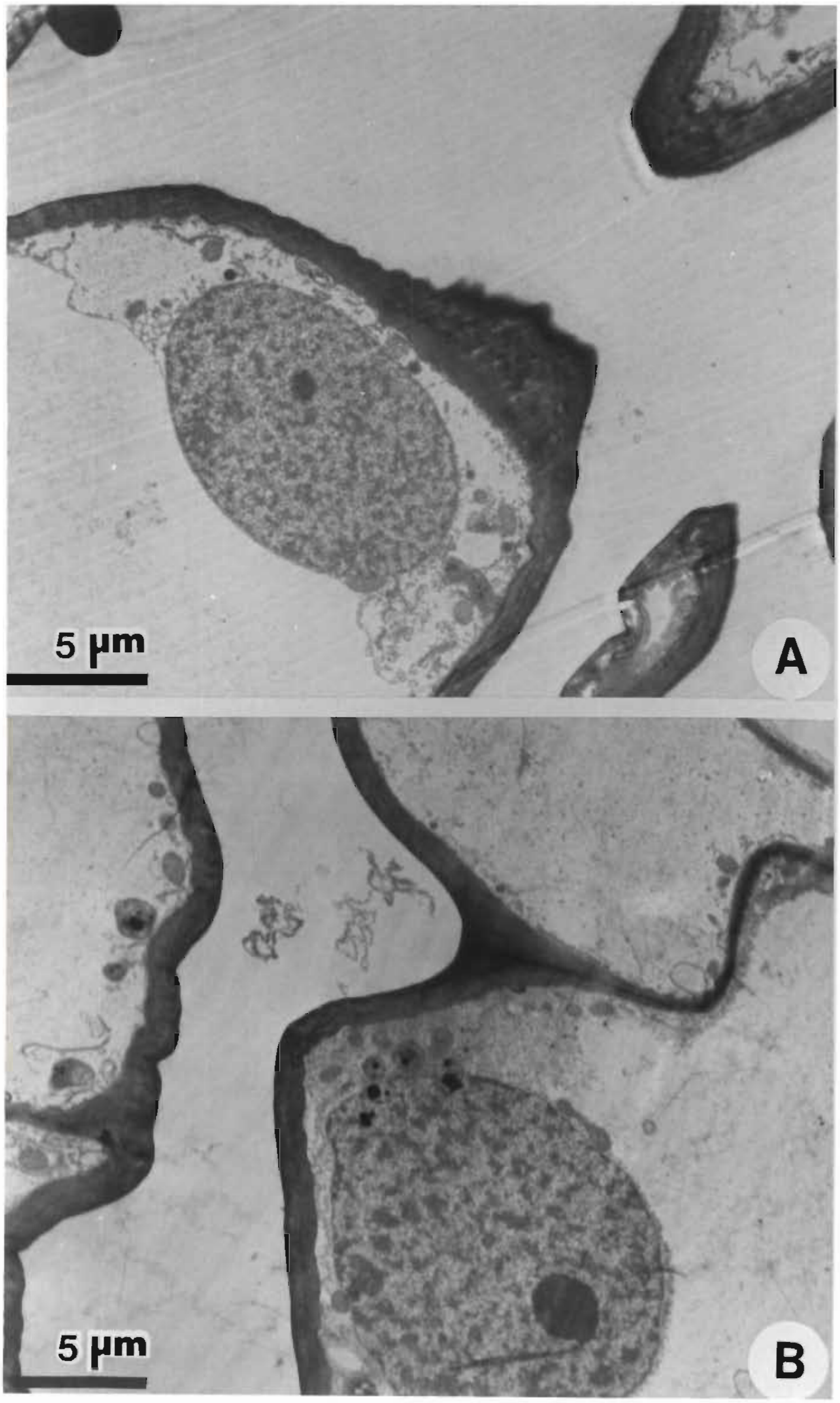

5 µm
A
5 µm
B

Figure 3.6 Electron micrographs of callus cells produced on Clivia leaf explants. (Large nuclei with minimal cytoplasm).

Leaf explants have been successfully used in monocotyledon culture by SEABROOK, CUMMING and DIONNE (1976) and HOSOKI and ASAHIRA (1980). (Other examples of the use of leaf explants are listed in the previous chapter). As a result of the relative availability of leaf material, a large amount of research was directed towards the usage of leaf explants. Table 3.12 records the culture response of the different ages and positions of leaf explants.

Table 3.12 Culture response of Clivia leaf explants

Explant		% callus production	Remarks
Young leaf		15	Easily damaged by the sterilant
Leaf base		10	Difficult to sterilize, twin leaf bases showed no response.
Mature leaf position:			
	1	5	
	2	± 60-70	Large production of non-meristematic tissue
	3	± 60-70	Large production of non-meristematic tissue.
	4	± 20-30	Explant surface not always in contact with the medium.*
	5	0	Explant desiccated readily.

* Isolation of sterile tissue is inversely related to size (MURASHIGE, 1974).

Position 2 and 3 from older leaves (Figure 3.7 and 3.8) responded with the greatest callus production, however all subsequent attempts to induce plantlet formation was unsuccessful. It was reasoned that this callus was similar to the more or less natural "callus like" tissue that yielded buds on the leaves of Haemanthus (HALL and TOMLINSON, 1973). The fact that mainly mature tissue produced

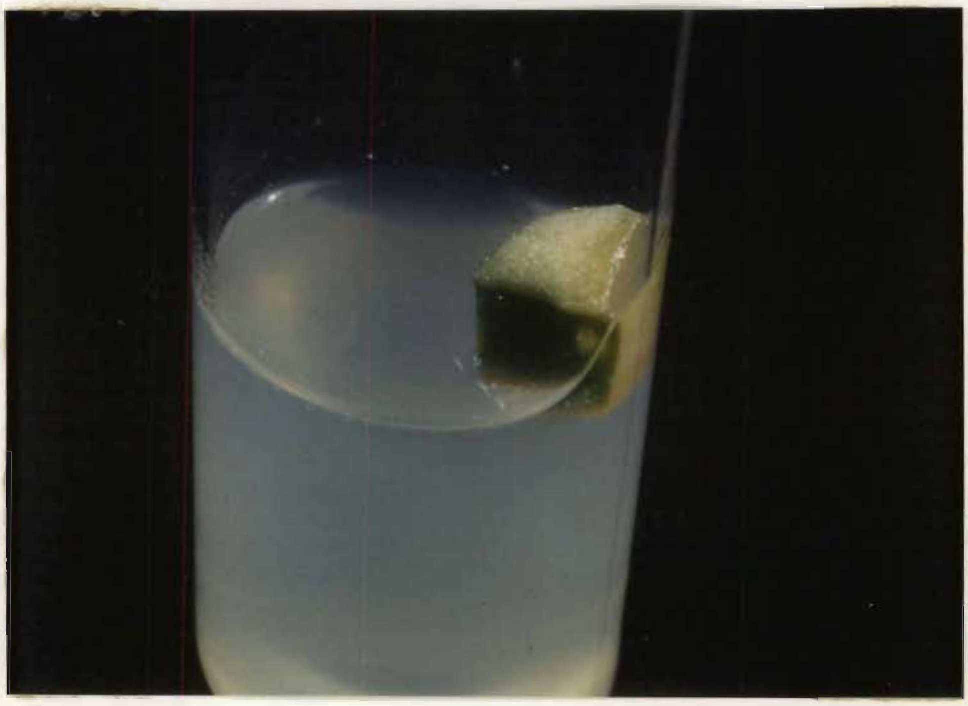


Figure 3.7 Clivia leaf explant and callus produced on the leaf explant taken from position 2.

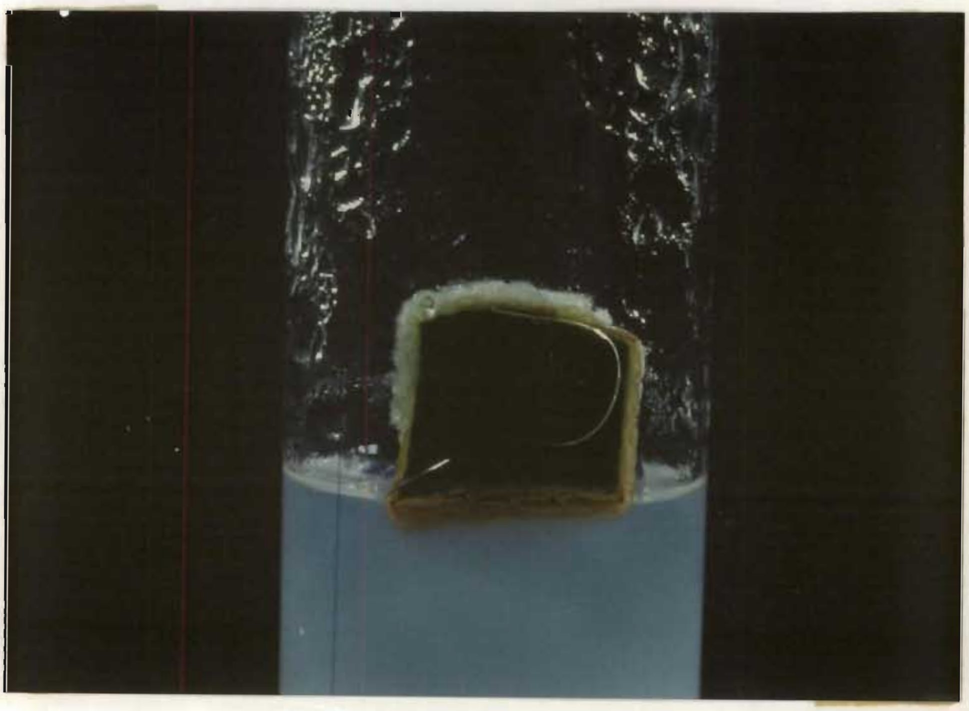


Figure 3.8 Clivia leaf explant and callus on a leaf explant taken from position 3.

callus is substantiated by TAKAYAMA and MISAWA (1980), however in their experiment juvenile tissue produced organs. PIERIK, STEEGMANS and VAN DER MEYS (1974) have reasoned that the origin of callus is unimportant as adult tissue rejuvenates in the callus phase. SEABROOK, CUMMING and DIONNE (1976) derived shoots from the leaf base explants of Narcissus. The harsh sterilization regime required to sterilize Clivia leaf bases resulted in reduction of explant vigour, while the retention of microorganisms in the axil of the bases resulted in a high percentage of contamination.

Variation in explant response as a result of leaf orientation (as found by GABRYSZEWSKA and SANIEWSKI (1984) was not recorded for Clivia leaves. Callus formed on the surface that was not in contact with the agar medium. The difference in leaf response between position 1 and positions 2 and 3 may be a result of the basipetal displacement of endogenous auxins as described by HARRIS and HART (1964). Or may be a result of the contribution of different cells (epidermis, parenchyma, vascular tissue) depending on the portion of the leaf the explant was derived from (KUKULCZANKA, KLIMASZEWSKA and PLUTA, 1977).

The inflorescence peduncle of Clivia responded identically in culture as did the leaf. As a result of the failure of the leaf/leaf callus to produce plantlets, the use of these explants was discontinued.

The use of flowers as a source of explants for monocotyledon tissue culture is not extensive, and the reversal of the floral organ to a vegetative state is rare (KONAR and KITCHLUE, 1982). However, KAUL and SABHARWAL (1972), BAJAJ and PIERIK (1974), HEUSER and APPS (1976) and PAGE and VAN STADEN (1986) have all successfully used flowers as a source of explant for culture. Floral buds were utilized as explants for the successful culture of Clivia nobilis (MIN and JINSHENG (1984). Table 3.13 and Figure 3.9 (a, b, c, d) indicate floral explant response in culture.

Table 3.13 Floral organ explants as used for Clivia tissue culture

Explant		Callus	Plant production	Remarks
Petals stage	1 (1-3 mm)	x		
	2 (3-10 mm)	x		
	3 (10-15 mm)	x		
	4 (15-20 mm)			Extension growth of petal, formation of anthocyanin as in normal flower.
	5 (>20 mm)			
Ovary		x	x	
Pedicel		x	x	Plant production via callus
Filament		x		See anther culture
Style				Extension growth, no callus
Entire bud		x	x	Plant production via callus

As indicated in the above results all floral explants excluding the style responded well in culture. The petals at stages 1 - 3 produced malformed petal and callus tissue (Figure 3.9c), whereas after a specific "size" (stages 4 and 5) excised petals in culture developed normally on the media with the production of anthocyanins in the petal tissue. It is unusual for petals to give a morphogenetic response in culture as in most cases floral explant response is normally observed with the pedicel and peduncle. However, buds have also been used as explants and given morphogenetic response in culture (HUSSEY, 1975a; SEABROOK, CUMMING and DIONNE, 1976; DUNSTAN and SHORT, 1979; HOSOKI and ASAHIRA, 1980; NAKAMURA and KELLER, 1982). CARTER (1984) after an anatomical

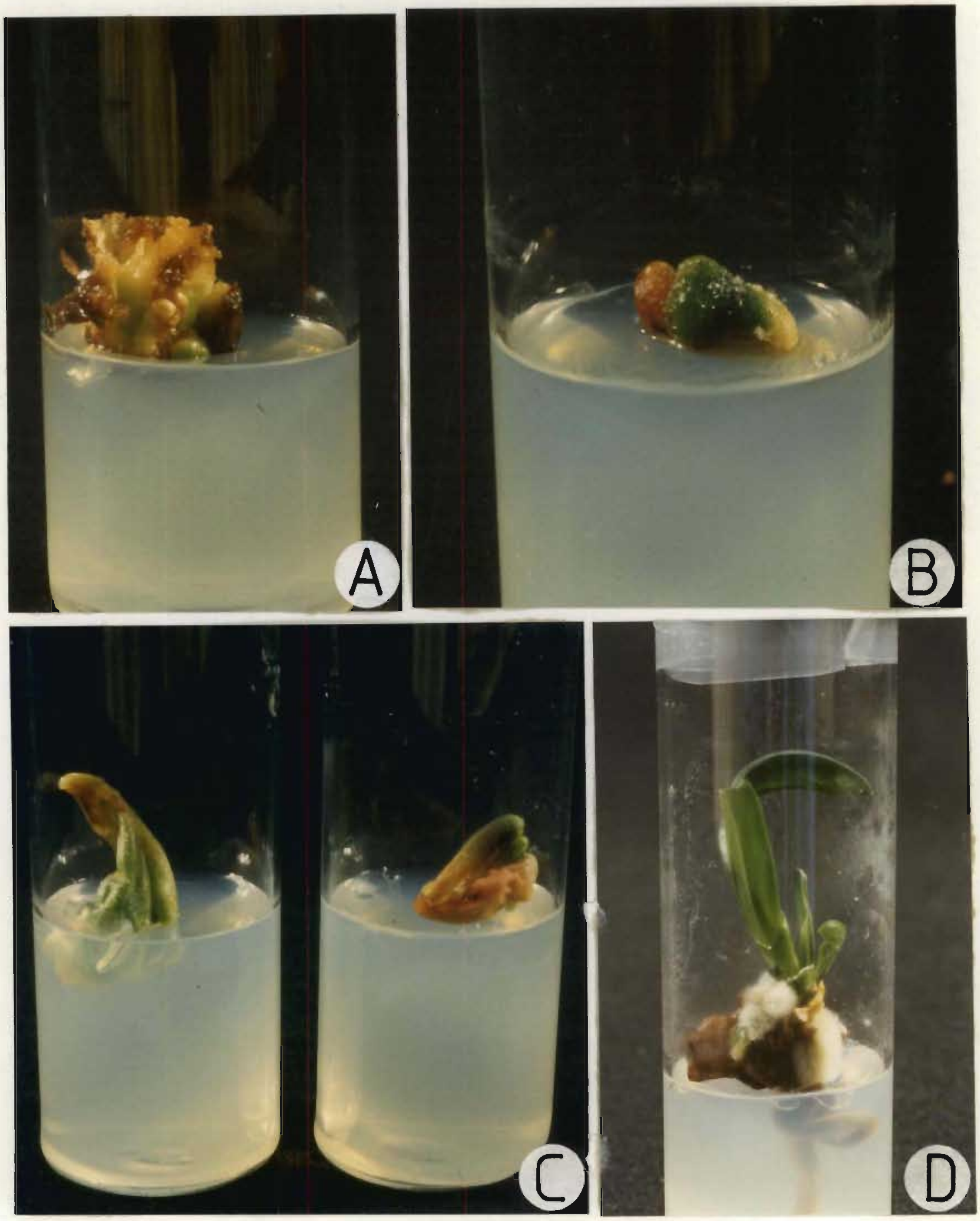


Figure 3.9 Clivia floral explant response in culture

a) callus produced on the basal portion of a floral bud

b) callus derived from the pedicel

c) malformed petal callus from a stage 2 petal

d) plantlet produced from callus tissue derived from the zone between the petal, ovary and pedicel investigation reported that young inflorescence explants may be more amenable to tissue culture propagation of Clivia (Figure 3.10). It appears that flower bud age is vital with respect to their *in vitro* response in culture, this feature having also been reported by a number of other researchers (PIERIK and STEEGMANS, 1975b; HUSSEY, 1976a; PAGE and VAN STADEN, 1986). KRIKORIAN and KANN (1985) recommended the use of buds between 0,5 - 6 cm large, once pollination has occurred, the flower tissue is no longer able to revert to the "vegetative" condition! (KAUL and SABHARWAL, 1972; PAGE and VAN STADEN, 1986). Callus tissue derived from the zone between the petal, ovary and pedicel of a stage 2 bud became embryogenic (Figure 3.9d). The exact origin of the callus could not be ascertained but it was evident that major zones for callus production in floral tissue came from either

i) the region between the petal and ovary
ii) the ovary, or
iii) The pedicel.

STIMART and ASCHER (1982b) and LIU and BURGER (1986) were able to culture Freesia and Lilium plantlets from pedicel explants respectively. LIU and BURGER (1986) recommended the use of reproductive tissue for culture, as it is relatively clean and there is the potential for obtaining large numbers of plants of a spontaneous mutant. These authors also found that the region of major plantlet regeneration from floral explants was from the pedicel explant taken closest to the receptacle, a feature also apparent in Clivia culture. STIMART and ASCHER (1982b), using pedicel callus from Freesia, found that true to type plantlets could be generated even after 27 months in culture.

NAKAMURA and KELLER (1982) reported that no growth regulator regimes could change the morphogenetic response in inflorescence cultures, whereas HOSOKI and ASAHIRA (1980) showed that benzyladenine supplementation was indispensible

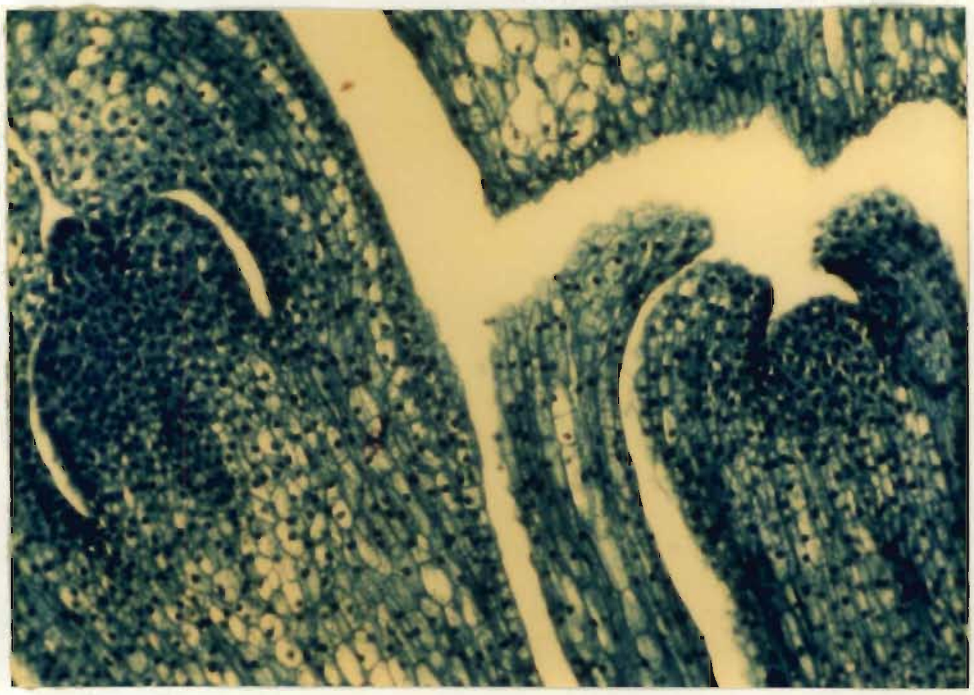


Figure 3.10 Light microscopy of a Clivia floral bud (LS) used for culture, (note multiple meristems)

for bud formation. HEUSER and APPS (1976) recommended equivalent amounts of cytokinin and auxin. PIERIK, STEEGMANS and VAN DER MEYS (1974), GROENEWALD, WESSELS and KOELEMAN (1977) and LIU and BURGER (1986) suggested that a higher cytokinin to auxin ratio is beneficial for callus and plantlet formation in monocotyledons. Clivia inflorescence cultures were markedly affected by hormone regimes, with both equal and higher concentrations of cytokinin to auxin proving stimulatory, while benzyladenine was the most effective cytokinin.

Subsequent experiments using "developed ovaries" (berries) were performed to maximise callus production. The results are tabulated and displayed in Table 3.14 and Figure 3.11; 3.12; 3.13 and 3.14.

Table 3.14 Clivia berry explant response to a cytokinin / auxin hormone grid. (A full strength MS medium plus 1 gℓ^{-1} PVP and 40 gℓ^{-1} sucrose was used).

	concentration BA mg ℓ^{-1}		
concentration NAA mg ℓ^{-1}	0	1	5
0	1	1	3
1	2	4	-
5	3	-	4*

Key

1 no callus
2 fine yellow non embryonic callus
3 yellow/green marginally embryogenic callus
4 yellow/green/red callus embryogenic.

* greatest amount of callus produced.

As depicted in Table 3.14 and Figures 3.11 to 3.14, it is from this explant source that embryogenic callus arose from both var citrina and var miniata. As mentioned in material and methods, three age groups of berries were used as explants. Although stage 1 produced callus, this callus was not embryogenic, while callus derived from stage 3 did not produce callus as the fruit wall tissue had begun to degenerate, with an increase in anthocyanin production. [It is interesting to note that mature berries of var *miniata* turn red on ripening, while berries from self-pollinated var *citrina* turn yellow on ripening. Embryogenic callus tissue derived from the fruit wall produced small amounts of the anthocyanin characteristic of the parent]. In most fruits, cell division is retarded at an early phase of development. Using grape berries HAWKER and DOWNTOWN (1973) found that only tissue from young fruits with immature seeds are capable of producing callus, while SOMMER (1962) showed that peach pericarps could be induced to produce callus even after the fruit cells had stopped growing. LAVEE (1977) showed that the mesocarps of young olive fruits have a lag phase before callus formation occurs. As the fruit develops this lag phase is shortened and callus production increases, until the final "green ripening" (and oil accumulation in the fruit) when the mesocarp loses its growth potential. This trend is closely followed in the fruit wall of the *Clivia* berry, resulting in maximum callus formation at stage 2 of fruit development. Callus developed into small "globular" or embryogenic structures (Figure 3.11c and 3.11d) which further developed into rooted plantlets (Figure 3.12). As indicated in Figure 3.11c between 30 - 40 embryoids can develop on a single "slice" of fruit wall tissue.

The following hormone treatments also resulted in callus formation and embryoid production on fruit wall explants 1 mg ℓ^{-1} BA ; 0,5 mg ℓ^{-1} NAA
1 mg ℓ^{-1} BA ; 1 mg ℓ^{-1} NAA
5 mg ℓ^{-1} BA ; 5 mg ℓ^{-1} NAA indicating
the necessity of both cytokinin and auxin for maximum callus formation and differentiation. High levels of either cytokinin or auxin alone, resulted in the formation of callus tissue that was not embryogenic. Hormonal supplementation was

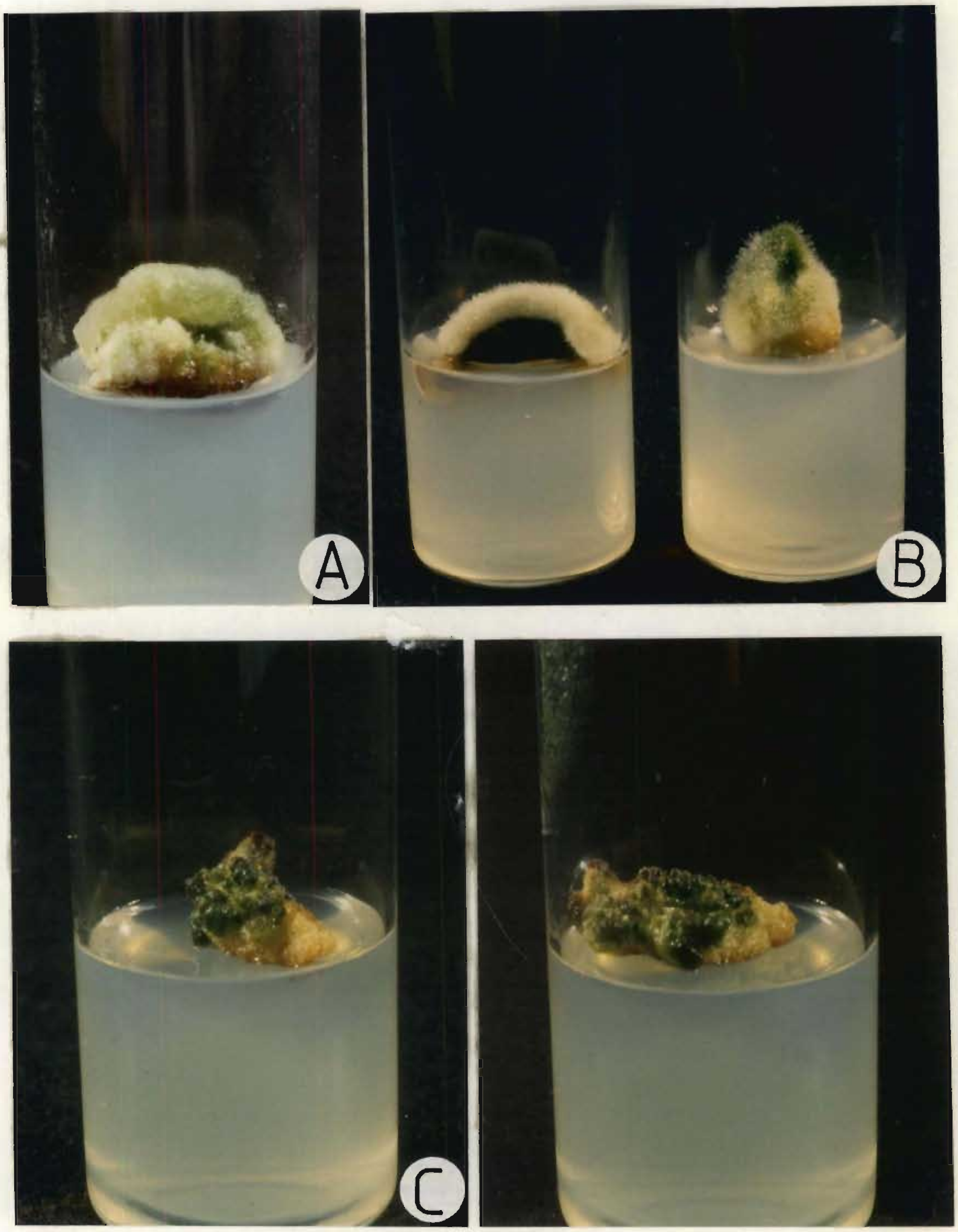


Figure 3.11 Tissue culture of Clivia berries

a and b) stage 1 callus on Clivia berry explants taken during stage 2 of berry development;

c and d) ± 30 - 40 "globular" or embryonic nodules on berry explants, each nodule can develop into a rooted plantlet if separated

Figure 3.12 Multiple plantlet production of Clivia miniata var citrina using berry explants

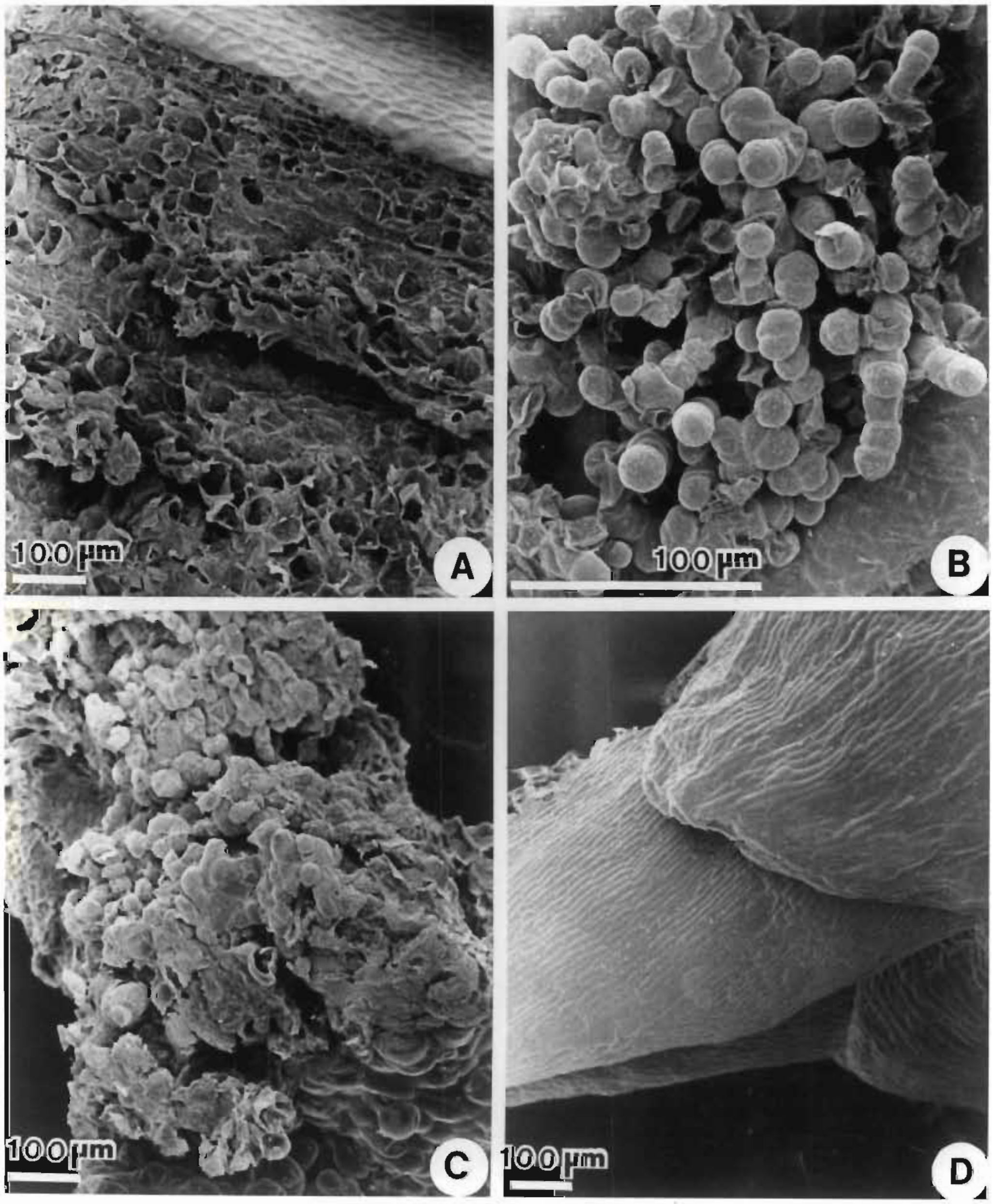

100 µm
A
100 µm
B
100 µm
C
100 µm
D

Figure 3.14 Scanning electron microscopy of Clivia berry explants

a) surface structure of fruit wall
b) stage 1 callus on explant (appear crystaline, see Figure 3.11a)
c) surface structure of mature embryogenic Clivia callus
d) scan of emerging leaf produced in vitro

also necessary for the development of olive mesocarp callus, where both cytokinins and auxins were necessary for callus growth and induction, with equal amounts of the two hormones proving optimal (LAVEE, 1977).

Ovary / fruit walls have often been successfully used as explants for monocotyledon tissue culture (HUSSEY, 1975a; SEABROOK, CUMMING and DIONNE, 1976; HOSOKI and ASAHIRA, 1980; KRIKORIAN and KANN, 1985). HUSSEY (1975a) used ovary wall tissue to induce plantlet formation in Ipheion, Hyacinthus, Muscari, Ornithogalum and Scilla, while callus formation in Narcissus could only be achieved using ovary tissue. Although Clivia callus was obtained using other explants the most successful explant in terms of multiple plantlet production was undoubtedly the ovary / fruit wall. However plantlet regeneration using this source of explant was found to be slow.

HUSSEY (1975a) emphasized the desirability of minimizing callus growth to avoid phenotypic and genotypic variation. KRIKORIAN and KANN (1985) using Hemerocallis as a model showed that complete stability in karyotype and phenotype in callus masses can be maintained for periods up to a year. Although there was a large amount of callus produced on the fruit wall, anatomical studies were not unequivocably able to show whether the plantlets were derived from the callus tissue produced on the surface of the fruit wall, or as in the case reported by HUSSEY (1975a), directly on the ovary tissue. In either case it can be assumed that by using this method of culture a high percentage of the resulting plantlets will be genetically identical to that of the parent, resulting in a viable technique for the culture of the rare Clivia miniata var citrina plant.

Unfortunately there are two major drawbacks if one wishes to apply this technique for commercial application i) fruit wall material is only seasonally available, and

ii) although the process of callus induction is fast (7-14 days), subsequent embryoid formation is painfully slow (8 - 12 months).

This drawback in part can be negated by maintaining a callus stock derived from the embryogenic tissue. The response of Clivia miniata and Clivia nobilis (as reported by MIN and JINSHENG, 1984) in culture, appears to be almost identical!

Germination of Clivia seeds in culture proved very successful with a germination percentage of between 60 - 80%. This in vitro germination provided an ideal source of sterile explant material. Seed embryo culture can be used advantageously as explants for the production of callus stocks and for rapid production of seedlings from seeds that normally have germination / establishment difficulties. "Embryo rescue" can be used in cases where post-zygotic incompatibility may affect the production of desired hybrids (a feature that may be present in self-pollinated var citrina, as seed set is small because there is a high percentage of "abortion") (GEORGE and SHERRINGTON, 1984; KRIKORIAN and KANN, 1985). Although embryo culture may not lead to rapid large scale propagation, it is especially useful for plant breeders.

Excised embryos were utilized as a source of explant material, however, if var citrina is heterogenous then usage of embryos for culture would be subject to genetic "uncertainty". Table 3.15 represents the response of excised embryos to various hormonal treatments, while the trends are pictorially demonstrated in Figure 3.15.

Once again equal concentrations of cytokinin and auxin resulted in the formation of multiple shoots (Figure 3.15c). The process of callus formation and multiple shooting can be enhanced by making sections in the embryo axis. If entire embryos were inoculated onto cultures normal development occurred. Or alternatively

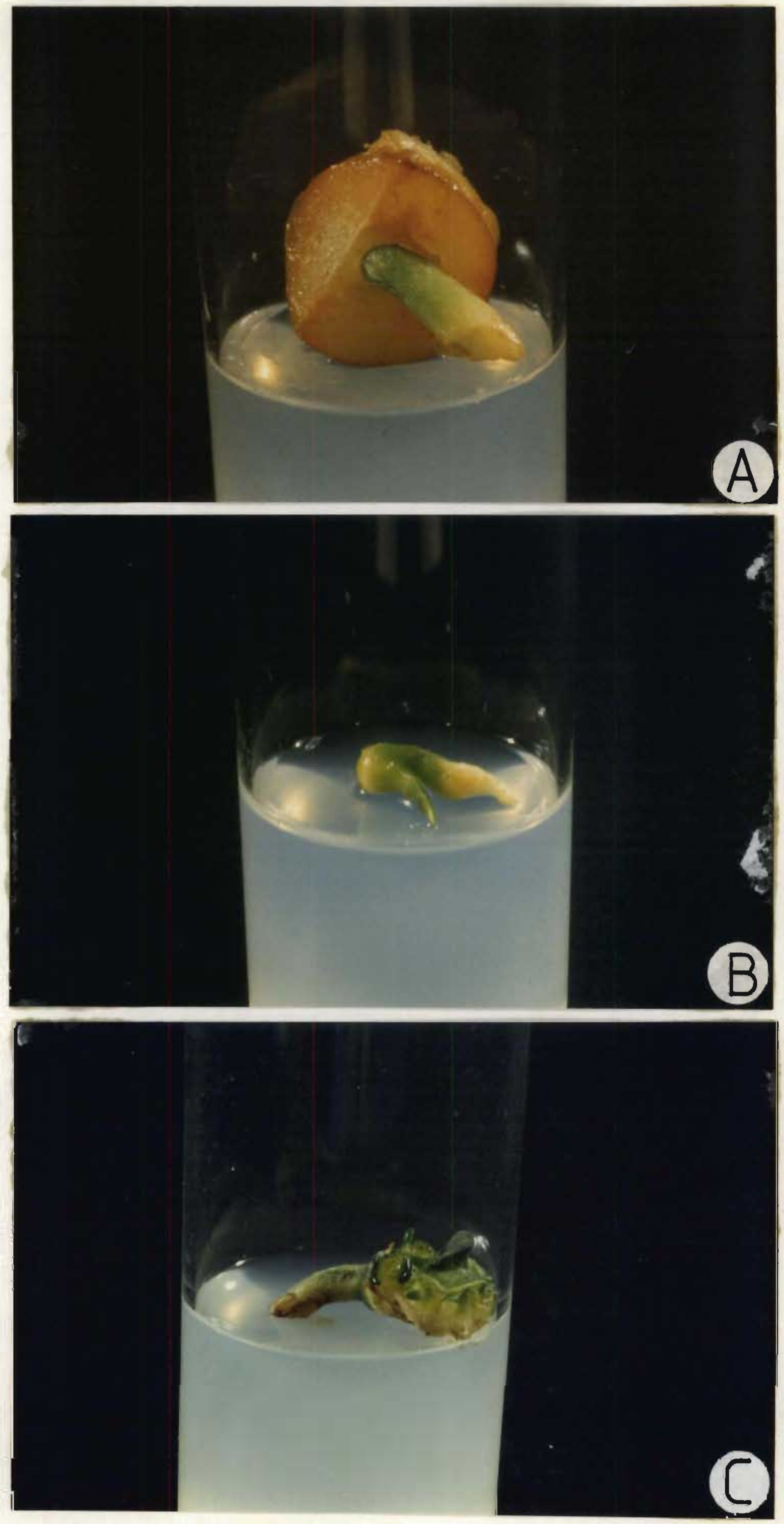


Figure 3.15 Clivia embryo explants

a) emerging embryo from mature endosperm tissue

b) isolated embryo in culture after three weeks

Table 3.15 Clivia embryo explant response to a cytokinin / auxin hormone grid. (A full strength MS medium, plus 1 $g\ell^{-1}$ PVP and 40 $g\ell^{-1}$ sucrose was used).

concentration NAA mg ℓ^{-1}	concentration BA mg ℓ^{-1}		
	0	1	5
0	1	1	2
1	1	3	-
5	1	-	1

Key

1 normal development

2 deformed embryo plus callus

3 multiple shooting

in the presence of high concentrations of hormones, deformation occurred at the radicle end. GREEN and PHILLIPS (1975) showed that embryos from mature kernels produced only callus whereas embryos from young kernels produced both plants and callus. This trend was mimicked in Clivia provided that the embryo was intact. In immature seeds the endosperm tissue was "soft and runny" and isolation of the embryo was difficult, while in mature seeds endosperm tissue (Figure 3.15a) was easy to section facilitating the removal of the entire embryo . Endosperm tissue did not respond in culture.

One must at this stage begin to consider the theory of totipotency. Are all explants equally viable, or is this a feature restricted to specific tissues at specific times of the year? With Clivia only fruit wall tissue, floral tissue and excised embryos showed a morphogenetic response in culture.

3.3.4 Hormones

The response of Clivia leaves and rhizome explants were tested against a 4 by 4 hormone grid. The results are tabulated in Table 3.16. The most critical organic components of propagation media are said to be the cytokinins and the auxins.

As discussed previously rhizome explant response in culture was very poor, and from Table 3.16b it is evident that there is no clear pattern with respect to rhizome explant response and hormonal treatments. This lack of response may be a result of excessive sterilization or the origin of the explant.

Leaf explant response to hormonal treatment does not show any trends apart from the fact that cytokinin appears to stimulate callus production to a greater degree than does auxin. Subsequent experimentation using a variety of hormones (2,4-D; IBA: 2iP; kinetin) showed the same lack of response. There is a great deal of variability between plants and the hormones necessary for morphogenesis (HUSSEY, 1975a), and the hormonal requirement of each type of explant should be determined (HUGHES, 1981). The use of 2,4-D was avoided if possible because of its possible role in chromosomal aberration and suppression of morphogenesis (MURASHIGE, 1974; GRESSHOFF, 1978).

In general most monocotyledons require a high auxin to cytokinin ratio or auxin alone to induce callus formation (EVANS, SHARP and FLICK, 1981; GEORGE and SHERRINGTON, 1984). However, there are cases where equal amounts of auxin to cytokinin or higher cytokinin to auxin ratios may induce callus (HEUSER and APPS, 1976; GROENEWALD, WESSELS and KOELEMAN, 1977). Unfortunately there was a very poor response to hormone combinations by the various Clivia explants. Table 3.17 shows the trends displayed by Clivia explants in hormonal treatments.

Table 3.16 Clivia explant response to a 4 by 4 BA/NAA hormone grid. (A full strength MS medium supplemented with 8 g ℓ^{-1} agar, 30 g ℓ^{-1} sucrose was used)

a) Clivia leaf explant response (% response in brackets)

concentration BA mg ℓ^{-1}

concentration NAA mg ℓ^{-1}	0	0,1	1	5
0	0	C(20)	0	C(20)
0,1	0	C(20)	C(10)	C(70)
1	C(20)	C(60)	C(10)	C(20)
5	C(40)	C(20)	0	C(50)

Key:

0 = no response
C = callus
R = roots
S = shoots

b) Clivia rhizome explant response (% response in brackets)

concentration BA mg ℓ^{-1}

concentration NAA mg ℓ^{-1}	0	0,1	1	5
0	0	C(20)	0	0
0,1	C(20)	0	0	0
1	0	0	C(20)	0
5	0	0	0	C(20)

Table 3.17 Explant response to hormonal ratios. (A positive result was recorded if the explant produced callus or plantlets)

Explant	Auxin > Cytokinin	Auxin < Cytokinin	Auxin = Cytokinin
Leaf	x	x	x
Rhizome	x	x	x
Fruit	?	x	x
Embryo		x	x

In general Clivia explants responded to a greater degree when equal ratios of cytokinin to auxin or when cytokinin was applied at a higher concentration. This response is contrary to the general trends normally found for monocotyledons. It is however evident, that the hormonal requirements of explants are governed to a large extent by the type of explant, and the age of the explant. This feature may be a result of the type of cells present, or alternatively the endogenous levels of growth regulators within the explant.

3.3.5 Subculture experiments

Using Clivia callus derived from filament, flower and ovary tissue, a 4 by 4 hormone grid was tested to establish a hormone supplemented medium suitable for the maintenance and bulking up of the preformed callus (Table 3.18).

The pattern of growth for callus subcultures at various hormone combinations, shows that at high concentrations of cytokinin to auxin (5 : 1) callus growth is markedly stimulated as was found by PIERIK, STEEGMANS and VAN DER MEYS (1974) and GROENEWALD, WESSELS and KOELEMAN (1977). The stimulation of growth occurs within the first two weeks of culture, thus requiring subculture of the Clivia callus on a fortnightly basis. Satisfactory growth of callus was also achieved on the Murashige and Skoog medium supplemented with the following hormones (Table 3.19).

Table 3.18 *Clivia* callus response to a 4 by 4 hormone grid. (A full strength MS medium, supplemented with 8 g ℓ^{-1} agar, 30 g ℓ^{-1} sucrose was used)

concentration NAA mg ℓ^{-1}	concentration kinetin mg ℓ^{-1} 0	0,1	1	5
0	D	D	MP	MP
0,1	M	M	MP	MP
1	R	<2	<2	>3
5	<2	<2	<2	<2

Key:

D : callus died
M : callus growth maintained
MP : callus growth maintained with little proliferation
<2 : accelerated growth ± twice the original callus mass
>3 : accelerated growth more than three times the original mass
R : root production

Table 3.19 *Clivia* callus growth on different hormones

Hormone combination	Callus growth x 1	x 2
0,5 mg ℓ^{-1} BA, 0,1 mg ℓ^{-1} 2,4-D	x	
0,25 mg ℓ^{-1} BA, 2,5 mg ℓ^{-1} NAA	x	
2,5 mg ℓ^{-1} BA, 5 mg ℓ^{-1} NAA	x	
0,5 mg ℓ^{-1} kinetin 2 mg ℓ^{-1} NAA	x	
1 mg ℓ^{-1} kinetin 0,5 mg ℓ^{-1} NAA	x	
5 mg ℓ^{-1} 2iP, 0,1 mg ℓ^{-1} NAA	x	

A surprising result was achieved using callus derived from leaves. When subcultured onto a medium containing 10 mg ℓ^{-1} kinetin and 1 mg ℓ^{-1} NAA, prolific root production occurred (Figure 3.16). Induction of shoots on these roots was not achieved, however root production on shoots produced in culture did not require hormonal supplementation. DURMISHIDZE, GOGOBERIDZE and MAMALADZE (1983) found that vigourous rhizogenesis resulted no matter what nutrient media was used, on callus obtained from buds.

A danger of using subcultured material for extended periods of time lies in the possibility of a reduction in morphogenetic potential, or an increase in the number of genetically different plants as the culture period is extended (MURASHIGE, 1974). These variations may arise as a result of a decrease in endogenous growth regulator levels, or the loss of organised centres by repeated subcultures (HUGHES, 1981).

3.3.6 Nutrient and environmental manipulation

Experiments using differing nutrient media gave no conclusive results, with the full strength Murashige and Skoog medium providing the explants with sufficient nutrients at non-toxic quantities. Murashige and Skoog's medium was used for all future experiments. Growing cultures in light, low light or dark conditions did not yield any significant results. Clivia leaf explants grown in total darkness resulted in the loss of chlorophyll from the explant after three weeks with subsequent necrosis. Use of 6 - 12 month old seedlings was unsuccessful as the sterilization regime required to achieve aseptic culture killed the explants. Seedlings grown in vitro gave limited response in culture when used as explants.

Figure 3.16 Rhizogenic Clivia callus

3.3.7 Anther culture

Despite the precautions taken, plant regeneration from anther and microspore culture was unsuccessful. Inclusion of increased amounts of boric acid was attempted to stimulate pollen growth, however no germination or plantlet regeneration was achieved. The failure of the cultures may be a result of a number of factors;

i) the use of non-purified agar (KOHLENBACH and WERNICKE, 1978);
ii) failure to add charcoal to media which acts by removing inhibitory substances (WENZEL, HOFFMANN and THOMAS, 1977)
iii) the lack of anther pretreatments especially low temperature pretreatments (SUNDERLAND and ROBERTS, 1977); and
iv) the physiological status of the mother plant.

The use of a liquid medium has been advocated for anther culture, with advantages including; the avoidance of nutrient competition between anther tissue and embryogenic spores, and a means of overcoming the inhibitory effects of anther wall components (WERNICKE and KOHLENBACH, 1976; SUNDERLAND and ROBERTS, 1977; SHARMA, FIROOZABADY, AYRES and GALBRAITH, 1983).

"Despite progress made in anther culture the exact mechanisms that regulate androgenesis are not known and yields of haploid plants is poor even in the best responding plant species" (SHARMA, FIROOZABADY, AYRES and GALBRAITH, 1983). Further study into anther and pollen culture of Clivia with specific attention being paid to the above inhibitory factors is recommended.

In certain explants filaments were intentionally left on the anther explants. The filament responded by producing prolific callus in culture (Figure 3.17). The anatomy of this callus type was determined to ascertain the callus' potential for future culture experiments (Figure 3.17b and c). These investigations indicate that filament callus does have potential for manipulation in culture.

3.3.8 Protoplast culture

Isolation of protoplasts from leaf material of Clivia was relatively easy to achieve using enzymic degradation of the cell wall. A greater number of protoplasts could be derived from young leaves. When using mature leaves it was necessary to remove the cuticle which was difficult to achieve (scoring of the leaf surface to facilitate enzyme entry was unsatisfactory). After sequential washing and purifying the majority of protoplasts (60 - 80%) proved to be viable when stained using Evans blue dye. The protoplasts were plated out at concentrations between 1×10^4 and 5×10^4 per mℓ of medium (using both a substrate of solid Oxoid no 1 agar and "soft" agar slowly agitated on a horizontal culture wheel). The protoplasts (Figure 3.18) when maintained in the mannitol solutions did not suffer from osmotic stress. Once in culture wall regeneration occurred within 48 hours and a certain amount of cell division occurred within the first week (5 - 10 cells per protoplast). No further growth or division occurred after the initial cell divisions, despite using differing hormone combinations. Further attention must be paid to the plating density of the protoplasts. EVANS and COCKING (1977) found that the optimum plating efficiency for tobacco protoplasts was about 5×10^4 protoplasts per mℓ with protoplasts failing to divide at one tenth the concentration. Protoplast culture response may have been affected by the physiological condition of the explant (VASIL and VASIL, 1980) and cell damage may have occurred when mesophyll cells were in contact with the enzyme solution (DODDS and ROBERTS, 1985). Clivia leaf explants did not respond in culture! To use the protoplast technique one must be able to readily isolate protoplasts in large numbers and culture them so that they are able to regenerate cell walls and form colonies.

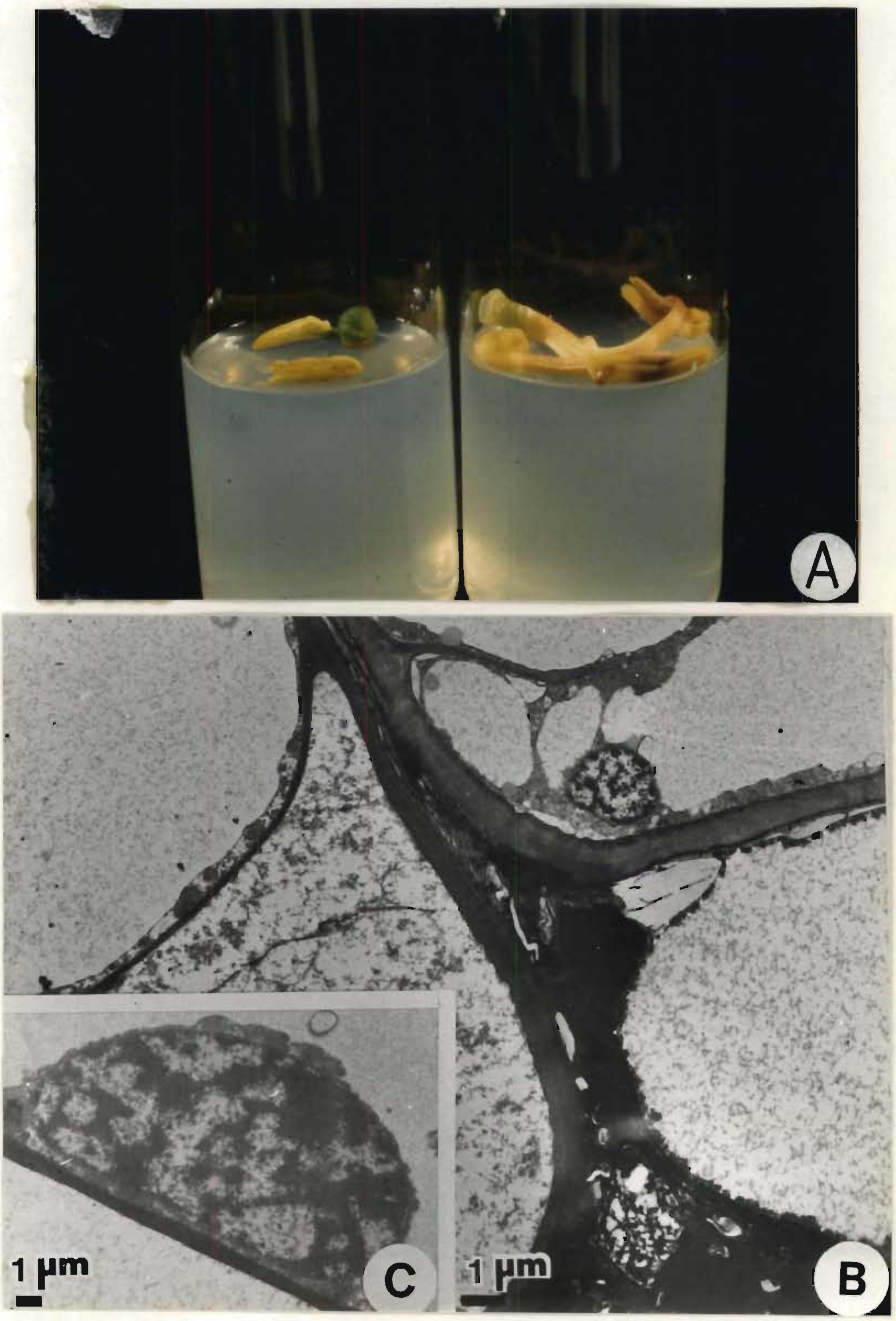


Figure 3.17 Clivia filament culture

a) callus production on filaments *in vitro*

b and c) anatomy of filament callus (may be used for further culture

Figure 3.18 Clivia protoplasts (produced by enzymic degradation of the cell wall)

The described technique provides an adequate system for the ready isolation of protoplasts in large numbers, but subsequent regeneration could not be achieved.

The technique of protoplast culture offers a variety of advantages for plant genetic manipulation. Further in-depth investigation into this in vitro technique is recommended because of its obvious advantages.

3.3.9 Hardening off

The final step in in vitro culture involves the hardening off of the plants produced in culture. Without a successful technique for the transplanting of the in vitro grown plantlets into the soil, tissue culture techniques for rapid plant multiplication are useless. Hardening off of Clivia plants was achieved using the described technique. In most cases the in vitro grown Clivia plantlets had a well developed cuticle and subsequent hardening off could be easily achieved without necessitating the use of the involved procedures of MAENE and DEBERGH (1987). It was reported by NOVAK and PETRU (1981) that in most cases, members of the Liliaceae, Iridaceae and Amaryllidaceae can be directly transferred into the soil, a feature also shown with Clivia. Mist sprays were used to assist acclimatization (ZIV, 1979). Normally plantlets produced in vitro have poorly developed cuticles (SUTTER and LANGHANS, 1979). The cuticle of Clivia contains substantial amounts of non-ester bonds. Out of a number of studied plants, the upper cuticular membrane of Clivia miniata was shown to be one of the least permeable to water (MERIDA, SCHONHERR and SCHMIDT, 1981; SCHMIDT, MERIDA and SCHONHERR, 1981; SCHMIDT and SCHONHERR, 1982). This factor contributed to the ease with which Clivia plantlets could be transplanted from in vitro conditions into normal in vivo situations. Non-rooted plantlets could also be hardened off successfully after applying a rooting powder (SERADIX) to the shoot explants. (The shoot was left for three weeks in the mist house within which time root development had occurred). This feature ties in closely with the suggestion of DEBERGH and MAENE (1981) where rooting and initial growth *in vitro* produced shoots can be achieved under *in vivo* conditions. However, root production on shoot explants in culture occurs spontaneously. Using the above hardening off technique, 80% of the rooted explants could be successfully hardened off and subsequent growth under the standard growing conditions for mature *Clivia* plants was possible.

3.3.10 Conclusion

Despite the use of a variety of explants as well as numerous nutrient and environmental manipulation experiments, only a limited amount of success was achieved using very specific explants. Plant regeneration was achieved using flower and fruit explants, and only minimal *in vitro* response was achieved using the other types of explants. The most promising technique involves the use of protoplast culture, which requires further study.

It is interesting to note that the results obtained from these experiments closely correlated with those reported by MIN and JINSHENG (1984), who were able to regenerate *Clivia nobilis* plantlets from flower and ovary explants. Is this a feature common to all members of the *Clivia* genus?

3.3.11 *Gloriosa* and *Sandersonia*

As yet only one report has been found concerning the *in vitro* culture of *Gloriosa* and *Sandersonia*. There is, however, extensive literature concerning the flowering process, corm size, corm formation and plant production. CUSTERS and BERGERVOET (1984) found that with *Nerine* and *Gloriosa*, two "new" florist's crops, clones from breeding programs showed a wide variation in micropropagation ability. This report was included in a meeting of the Netherlands Society for Plant Cell and Tissue Culture, and no details were published. Because of the similarity of Gloriosa and Sandersonia with regards to their growth habits, biochemistry and the unavailability of the plants and cut flowers, the two species have been grouped together in this chapter in order to compare the growth responses of these two members of the Colchicaceae.

3.3.12 Sterilization

Retention of viable contaminants on explant materials leads to rapid proliferation of the microorganisms when brought into contact with the nutrient medium, which in turn affects the growth of the explant. Initial sterilization experiments on both Gloriosa and Sandersonia obtained from their natural habitats, were unsuccessful due to rapid colonization of the medium by internal contaminants in the plants, both bacterial and fungal. Electron microscopy of the explant tissue showed a great deal of bacterial contamination within the corm explant (Figure 3.19). This contamination was partially a result of damage caused on removal of the plant from the soil. Between the removal of the plant from the soil to the actual culture of the plant (3 - 24 hours) invasion of microbes through the damaged surface of the plant occurred, resulting in a 95 - 100% loss due to contamination. This percentage loss was unacceptable and a pretreatment stage 0 (DEBERGH and MAENE, 1981) was incorporated as a necessary part of the sterilization regime. Subsequent sterilization of pretreated plants (12 months after transplanting) and seedlings, subjected to the prescribed sanitary techniques showed sterilization percentages ranging from 60 - 80%. This highlights the obvious advantages that can be obtained using stringent pretreatment of mother plants. It is advocated that the inclusion of a stage 0 should be a prerequisite for all long term culture experiments, as it provides not only plants with reduced potential for contamination, but also more standardized plant material, and the "history" of the plant is known (DEBERGH

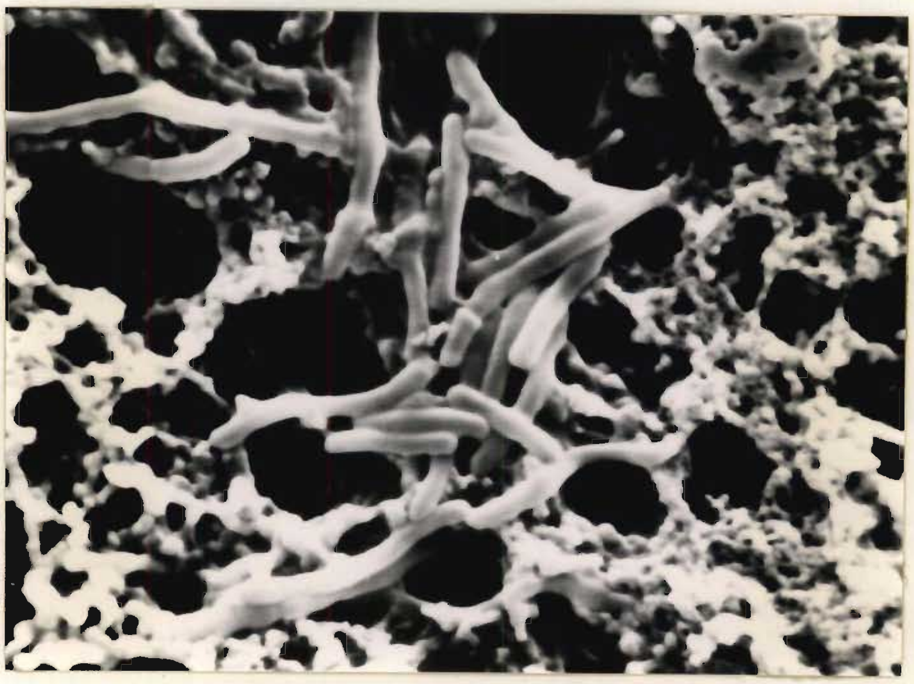


Figure 3.19 Typical scanning electron micrograph of a Gloriosa corm explant prior to culture, (contamination mainly due to rod shaped bacteria)

and MAENE, 1981). The problems of contamination in what appear to be symptomless plants is a world-wide phenomena (KNAUSS, 1976; HOLDGATE and AYNSLEY, 1977; DEBERGH and MAENE, 1981). From personal communication and a review of the literature it becomes apparent that the degree of sterilization required to decontaminate the explant can be related to the prevailing climatic conditions of the country. Sterilization requirements for tissue culture performed in cold temperate climates are not as vigourous as those required for aseptic culture in tropical and sub-tropical regions.

An antibiotic (Novostrep) was used in an attempt to lower the amount of bacterial contamination as advocated by MURASHIGE (1974). The inclusion of an antibiotic did not improve the success of the sterilization treatment. DEBERGH and MAENE (1981) found that antibiotics did not yield satisfactory results, and often the inclusion of an antibiotic inhibits the growth of bacteria without eliminating them (FISSE, BATLLE and PERA (1987). The use of antibiotics in the sterilization technique was discontinued.

HUSSEY (1977b) found that when the leaves and inflorescences of *Gladiolus* die down the overlapping leaf bases of the corm dry out, and become thin and membranous. In this condition contamination on the corm, taken from the soil, is difficult to remove and aseptic culture is virtually impossible. A similar feature was observed when *Gloriosa* and *Sandersonia* corms become dormant as the tunic becomes membranous, and thus where possible only actively growing corms were utilized for tissue culture as advocated by HUSSEY (1977b).

Results of sterilization treatments performed on *Gloriosa* and *Sandersonia* corms are summarized in Table 3.20a and b. (Ethanol was not utilized as a sterilant because of the poor results obtained with *Clivia*).

Table 3.20a Percentage of <u>Gloriosa</u> explants sterile after four weeks following sterilization in H_2O_2, NaOCl and $HgCl_2$. (A full strength MS media, 8 g ℓ^{-1} agar, 30 g ℓ^{-1} sucrose, 1 mg ℓ^{-1} kinetin and 1 mg ℓ^{-1} 2,4 D was used.)

Sterilant	Time (mins)	% explants sterile after 4 weeks in culture			
		Corm	Leaf/stem	Flower/Ovary	Seed
10% H_2O_2	10	-	60	-	23
	20	15	60	-	-
1% NaOCl	10	5	50	74	91
	15	10	60	60	94
	30	35	-	-	-
2% NaOCl	10	5	57	72	95
	15	73-90	73	-	-
	30	65	-	-	-
3% NaOCl	10	30	77	80	95
	15	74	87	-	-
	30	75	-	-	-
0,1% $HgCl_2$	10	55-65	83	95	100
	20	80	90	93	100
	30	80*	-	-	-

* Dead

Table 3.20b Percentage of <u>Sandersonia</u> explants sterile after four weeks following sterilization in H_2O_2, NaOCl and $HgCl_2$

Sterilant	Time (min)	% explants sterile after 4 weeks in culture			
		Corm	Leaf/Stem	Flower/Ovary	Seed
10% H_2O_2	10	15	21	69	93
	20	17	16	73	97
1,75% NaOCl	15	72	67	79	100
	30	80	-	-	-
0,1% $HgCl_2$	15	82	-	-	-
	30	90*	-	-	-

* Dead

As expected the corms of Gloriosa and Sandersonia proved to be the most difficult to sterilize. Roots excised from the corm were useless for tissue culture as the roots arising from the corm are thin, and the sterilization regime necessary to decontaminate them proved fatal to the growing cells. Seventy-three percent of Gloriosa corm explants were freed from contamination when sterilized in 2% NaOCl for 15 minutes. Higher concentrations of NaOCl (3%) or longer periods of time (30 minutes) resulted in elevated sterilization, but the percentage of viable explants decreased. The use of mercuric chloride was effective in decontaminating corm explants, however a large number of explants were killed by the sterilant or alternatively explant vigour was markedly reduced. Sterilization of Sandersonia corms was achieved using a similar concentration of sodium hypochlorite, but unfortunatley numerous sterilization regimes could not be tested due to the limited plant material available.

A 95% contamination figure was recorded when culturing the red ripe seeds of Gloriosa. Removal of the soft fruit wall tissue and subsequent soaks in sterilant resulted in 80 - 100% decontamination.

Sterilization of aerial organs (stems, leaves, flowers and ovaries) was easier to achieve than underground organs. NaOCl (1 - 2%) and H_2O_2 (10%) proved to be adequate sterilants to achieve more than 66% sterilization. In all future experiments a compromised sterilization regime was utilized for stems and leaves, (these organs were soaked in 1,75% NaOCl (half-strength Jik) plus Tween 20 for between 15 - 20 minutes, while corm material was sterilized in either 2% NaOCl for 15 minutes or 0,1% $HgCl_2$ for 10 - 15 minutes). With the inclusion of stage 0 the advantage of the Benomyl soak during presterilization was not evident, however this step was retained as a precaution, and the use of Tween 20 was included as a matter of course.

As was the case with Clivia, it was evident that for the successful establishment of tissue cultures an integrated sterilization regime is required, and an optimum sterilization technique should be established for each species and each explant of that species (Table 3.21).

Table 3.21 Optimum sterilization procedures for Gloriosa and Sandersonia explants

Explant	Sterilant and time of soak (min)	
	Gloriosa	Sandersonia
Leaf / Stem	10% H_2O_2 (20 minutes); 2% NaOCl (15 minutes)	1,75% NaOCl (15 minutes)
Flower	1% NaOCl (10 minutes)	10% H_2O_2 (20 minutes); 1,75% NaOCl (15 minutes)
Seed	2% NaOCl (10 minutes)	10% H_2O_2 (20 minutes); 1,75% NaOCl (15 minutes)
Corm	2% NaOCl (15 minutes); 0,1% $HgCl_2$ (20 minutes)	1,75% NaOCl (15 minutes); 0,1% $HgCl_2$ (15 minutes)

The optimization of a sterilization procedure is important as the use of a single procedure may be insufficient or damaging to that specific explant resulting in the discarding of that explant as a potential regenerative source. Not only should the researchers approach to the sterilant and time of sterilization be empirical, but the "attitude" on the flow bench can be crucial. The development of an economical, neat and aseptically sound bench technique is strongly advocated, especially in countries where contamination poses a serious threat to the successful application of tissue culture techniques.

3.3.13 Explant origin

Virtually every organ can serve as a source of explants, and the type of explant may be a simple organ or a complex reproductive structure. The response of the various explants from the two study species is tabulated in Table 3.22.

Table 3.22 The response of different Gloriosa and Sandersonia explants in culture

Explant	Callus	Direct organogenesis	Indirect organogenesis
Root			
Corm	GS	GS	GS
Meristem		G	
Leaf	G		G
Inflorescence pedicle	GS		
Flower	GS		
Fruit / Capsule / Ovary	GS		GS
Seed / Embryo	GS	GS	GS

G = positive response by Gloriosa
S = positive response by Sandersonia

As was the case for Clivia all explants (except the roots) responded in culture. Meristem isolation was achieved using Gloriosa apices and use of this source of explant definitely has the advantage of producing disease free plants in vitro, however the in vitro return from this source is not viable for commercial institutions and skilled manipulation is required to extract the meristem in an undamaged stage.

The general consensus is that the basal part of a leaf shows the highest regenerative ability in culture probably due to the basipetal transport of auxin (HARRIS and

HART, 1964). This was also the case with Gloriosa leaves (Figure 3.20b). Induction of callus from leaves was achieved using media supplemented with kinetin and 2,4-dichlophenoxyacetic acid (Figure 3.20a) as well as benzyladenine and napthaleneacetic acid (Table 3.23).

Table 3.23 Gloriosa leaf explant response to a 4 by 4 hormone grid. (A full strength MS medium 8 g ℓ^{-1} agar 30 g ℓ^{-1} sucrose was used)

Concentration NAA mg ℓ^{-1}	concentration BA mg ℓ^{-1}			
	0	0,1	1	5
0	C(20)		EPC(80)	
0,1		C(10)	C(80)	C(20)
1	C(40)		C(80)	C(20)
5	C(40)		C(80)	C(40)

Key:

E	:	extension growth
P	:	plant formation on callus
C	:	callus (% response in brackets)

HUSSEY (1975a) was able to induce plantlets on the developing leaves of four Liliaceous species, Hyacinthus, Muscari, Ornithogalum and Scilla, but no plantlets were induced on the leaves of the Iridaceae or Amaryllidaceae. KATO and KAWAHARA (1972) and FINNIE and VAN STADEN (1986) found that if the leaf explant contained a portion of the main vein, then regenerative ability was enhanced. Although Gloriosa leaves are not large, basal portions of the leaf containing the main vein were by far the most regenerative (Figure 3.20b). Explants taken from the apical portion of the leaf did not respond well in culture, this being partially due to differentiation and specialization of the leaf apex in the form of a tendril (FRENCH, 1977). Seasonal fluctuation of Gloriosa leaf explants in culture was not observed (KUKULCZANKA, KLIMASZEWSKA and PLUTA, 1977) (provided the leaves were not senescing) as the aerial portions of Gloriosa only appear in spring and summer. It is interesting to note that the leaf explant response appears to be cytokinin dependent, as increasing levels of auxins do not markedly affect the leaf's ability to initiate callus. One mg ℓ^{-1} BA resulting in the maximum stimulation of callus with concentrations above and below this level being either inhibitory or ineffective. Generally monocotyledonous plants require a high auxin to cytokinin ratio or auxin alone for callus induction (EVANS, SHARP and FLICK, 1981; GEORGE and SHERRINGTON, 1984). However, there are reports where callus and plantlet formation in monocotyledons has been stimulated by high cytokinin to auxin ratios, as was the case with Gloriosa (PIERIK, STEEGMANS and VAN DER MEYS, 1974; NEL, 1983; FINNIE and VAN STADEN, 1986).

Only limited wound callus was derived on Sandersonia leaves.

Stem explants of Gloriosa and Sandersonia produced crystalline callus on the cut surfaces of the stem, this callus was similar to that found using Clivia leaves. Use of this organ as a source of explants was discontinued. Response of stem tissue to hormonal supplementation was not consistent, callus regeneration occurred in the presence of cytokinin or auxin or both. Root regeneration occurred on stems grown in 0 mg ℓ^{-1} kinetin and 5 mg ℓ^{-1} NAA. In order to have sufficient stem explant material for experimentation, use was made of mature plants and it was found that the stems were "woody" and difficult to section, indicating that the stem material was well differentiated. Greater success in culture may be achieved using younger stem tissue or alternatively immature shoot tips (YU and MEREDITH, 1986).

Flower tissue of both Gloriosa and Sandersonia produced callus in culture as did the pedicel. No plantlet regeneration from the tissue source was achieved even though a number of researchers have recommended flower buds and pedicels as ideal explants for monocotyledon tissue culture (HUSSEY, 1976c; FRETT and DIRR, 1983; LIU and BURGER, 1986; PAGE and VAN STADEN, 1986; TAEB and ALDERSON, 1987). As was the case in Clivia floral culture, small buds (stage 1 - 3) produced callus, while normal petal development occurred in explants bigger than a specific size.

Ovary tissue provided a source of callus tissue for both species. A fortuitous event was the growth of callus from immature seeds in a Sandersonia ovary, on a media supplemented with 2,5 mg ℓ^{-1} kinetin and 0,5 mg ℓ^{-1} NAA. (Seed germination of Sandersonia was not achieved). The growth of these "seedlings" which formed callus , provided a rapidly multiplying source of callus which was utilized for the majority of the subsequent subculture experiments. In the case of Clivia, fruit wall tissue provided the best source of explants for plantlet regeneration, while no reaction was recorded for Gloriosa and Sandersonia fruit wall tissue (irrespective of age).

As mentioned previously embryo rescue was one of the earliest and most widely used aseptic culture procedures and it dates back to 1945 (KRIKORIAN and KANN, 1985). Removal of embryos from the study species was difficult due to the hardness of the seed coat. Soaking of the seed to soften the coat was unsuccessful. Thus the technique of in vitro seed germination was utilized, and once radicle emergence occurred the developing embryos could be further manipulated. A marked difference in the in vitro germination ability of the seeds of the two species was observed (0% in Sandersonia, 95% in Gloriosa). From personal communications it is apparent that seed germination in Sandersonia is very poor. Attempts were made to germinate the available seeds using standard dormancy breaking methods (gibberellic acid and light treatments, stratification and scarification). These proved unsucessful.

Using seedlings / emerging embryos as explants material, callus could be derived using a medium rich in 2,4-dichlorophenoxyacetic acid. The majority of the callus was derived from the radicle region of the seedling / embryo. Callus and multiple plantlet production was achieved using embryo explants on a medium supplemented with 1 mg ℓ^{-1} BA and 1 mg ℓ^{-1} NAA. On media where 2,4-dichlorophenoxyacetic acid was present (1 mg ℓ^{-1}) the source of cytokinin was irrelevant, and prolific callus was produced. This callus derived from 2,4-dichlorophenoxyacetic acid treatments was utilized for the majority of subculture experiments. RADOJEVIC, SOKIC and TUCIC (1987) experimenting with Iris embryos found that embryonic callus was only induced on a medium containing 2,4-dichlorophenoxyacetic acid, and somatic embryos could be induced on the same medium and the resultant plantlets had the normal diploid number. Explant response and the associated effect of age of the seed as described by GREEN and PHILLIPS (1975) was also evident in Gloriosa.

Gloriosa seeds germinated on a Murashige and Skoog medium containing no hormones were grown in low light (0,5 $\mu Em^{-2}s^{-1}$) for 12 months. After this period it was found that there was an average of five cormlets produced from each seed. Although the corms / plantlets were achlorophyllous, the procedure allows for prolific sterile corm production for subsequent experimentation and commercial exploitation, due to the exponential nature of the culture growth. There is a drawback to the procedure in that the genetics of the seed is unknown (KRAUSE, 1986). Should the plant be true breeding this system is more suitable than the seed production technique described by KRAUSE (1986), in that there is a five-fold increase from a single seed, and each of the in vitro corms produced is at the same developmental stage as is the one year old corm.

In vitro experimentation using corms proved to be by far the most reactive explant, and multiple production of Gloriosa and Sandersonia plantlets was achieved. The "types" of corms used for explants and the position from which the explants were taken are shown in Figure 3.21. The developmental sequence of *Gloriosa* corms in culture is displayed in Figure 3.22 and that of *Sandersonia* in Figure 3.23. The developmental sequence can be summarized into the following stages:

1) initial growth from meristematic apex
2) multiple shoot regeneration from corm explant
3), 4) and 5) extension growth of the multiple shoots with prolific leaf production, between 2-8 plantlets formed per tube
6) the development of cormlets on the shoot, the production of cormlets may be directly associated with the shoot present, or alternatively depending on the nutritional and environmental conditions in which the explant is growing. Cormlet development may occur without the development of the aerial leaves (forming a chain of cormlets). Nutritional and environmental manipulation experiments will be discussed in a following section.

The most important factor controlling corm explant response in culture, was the origin of the explant taken on the corm (Figure 3.21c). Table 3.24 clearly shows the variation in culture response to different explant portions.

Table 3.24 Culture response of *Gloriosa* and *Sandersonia* corm explants from specific regions

Corm explant position	Media	
	kinetin / 2,4-D	BA / NAA
1	callus	plantlets
2	callus	roots
3	callus	plantlets
4	callus	roots
5	callus	plantlet

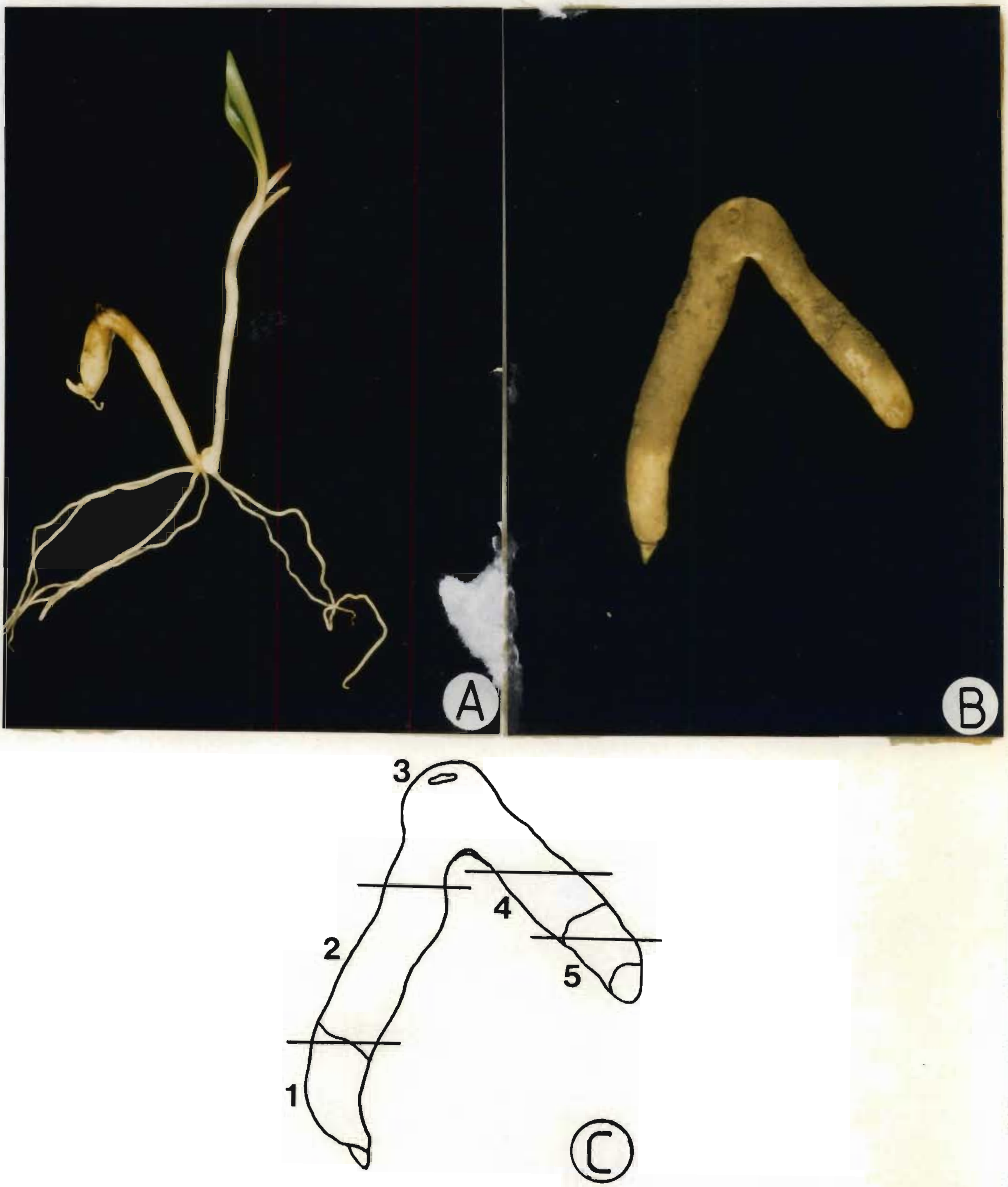


Figure 3.21 Corms used for tissue culture

a) Gloriosa corm in its second growth season

b) four year old corm

c) diagramatic representation of a typical corm showing the five

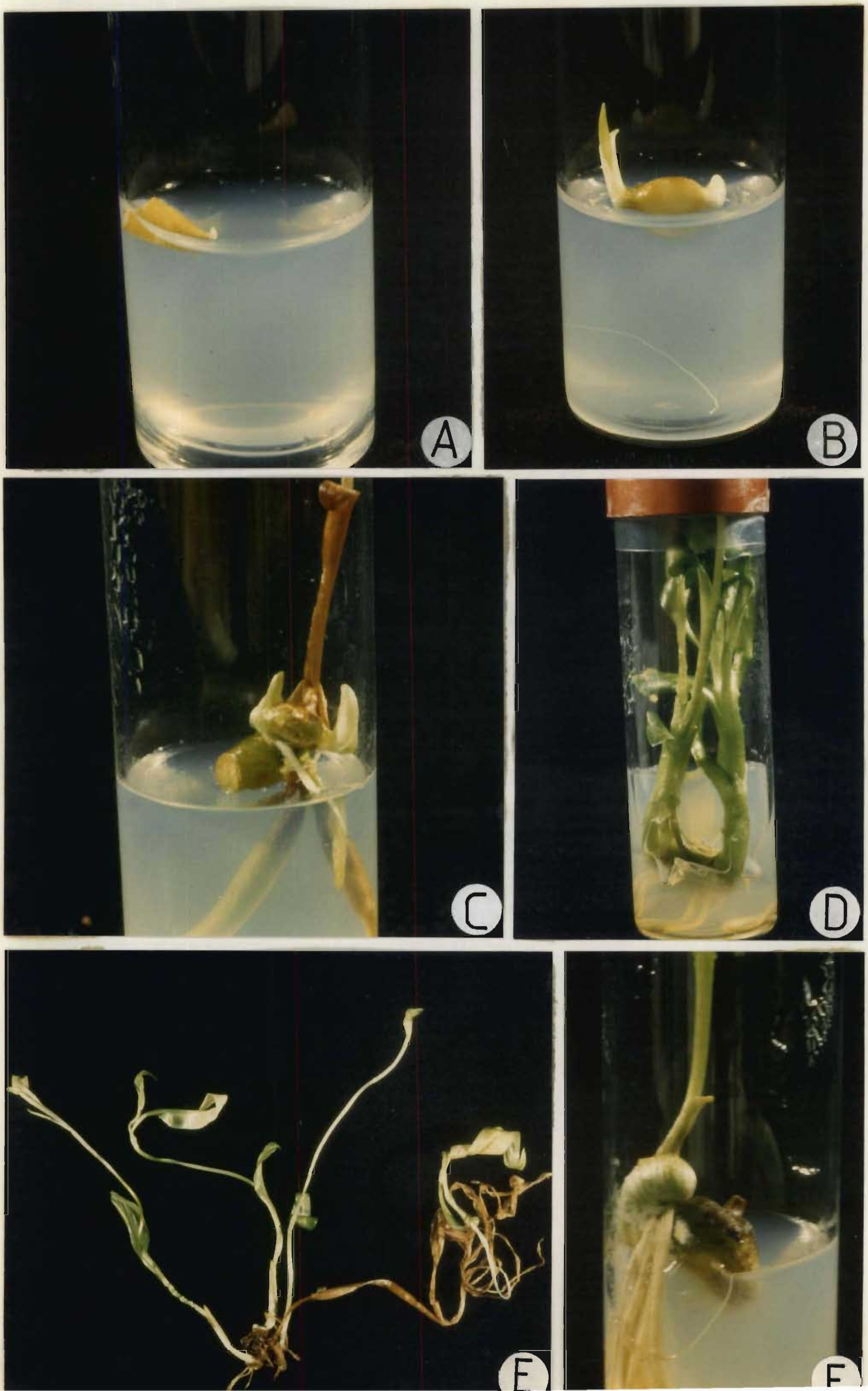

A
B
C
D
E
F

Figure 3.22 Developmental sequence of *Gloriosa* corm explants

a) initial growth from meristematic apex

b) multiple shoot regeneration from corm explants

c and d) extension growth of multiple shoots with prolfic leaf production

e) four plantlets produced in a single culture tube

f) development of corms

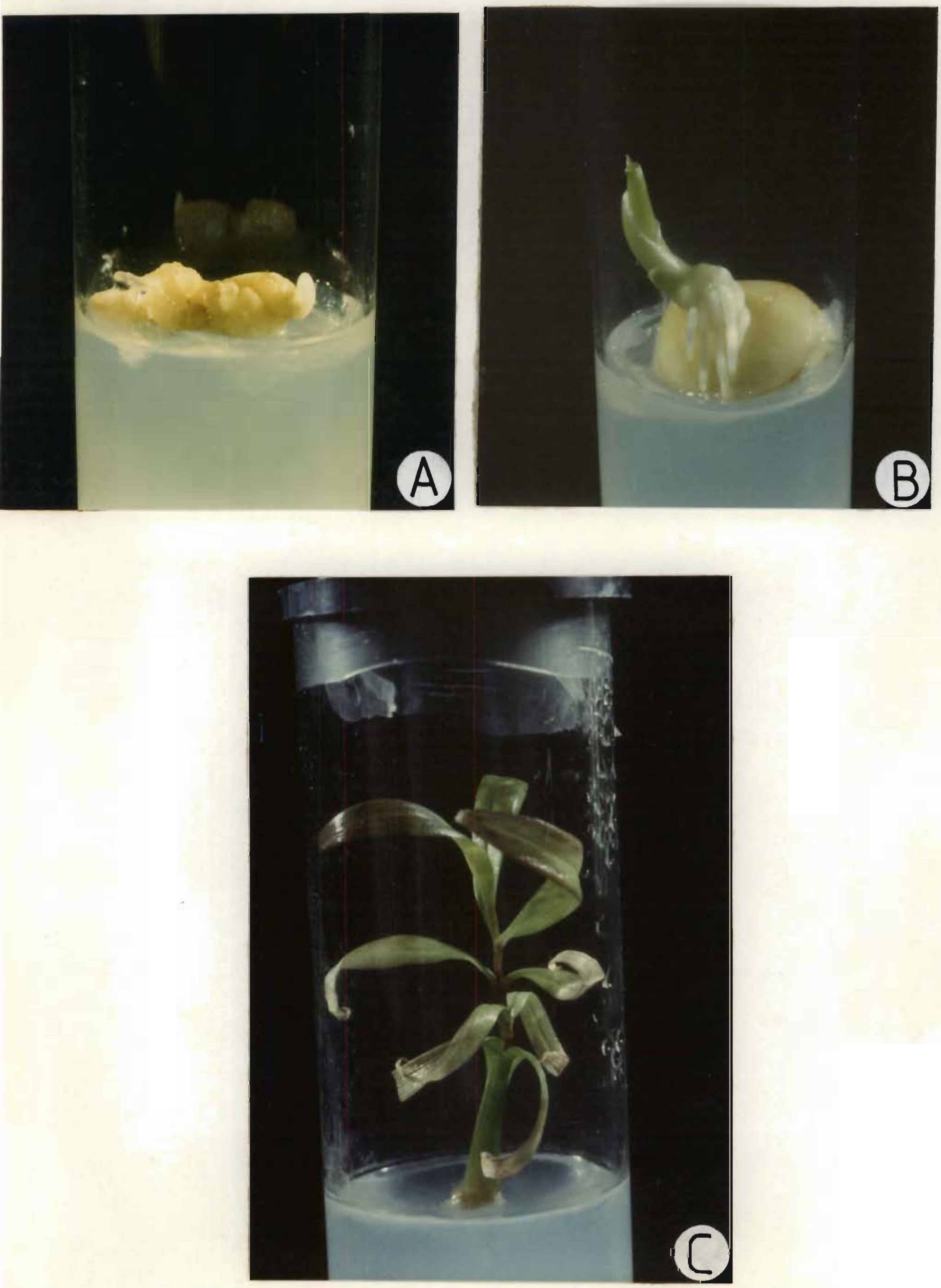

A
B
C

Figure 3.23 Developmental sequence of Sandersonia corm explants

a) initial growth from meristematic apex
b) extension growth
c) Sandersonia plantlet in vitro

Sixty percent of plantlets produced, originated from position 1 and 5 and the remaining 40% from position 3. Figure 3.24 pictorially shows the above trends with no plantlet production occurring on explants form positions 2 and 4 but extensive root formation.

TRAN THANH VAN (1977) and GRESSHOFF (1978) expressed the feeling that explant orientation can affect explant response in culture. In the case of Gloriosa and Sandersonia corm explant orientation had no effect on the response in culture. Plantlet regeneration was achieved no matter what the orientation of the explant, provided the explant was not damaged.

The response in culture described above was observed for both Gloriosa and Sandersonia. HUSSEY (1982a) stated that in the Liliaceae, Iridaceae and Amaryllidaceae, the induction of direct adventitious shoots in vitro is the principle method of propagation. An added feature is that the mode of regeneration is genetically stable and hence similar to axillary meristem propagation.

A further technique to produce Gloriosa and Sandersonia plants in vitro is by making repeated longitudinal sections through a sterile shoot. Each section was normally able to develop into an entire shoot.

In vitro culture of monocotyledonous plants from subterranean or storage organs is well documented in the literature. However, most of these studies involved the use of bulbs or bulb scales as explants and a few researchers working with corms (HUSSEY, 1975a; SIMMONDS and CUMMING, 1976; STEINITZ and YAHEL, 1982; PAGE and VAN STADEN, 1984). Due to the somewhat unique nature of the Gloriosa and Sandersonia corm (a stoloniferous corm) it is difficult to make direct comparisons between the techniques utilized by other researchers and the systems described above.

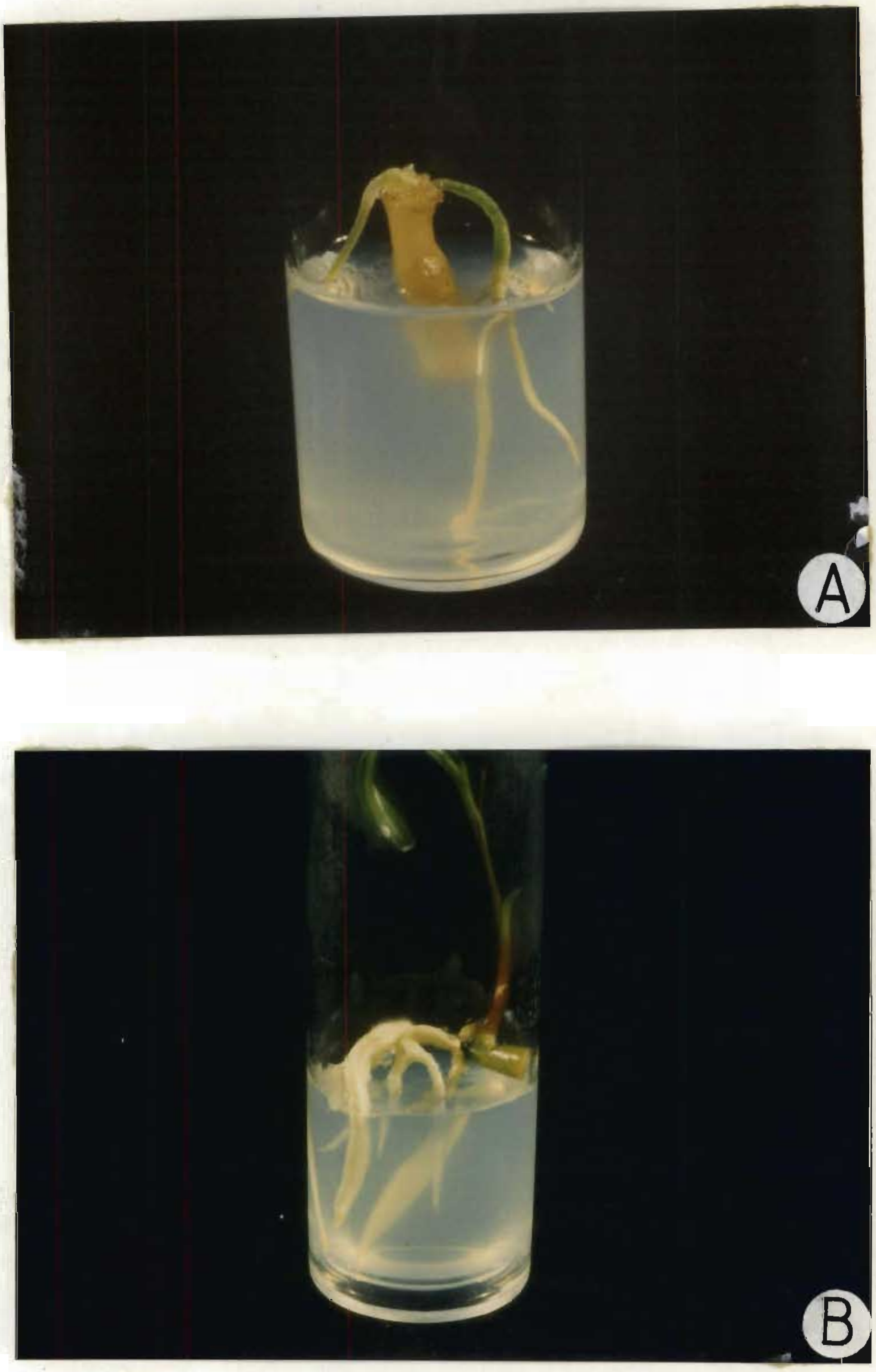


Figure 3.24 Response of the different corm explants in culture

a) extensive root formation on explants from position 2 or 4

b) plantlet production from a position 1 explant

The use of corm explants is recommended for rapid culture of both study species, especially for commercial institutions because:

i) The progeny is a result of adventitious shoot formation. (It can be argued that this type of culture is a form of meristem culture because of the utilization of the meristem apex of the corm and that subsequent development is a result of the in vitro release of axillary shoots from apical dominance as described by HUSSEY (1976b). However the occurrence of multiple plantlet regeneration from all portions of the explants taken from both positions 1 and 3 without direct intervention of the meristem would seem to rule this out), and as such is genetically stable. Intermediate callus production does not occur.

ii) The end product of this procedure is multiple cormlets. As the cormlets are easy to harden off, transport and transplant, it seems prudent to pursue the multiple production of corms rather than shoot production via tissue culture.

It has been reported that genotypes of certain monocotyledonous species may respond differently in culture (PIERIK, 1976; PIERIK, VAN LEEUWEN and RIGTER, 1979). Gloriosa and Sandersonia explants taken directly from the mother plant respond identically in culture, once again highlighting the similarity of the two species with regards to their growth habits, biochemistry and physiology.

3.3.14 Hormones

The response of Gloriosa corm explants was tested against a 4 by 4 hormone grid, the results are graphically displayed in Figure 3.25 a - e. Unfortunately due to the limited number of Sandersonia corms available a comparative study could not be attempted. The induction of plantlets from corms of Gloriosa appears

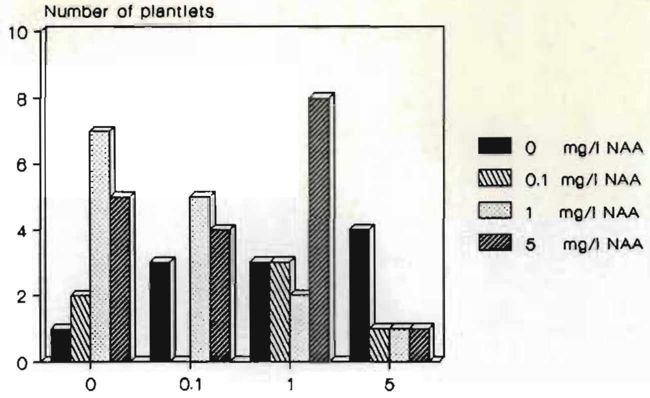


Figure 3.25a Number of entire plantlets.

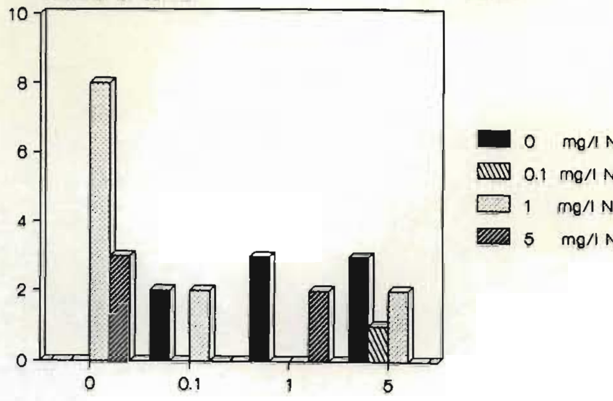


Figure 3.25b Number of corms.

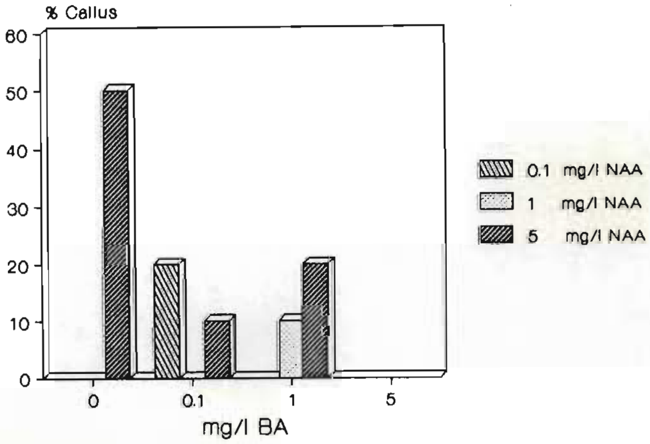


Figure 3.25c Callus production.

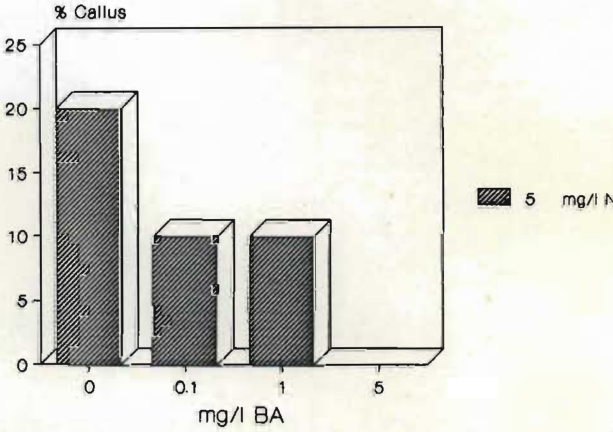


Figure 3.25d Crystalline callus production.

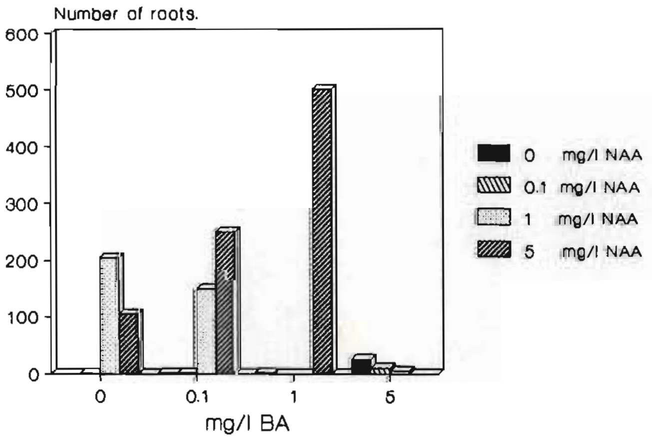


Figure 3.25e Root production.

Figure 3.25 *Gloriosa corm* explant response to be stimulated by the presence of auxins in the media. MII, MORI and IWASE (1974) feel that organogenesis is controlled by the concentration of auxin. The fact that Gloriosa plantlets can be formed in the absence of cytokinin, points to the possibility that monocotyledons have a cytokinin autonomy (SHERIDAN, 1968; GRESSHOFF, 1978). (However, increase in the amount of cytokinin alone increases plantlet formation!). Auxin applied at high concentrations (5 mg ℓ^{-1} NAA) in the absence of cytokinin became inhibitory to plantlet formation.

Alternatively increasing the amount of cytokinin alone in the media stimulates plant production above that of the control (0 mg ℓ^{-1} BA, 0 mg ℓ^{-1} NAA). TAKAYAMA and MISAWA (1979) also found that by increasing the cytokinin concentration plant production could be stimulated. This stimulatory role of the cytokinin may be due to a release of inhibition from apical dominance (MURASHIGE, SERPA and JONES, 1974; HUSSEY, 1976b, 1977b; DICKENS, KELLY, MANNING and VAN STADEN, 1986) or the cytokinin may have overcome or prevented dormancy in the corm explant (HUSSEY, 1976b, 1977b; ZIV, HERTZ and BIRAN, 1983). HUSSEY (1976b) stated that members of the Liliaceae and Amaryllidaceae require higher levels of cytokinin than the Iridaceae, and those species with a slow rate of natural propagation (Gloriosa?) especially require high levels of benzyladenine (HUSSEY, 1980). The cytokinin did not stimulate plantlet initiation in the presence of auxin, and at high concentrations of benzyladenine (5 mg ℓ^{-1} BA) the effects of the auxin was markedly reduced. At equal concentrations of the two hormones no stimulation in morphogenetic potential was observed.

In spite of what appears above as trends, the variation in the number of plantlets produced can be more directly related to origin and physiological status. In general however, more plantlets are produced when there is a higher auxin to cytokinin ratio.

The number of corms produced in culture (Figure 3.25b) can be directly related to the number of plantlets induced per corm explant, except in the case where auxin was applied at high concentrations, when an inhibition of corm formation was observed. The inhibition of corm formation by auxins, and stimulation of formation by cytokinin has also been shown by DICKENS, KELLY, MANNING and VAN STADEN (1986).

Callus and crystalline callus induction is stimulated by the presence of auxin especially at high concentrations (5 mg l^{-1} NAA) (Figure 3.25c and d). ZIV, HALEVY and SHILO (1970) maintained that auxin was essential for callus formation. BAJAJ, SIDHU and GILL (1983) found that low levels of auxin and cytokinin did not result in callus formation, but high auxin concentrations and cytokinin resulted in callus. With Gloriosa explants callus did occur at low levels of auxin and cytokinin, but in general callus formation is stimulated when the concentration of auxin is higher than the cytokinin (EVANS, SHARP and FLICK, 1981).

As was expected high levels of auxin stimulated rhizogenesis in Gloriosa corm explants (KAUL and SABHARWAL, 1972); BUI-DANG-HA, NORREEL and MASSET, 1975; STIMART, ASCHER and ZAGORSKI, 1980). High levels of cytokinin drastically suppressed root formation, clearly indicating the interaction between the two hormones (TAKAYAMA and MISAWA, 1979).

The use of 2,4-dichlorophenoxyacetic acid as an auxin source in the grid, resulted in the production of callus at the cut surface of the explant or malformation of the developing plantlets. There are two views as to the possible effects of this auxin on cultures;

i) it may result in chromosomal aberration and suppression of morphogenesis (MURASHIGE, 1974; GRESSHOFF, 1978);

ii) it induces embryogenesis and chromosomal aberration does not occur (RADOJEVIC, SOKIC and TUCIC, 1987).

To avoid potential complication of morphogenetic suppression of explants in media supplemented with 2,4-dichlorophenoxyacetic acid, this auxin was used exclusively for the induction and maintainance of callus.

What appears above as conflicting results, amplifies the fact that no simple formula can be devised to satisfy the hormonal needs of explants. Organogenesis is controlled by a number of complex interactions involving environmental, nutritional and plant related factors, which ultimately can be traced back to the status and origin of the explant at the time of culture.

3.3.15 Subculture experiments

Propagation by indirect organogenesis involves the induction and manipulation of callus. Callus cultures are useful in analysing the effect various factors have on organogenesis and embryogenesis. The regenerative capacity depends on a number of factors already discussed in the previous chapter. According to GEORGE and SHERRINGTON (1984) the variety of explants that can be used for the induction of callus in monocotyledons has a narrower range than for dicotyledons, with embryos, young leaves, stem nodes and inflorescences being the most frequently used source of explants. In the case of Gloriosa and Sandersonia all organs could serve as a source of callus, especially when the induction medium was supplemented with 2,4-dichlorophenoxyacetic acid (Figure 3.26). However the callus utilized for the majority of the subculture experiments was derived from embryos. YEOMAN (1973) stated that callus initiation is dependent on the culture environment and not on the origin of the explant, while EVANS, SHARP and FLICK (1981) maintained that callus initiation is explant dependent. From the results obtained for Gloriosa and Sandersonia callus initiation, it is apparent that the

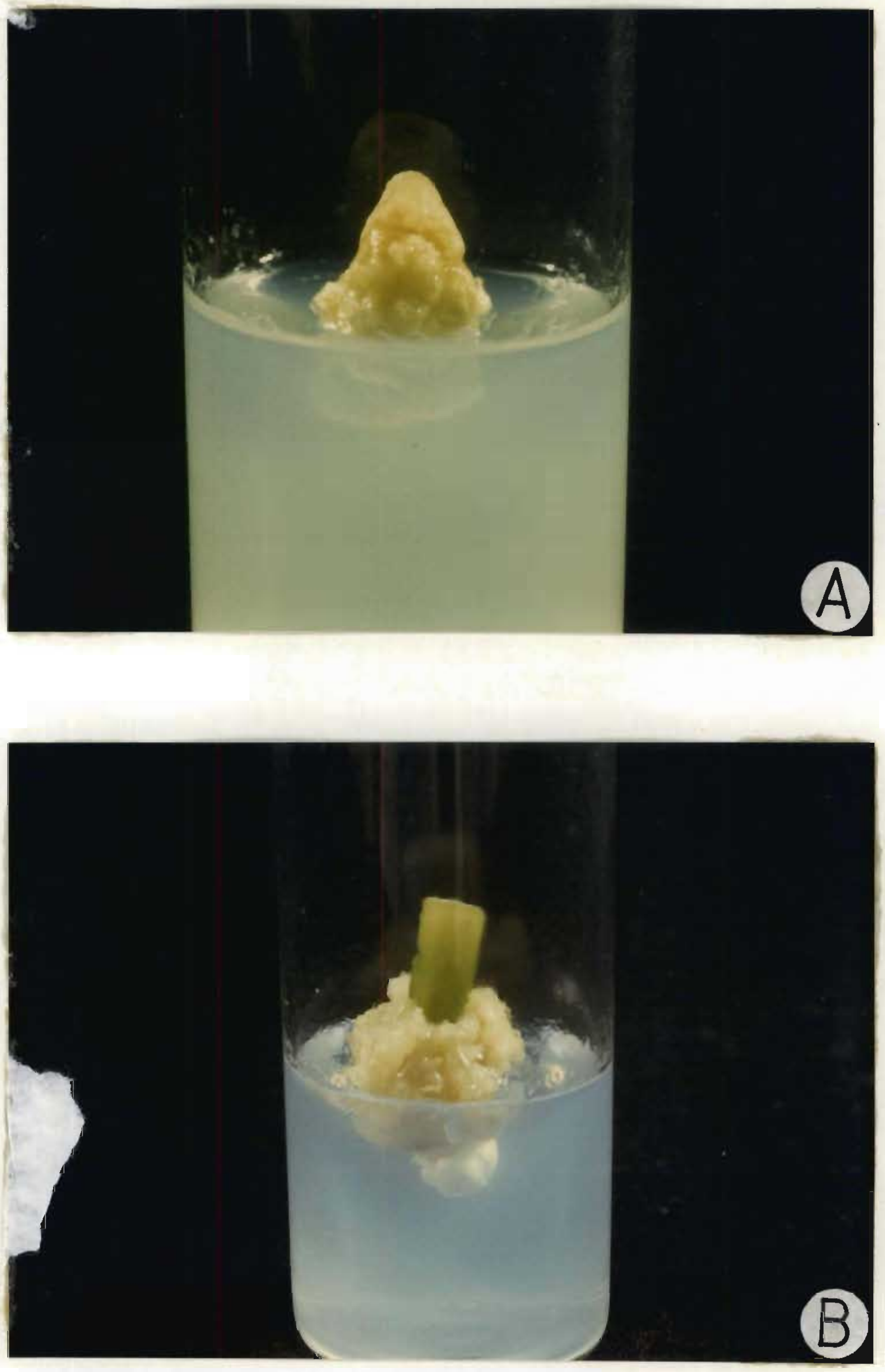


Figure 3.26 Callus production on media supplemented with 2,4-D

a) Gloriosa corm explant

b) Sandersonia corm explant

type of callus produced is explant dependent. Without the correct hormone combination callus initiation is retarded, but not entirely absent. The culture environment did not appear crucial (provided the explant was viable and in a suitable hormone combination) as light, temperature and nutrients did not affect callus induction. The origin, physiological age and status of the explant markedly affect callus initiation.

Gloriosa and Sandersonia callus was maintained on a medium supplemented with 2,4-dichlorophenoxyacetic acid (1 mg ℓ^{-1} kinetin, 1 mg ℓ^{-1} 2,4-D) and nodular non-friable callus was readily produced (Figure 3.27). Once callus was removed from the source of 2,4-dichlorophenoxyacetic acid rhizogenesis resulted, irrespective of the hormones and their relative concentrations (Figure 3.27c and d). Rhizogenesis occurred on media devoid of hormones. VAN STADEN, CHOUVEAUX, GILLILAND, McDONALD and DAVEY (1981) found that Protea callus would regenerate roots only, despite cultural variation. Often hard to root species only express their rooting capacity in vitro under suitable conditions via direct or indirect rhizogenesis. If rhizogenesis is considered a complex phenomena involving interactions between external factors, endogenous hormonal levels and nutrition, the auxin related factors appear to be of major importance (BRIDGLALL and VAN STADEN, 1985; NOUGAREDE, CHRIQUI and BERCETCHE, 1987). NOUGAREDE, CHRIQUI and BERCETCHE (1987) showed that napthaleneacetic acid and 2,4-dichlorophenoxyacetic acid leads to indirect rhizogenesis, however several cell cycles precede the recovery of rhizogenetic capacity. GROENWALD, WESSELS and KOELEMAN (1977) and BAJAJ, SUDHU and GILL (1983) found that by reducing the amount of auxin from induction levels, rhizogenesis results, a feature also shown in Gloriosa and Sandersonia.

The use of 2,4-dichlorophenoxyacetic acid as an auxin supplement is the subject of a great deal of scientific debate; a number of researchers avoid using 2,4-dichlo-

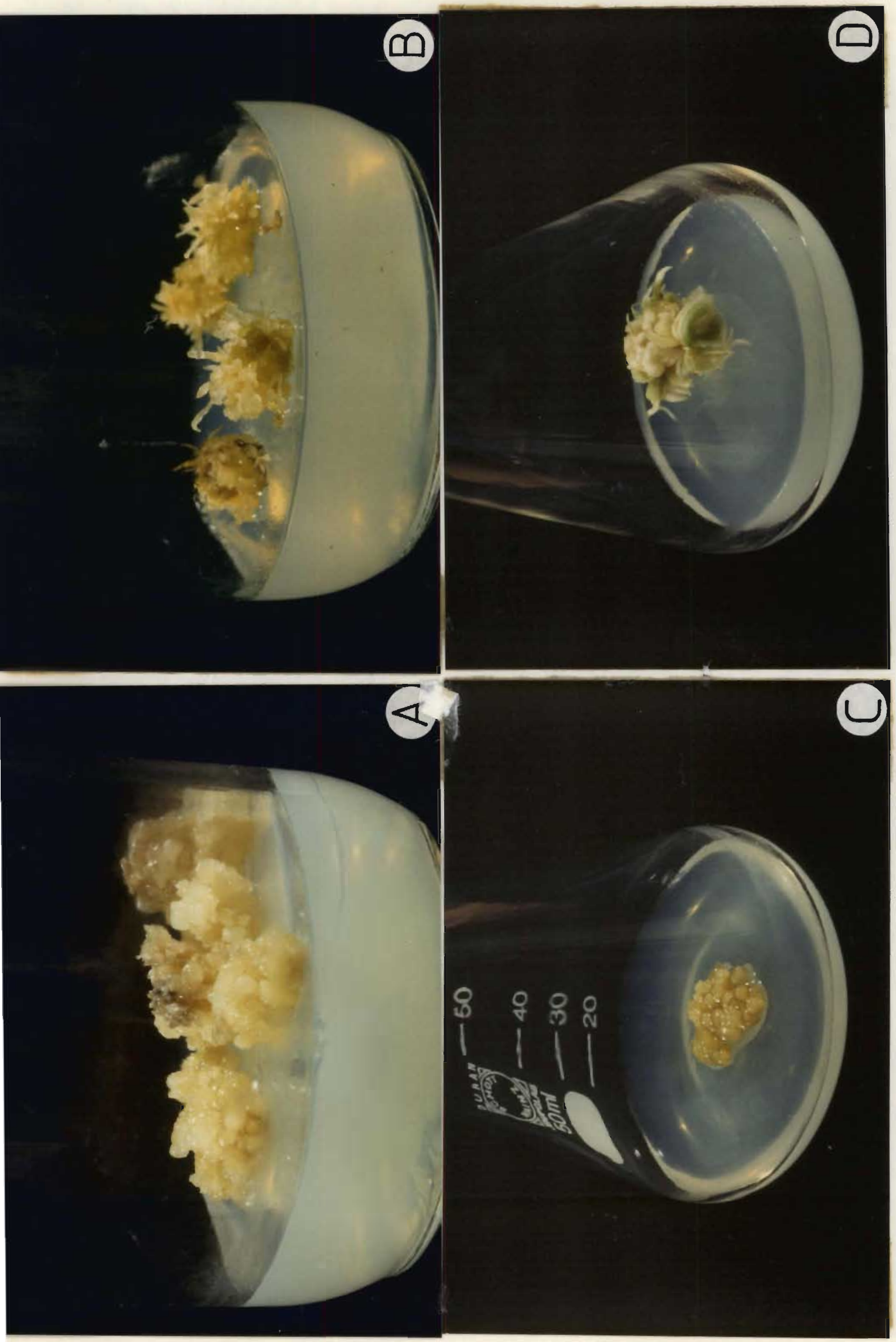

A
B
C
D
50
40
30
20
50ml

Figure 3.27 Callus used for subculture

a) <u>Gloriosa</u>

b) <u>Sandersonia</u>

c) callus maintained on 2,4-D

d) "root" production on media devoid of 2,4-D rophenoxyacetic acid because it may cause chromosomal aberrations, polyploidy and the suppression of organ formation (GRESSHOFF, 1978). MURASHIGE (1974) reported that 2,4-dichlorophenoxyacetic acid stimulates callus production but inhibits organogenesis. 2,4-Dichlorophenoxyacetic acid is regarded as being more active than napthaleneacetic acid (SEABROOK, 1980) and it also has been speculated that 2,4-dichlorophenoxyacetic acid acts primarily as an auxin but can also stimulate cytokinin synthesis. For callus induction 2,4-dichlorophenoxyacetic acid is often essential (JHA, MITRA and SEN, 1984). However, for plant regeneration it must be removed from the medium (EVANS, SHARP and FLICK, 1981). There is also a danger that 2,4-dichlorophenoxyacetic acid may be retained within the cultured cells for an extended period of time (MONTAGUE, ENNS, SIEGEL and JAWORSKI, 1981). Thus for these reasons 2,4-dichlorophenoxyacetic acid was utilized for callus induction and maintenance of cultures. Subsequent callus manipulation experiments were conducted in its absence.

Figure 3.28 indicates the amount of Gloriosa callus growth achieved on various hormone combinations using callus derived from a single stock. It is evident from these results that there is no trend with respect to Gloriosa callus growth and hormone combinations. The growth of Gloriosa callus utilizing natural hormones (zeatin and IAA) shows the same lack of growth trends (Figure 3.29) as does the callus grown on synthetic hormones. The only major difference was the increased amounts of chlorophyll produced in the cultures grown on the natural hormones. No shoot production was achieved on the callus grown in either of these two experiments. Multiple shooting was achieved from callus grown on a Murashige and Skoog medium supplemented with 2 mg ℓ^{-1} kinetin and 0,25 mg ℓ^{-1} NAA (Figure 3.30). Subsequent cultures taken from the same stock did not produce shoots. Using the same stock of callus used in Figure 3.28, the 4 by 4 experiment was repeated a year later (Figures 3.31 and 3.32 show the amount of callus growth

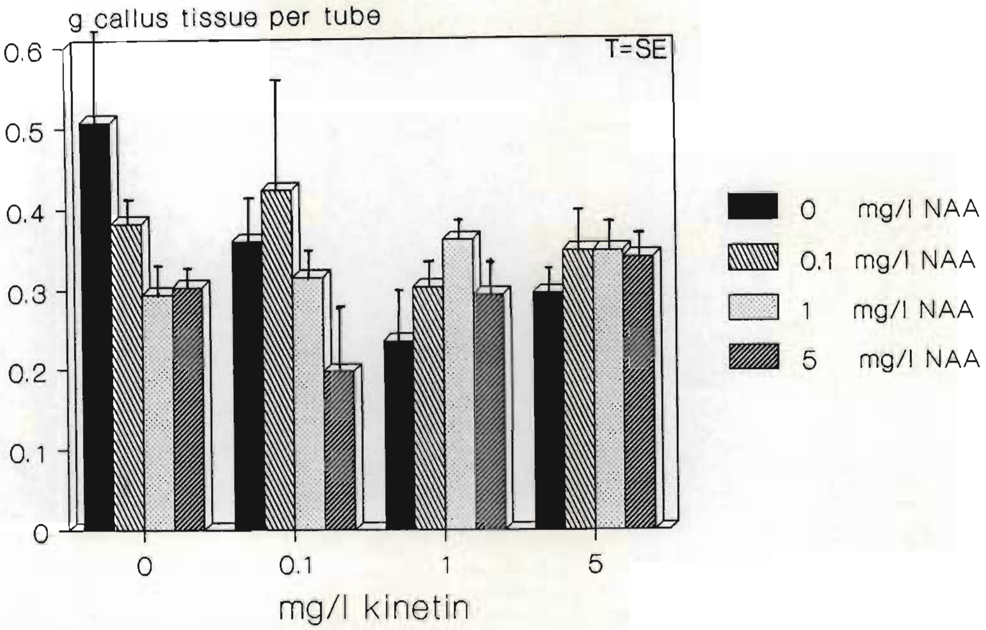


Figure 3.28 Amount of Gloriosa callus growth on a 4x4 synthetic hormone grid.

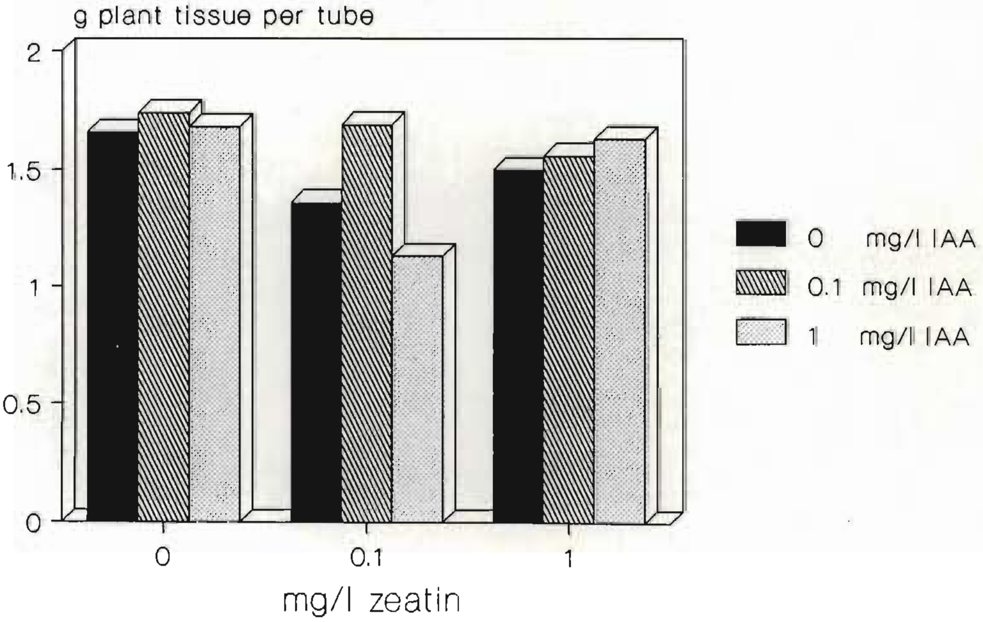


Figure 3.29 Growth response of Gloriosa tissue to a natural hormone grid.

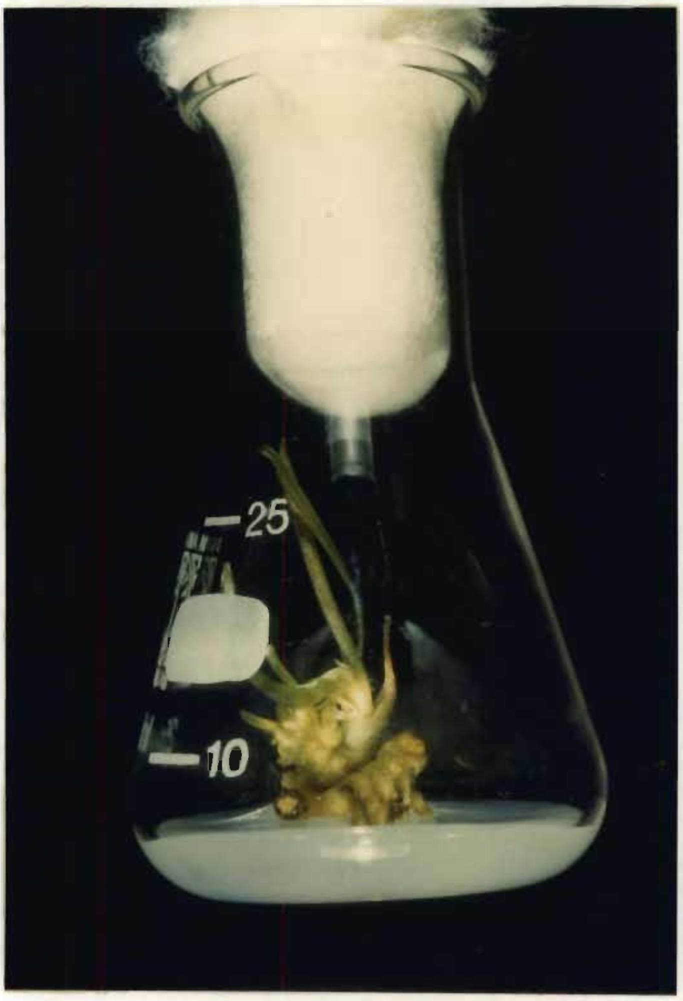


Figure 3.30 Multiple <u>Gloriosa</u> plantlet production using callus

and morphological response). As the amount of auxin (NAA) is increased the production of roots is suppressed while callus formation is stimulated (the increase in callus production (Figure 3.32) is shown as an overall decrease in the weight of plant material (Figure 3.31)). This phenomena again shows that high auxin concentrations with or without cytokinins stimulate callus formation, while low auxin and cytokinin concentrations do not induce callus proliferation (HOSOKI and ASAHIRA, 1980; BAJAJ, SIDHU and GILL, 1983). Reduction in the amount of auxin induces rhizogenesis (GROENEWALD, WESSELS and KOELEMAN, 1977; BAJAJ, SIDHU and GILL, 1983).

Extended subculture periods may lead to a reduction in the morphogenetic potential of the callus. HUGHES (1981) recorded that loss of morphogenetic response may be a result of several factors:

i) reduction in the endogenous levels of hormones;
ii) callus becomes habituated;
iii) chromosomal aberrations;
iv) loss of organised centres (meristemoids) through repeated subculture and unorganised cell divisions.

There is also the possibility that the failure of Gloriosa callus to produce shoots may be a result of hormone carry-over or retention that offset the subsequent experiments (NITSCH, 1968; MONTAGUE, ENNS, SIEGEL and JAWORSKI, 1981), especially the retention of 2,4-dichlorophenoxyacetic acid.

The origin of the callus tissue from the embryo axis may also have affected the callus' ability to produce shoots. GEORGE and SHERRINGTON (1984) reported a case where only the cells obtained from the micropylar end of the embryo sac were embryogenetically predetermined and these cells retain this capacity in subsequent generations. Gloriosa callus tissue was derived from the radicle portion

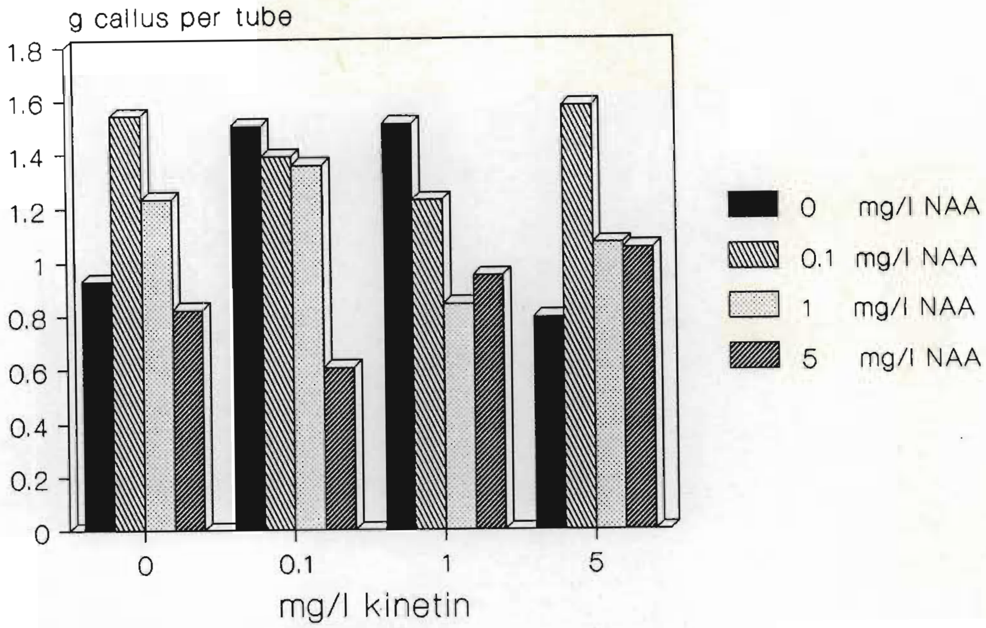


Figure 3.31 Amount of Gloriosa callus growth on a 4x4 synthetic hormone grid.

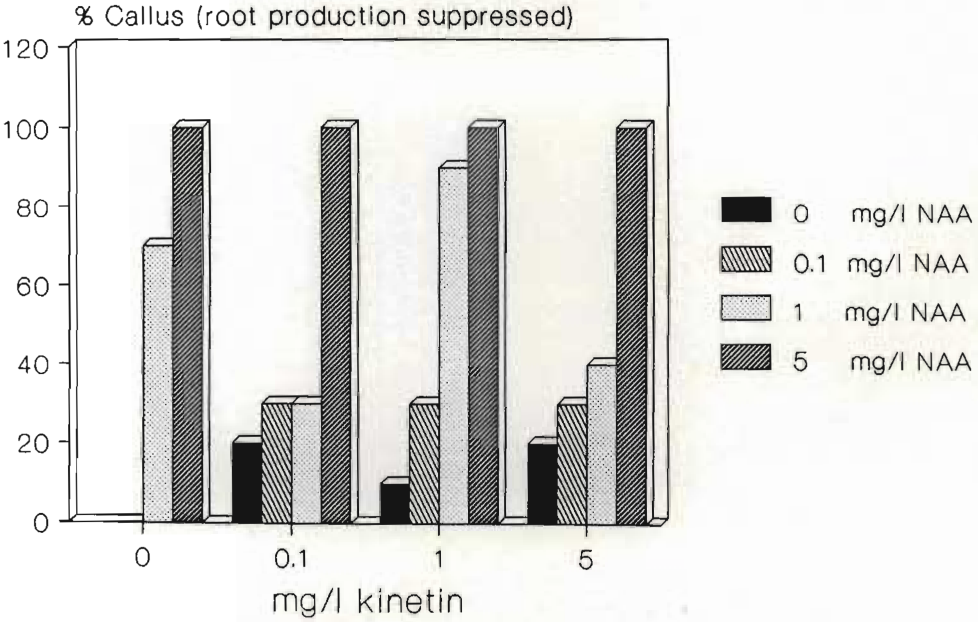


Figure 3.32 Morphological response of Gloriosa tissue to a 4x4 synthetic hormone grid.

of the embryo, and this may explain the callus' predetermined ability to produce roots and not shoots, even without hormone supplementation.

Sandersonia callus, on the other hand, showed a distinct response to hormone applications (Figure 3.33). Increasing the amount of auxin (NAA) results in an increase in the amount of plant material produced. This increase in plant material is a result of the production of both callus and roots. Contrary to what was found in Gloriosa, Sandersonia callus production was stimulated by high levels of cytokinin and low levels of auxin, while increases in the amount of auxin resulted in stimulation of root production, a feature also reported by SEABROOK, CUMMING and DIONNE (1976). This marked difference in Sandersonia and Gloriosa callus response in culture was unexpected, as the explant response of the two species was almost identical. The most feasible explanation for the variation in callus response is the fact that the callus originated from different explants. Gloriosa callus was derived from the root apex of the mature embryo of germinating seeds, while Sandersonia callus was derived principally from the immature ovules and ovary walls of pollinated flowers. This indicates that there is a variability in cultural requirement of tissue from different explants, explaining the fact that different types of explants respond differently in the same culture environment.

Experiments investigating the growth rate and maintenance potential of callus tissue was performed to determine optimum subculture time, and whether the organogenic potential of the callus is diminished over time. After prolonged subculture, changes often occur in callus cultures, hormone habituation, the loss of organogenic potential and changes in the external characteristics of the callus (THORPE, 1980). The normal sigmoid growth curve was obtained for Gloriosa callus (Figure 3.34). DODDS and ROBERTS (1985) found that x (the time for approximate subculture) ranges between 18 - 24 days for a typical callus culture.

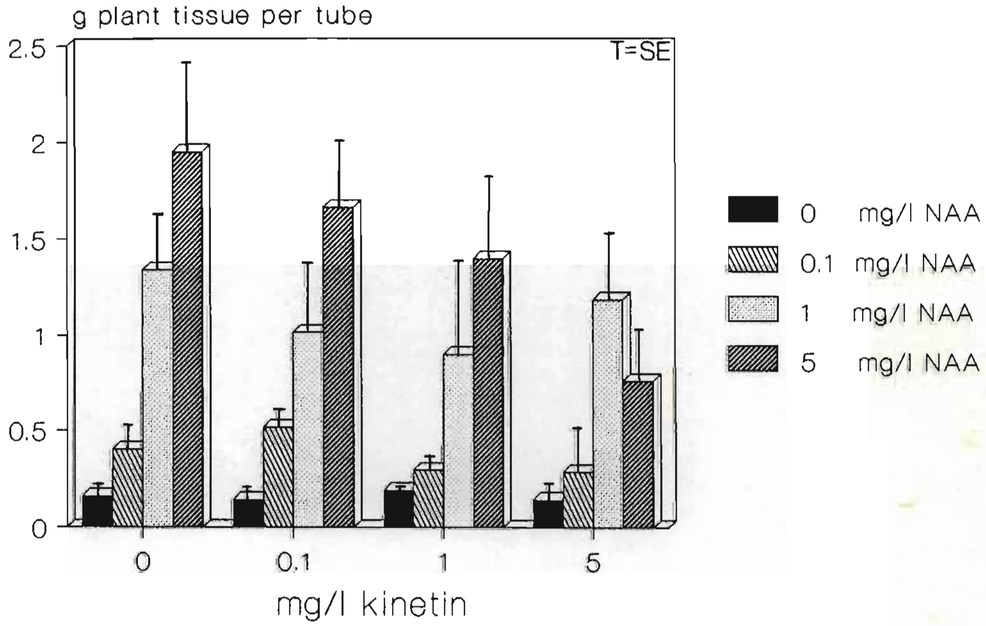


Figure 3.33 Amount of <u>Sandersonia</u> tissue growth on a 4x4 synthetic hormone grid.

YEOMAN and MACLEOD (1977) suggest that cultures maintained at 25°C on agar should be subcultured every 4 - 6 weeks. In the case of Gloriosa callus, x is between 55 - 65 days, so that callus is subcultured during the active growth phase, to minimise the lag phase after subculture due to presence of senescing callus. During the stationary phase the cultures began to senesce and turn brown.

The difference in growth response of new (± 3 month old, subcultured once) and old (± 30 months old, subcultured 12 times) callus is striking, with the growth rate of the new callus far exceeding that of the old callus (Figure3.35). The ability of new callus to produce plantlets and corms adds weight to the argument that after prolonged subculture the morphogenetic potential of callus is gradually lost (HUGHES, 1981). For successful production of plantlets from callus cultures, initiation of new callus should be routinely performed. This not only allows for the production of organogenic callus, but also reduces the potential amount of chromosomal aberration by reducing the period for which callus is in culture. It is possible that prolonged exposure to 2,4-dichlorophenoxyacetic acid was probably the primary cause of the failure of old callus to produce shoots and corms, because of the retention of the organogenic suppressing 2,4-dichlorophenoxyacetic acid and the possibility of resulting chromosomal damage.

The morphology and anatomy of the "roots" produced from callus cultures above, for both Gloriosa and Sandersonia, was similar to that of the corm grown in vitro. Hardening off of the roots could be easily achieved, however shoot regeneration from these roots in vivo did not occur. The failure of the roots to produce shoots may have been due to:

i) the inability of these roots to produce shoots; or

ii) complications as a result of dormancy (affecting root and shoot growth).

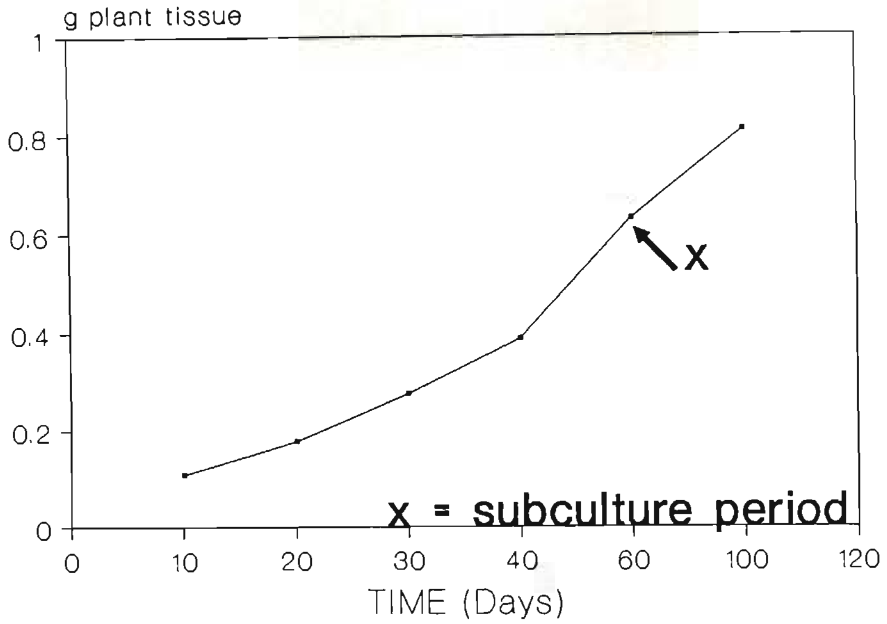


Figure 3.34 Growth rate of <u>Gloriosa</u> tissue over a 100 day period.

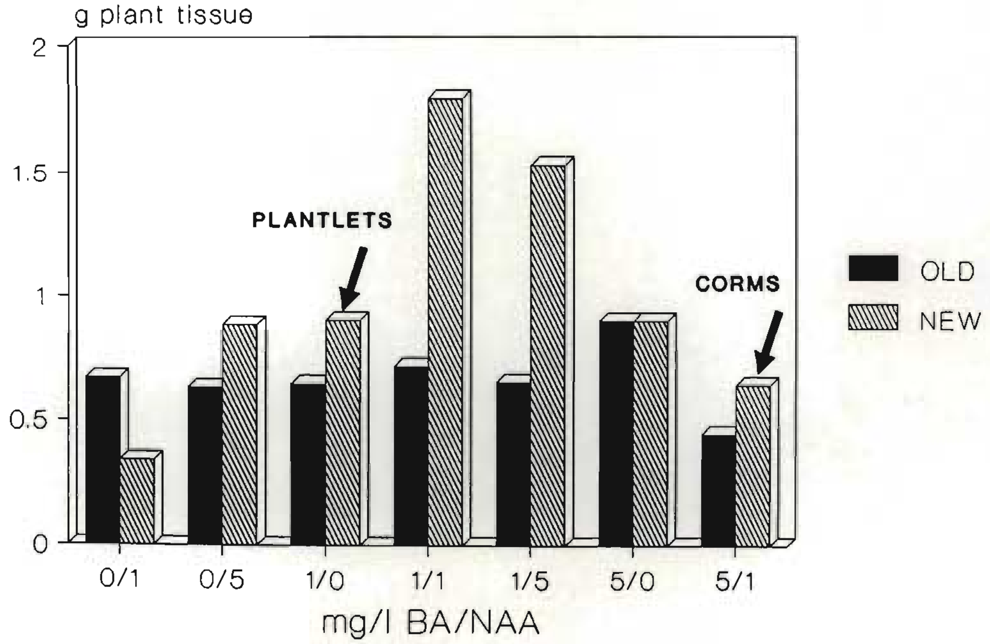


Figure 3.35 Growth response of NEW and OLD, <u>Gloriosa</u> tissue in culture.

Further experimentation is required to ascertain the true nature of the lack of response of roots to produce shoots. Utilization of the callus route has numerous advantages. Large quantities of callus and roots can be produced, however the use of corm explants for multiple plantlet production is recommended. Callus culture and subsequent root production does have potential for the establishment of suspension cultures and the production of colchicine in vitro, especially as the majority of the plant material produced in culture is in the form of roots, and colchicine levels are highest in the subterranean portions of the plant.

When callus is grown in an agitated liquid medium it forms a suspension composed of free cells, cell aggregates with cell numbers ranging from a few to several hundred, depending on the friability of the callus (NARAYANASWAMY, 1977). Gloriosa and Sandersonia callus was grown in an agitated liquid Murashige and Skoog medium with either; 1 mg ℓ^{-1} kinetin, 1 mg ℓ^{-1} 2,4-D
0,1 mg ℓ^{-1} kinetin, 0,1 mg ℓ^{-1} NAA
0,1 mg ℓ^{-1} kinetin, 1 mg ℓ^{-1} NAA
1 mg ℓ^{-1} kinetin, 0,1 mg ℓ^{-1} NAA or
1 mg ℓ^{-1} kinetin, 1 mg ℓ^{-1} NAA.

The response of callus growth in the liquid medium is recorded in Figure 3.36 and Figure 3.37, with 1 mg ℓ^{-1} kinetin and 0,1 mg ℓ^{-1} NAA providing a medium that accelerated growth to a greater degree than the other four media. Because of the non-friable nature of the callus a true suspension was not achieved, with large "clumps" of cells in the suspension. To improve the structure of the suspension, callus was pretreated with 0.5% pectinase and 0,1% macerase overnight to reduce the callus to single cells prior to inoculation. Although the enzyme treatment improved the suspension, a large percentage of the cultures become infected with a yeast, and therefore this step was discarded. The cultures were inoculated at a density of 1 g per 100 mls of medium and agitated on an orbital

Figure 3.36 Suspension culture of Gloriosa callus

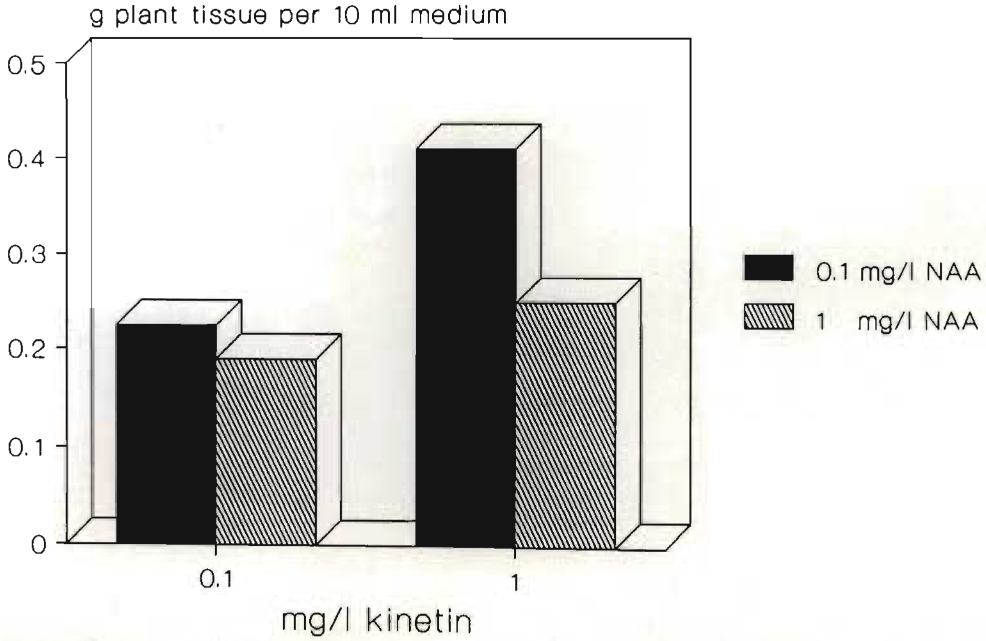


Figure 3.37 Growth of Gloriosa callus in liquid culture (suspension).

shaker at 150 rpm. There was a long lag phase when only 1 g was utilized to initiate cultures. This lag phase could be reduced by increasing the amount of inoculum to 2 g or by "chopping" the callus into smaller pieces. HELGESON (1979) recommended that between 2 - 3 g of callus per 100 mls should be used to initiate culture. Sandersonia and Gloriosa response in suspension culture was the same. In liquid media with low levels of hormones (0 mg ℓ^{-1} kinetin, 0 mg ℓ^{-1} NAA and 0,1 mg ℓ kinetin, 0,1 mg ℓ^{-1} NAA) the callus readily produced roots (similar to those produced on solid media). Subculture of roots produced in liquid medium, back into a liquid medium did not initiate any more root production. It was found that only callus could serve as the inoculum for root propagation in liquid culture. (Root production in liquid media was more prolific than on a solid media, probably due to the increased surface area of the explant in contact with the medium. Callus production was greatest on a solid medium despite the supposed advantage that suspension cultures have a much faster rate of multiplication (DODDS and ROBERTS, 1985). An anatomical investigation of the suspension revealed a large percentage of parenchyma and xylem cells, indicating that differentiation had occurred in the suspension, (the xylem cells being derived from the formation of roots in culture).

Due to the failure of callus cultures to produce multiple plantlets, the technique of suspension culture for multiple plant production was not further pursued. Suspension cultures were utilized to produce colchicine in vitro.

Callus from both species exuded a clear exudate on the interface between the medium and the explant (Figure 3.38). The exuded substance may be a long chain polysaccharide (CONRAD, BINARI and RACUSEN, 1982). It was reported that certain monocotyledonous species release large amounts of polysaccharide into the medium after several weeks in culture. This is apparently a result of increased dictyosome numbers in the cultured material (CONRAD, BINARI and RACUSEN, 1982). This would explain the presence of the exudate. Gram stains were performed on the exudate and plant tissue to determine whether microbial interaction was involved. These tests proved negative. Results from electron microscopy are presented in Figure 3.38 with tissue taken from callus that produced exudate and callus that does not.

Often both callus and plantlet tissue derived directly from the explant was vitrified. The occurrence of the vitrification was not hormone related and was probably due to mechanical or chemical damage to the explant resulting from transfer on the laminar flow bench or during sterilization. Further causes of vitrification may have been the high humidity in the culture vessels (in order to discourage mites the tubes and caps were sealed with parafilm resulting in a great deal of free water within the tube) or high ethylene levels being introduced into the tube during flaming and then sealed in by the parafilm.

3.3.16 Nutrient and environmental manipulation

As found with Clivia, both Gloriosa and Sandersonia tissue showed no significant differences in their growth pattern when grown on the four different media. A full strength Murashige and Skoog medium was selected as the most suitable medium for tissue growth. An added advantage is that it can be obtained as a premix, and application of the above techniques can be applied by commercial concerns without necessitating complex media preparation. TAKAYAMA and MISAWA (1982/1983) found that bulblet differentiation was not affected by the concentration of Murashige and Skoog medium between one-eighth and full strength.

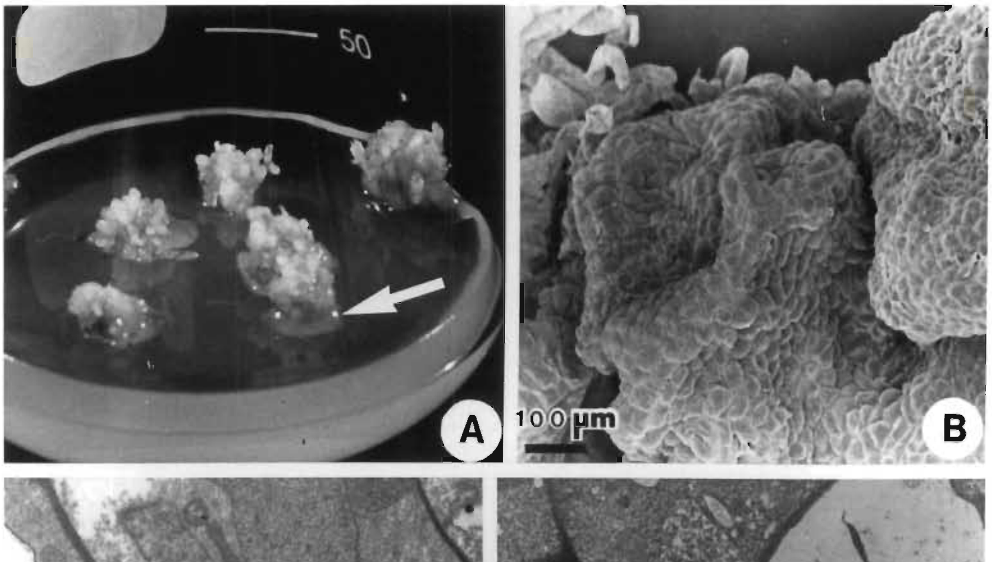

50
A
100 µm
B
5 µm
C
5 µm
D

Figure 3.38 Exuding and non-exuding callus

a) exudate at medium and callus interface
b) surface structure of exuding callus
c) anatomy of non exuding callus
d) anatomy of exuding callus (increased dictyosome activity was not observed)

Many factors have been shown to influence tuberization *in vitro* with the relative supply of sucrose being an important factor (SMITH and PALMER, 1970; FORSYTH and VAN STADEN, 1984). Table 3.25 shows the effect of sucrose and kinetin on corm formation in *Gloriosa* explants.

Table 3.25 The effect of sucrose and kinetin on corm formation of *Gloriosa* explants *in vitro*

Sucrose $g\ \ell^{-1}$	Kinetin $mg\ \ell^{-1}$	No of corms per tube	No of plantlets per tube	Formation of callus
20	0		2	x
	5		2	x
	10		1	
50	0		3	x
	5		2*	x
	10	3	3	
80	0			
	5		1*	x
	10	5	1	

* vitrified plantlets

Corm formation appears to be stimulated by high levels of sucrose (50 - 80 $g\ \ell^{-1}$) and high kinetin concentrations (10 $mg\ \ell^{-1}$) (see also Figure 3.25b where increasing cytokinin results in an increase in corm formation). It appears that corm response may be mediated by cytokinins (SMITH and PALMER, 1970) or a combination of sucrose and cytokinin as suggested by FORSYTH and VAN STADEN (1984). It is unlikely that sucrose alone can initiate corm formation, but has its effect through other factors probably a hormone. TRAN THANH VAN (1977) suggested that the sucrose effect on organ formation is due to changes in the osmotic potential. Sucrose levels that result in good callus growth may not be optimum for morphogenesis, and the effect of cytokinin and the efficiency of nitrogen ions may be related to sucrose concentration (GAMBORG, CONSTABEL and SHYLUK, 1974). To draw a comparison between corm formation and tuber formation can be difficult, however it appears that the high levels of sucrose and cytokinin stimulate corm formation. The sucrose effect may be related to a greater entry into the vascular tissue with rapid transport to the meristem, or the effect may be linked to the endogenous hormone levels within the explant (FORSYTH and VAN STADEN, 1984). An interesting feature is that galactose has been shown to stimulate ethylene production (COLCLASURE and YOPP, 1976) and it is possible that high levels of sucrose may have a similar effect on ethylene evolution, resulting in senescence of the explant with a hastening in the production of storage / overwintering organs.

Results from the sugar treatment on Gloriosa callus tissue are displayed in Figure 3.39 with the optimum sucrose concentration being ± 20 g ℓ^{-1}. Callus without a sucrose supplement died, while sucrose added at 40 g ℓ^{-1} and 80 g ℓ^{-1} showed a decrease in the amount of growth against that of 20 g ℓ^{-1}. This reinforces the statement that the carbohydrate requirements of explants can be satisfied by the incorporation of sucrose at a concentration of 2 to 3% (MURASHIGE, 1974). At 10 g ℓ^{-1} only callus was produced while at higher levels (20 - 80 g ℓ^{-1}) root regeneration was observed from the callus tissue. TAKAYAMA and MISAWA (1980) showed that high levels (90 g ℓ^{-1}) of sucrose had an inhibitory effect on the growth of scale leaves (as did 80 g ℓ^{-1} on the growth of Gloriosa callus), but the inhibition could be reversed on transfer to 30 g ℓ^{-1}.

Nitrogen availability may greatly affect survival and growth in culture, with nitrate being the most important form of nitrogen. Figure 3.40 shows the growth response of Gloriosa callus at differing relative levels of nitrogen (both NH_4NO_3 and KNO_3

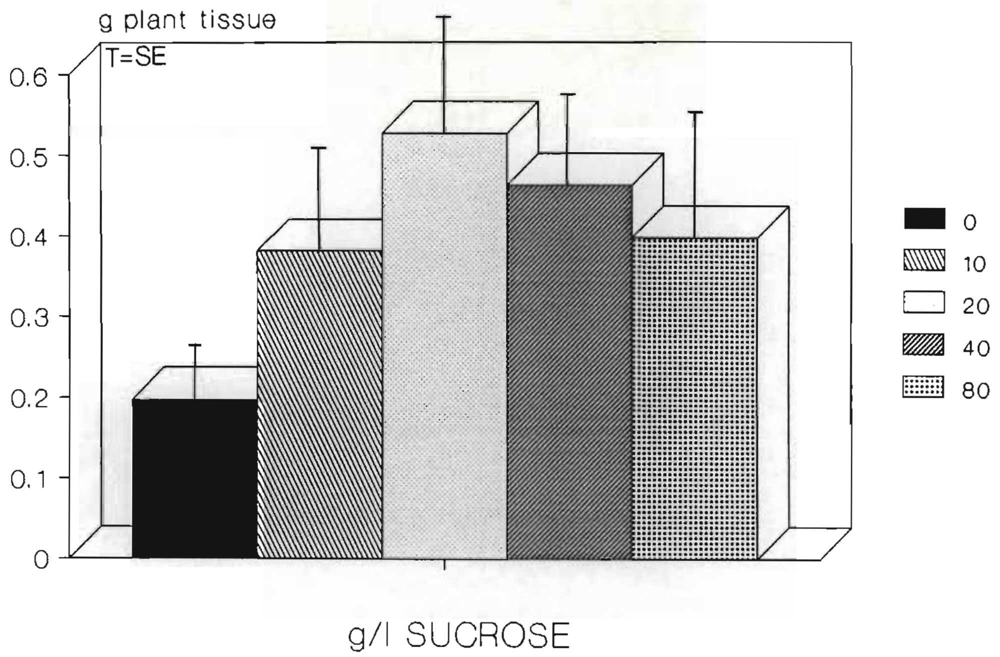


Figure 3.39 Growth of Gloriosa callus in differing concentrations of sucrose.

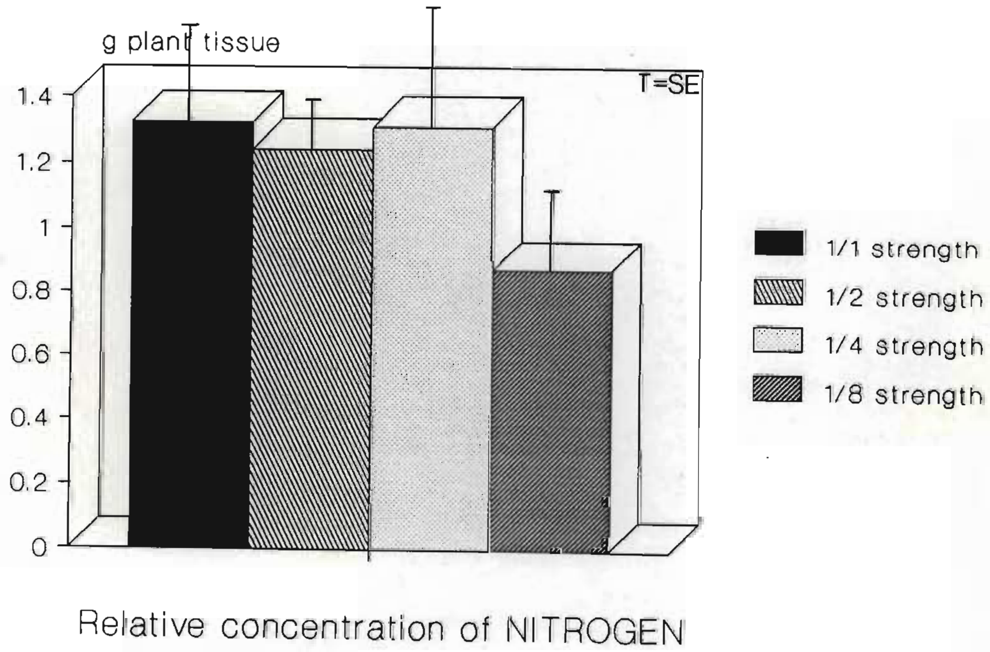


Figure 3.40 Growth of Gloriosa callus in differing relative levels of nitrogen.

were reduced). Nitrogen in a full strength Murashige and Skoog medium is added at a concentration of 1650 mg ℓ^{-1} NH_4NO_3 and 1900 mg ℓ^{-1} KNO_3 and it is obvious that at even a quarter strength there is sufficient nitrogen available in the medium to maintain good callus growth. However, at an eighth strength (206 mg ℓ^{-1} NH_4NO_3 and 237,5 mg ℓ^{-1} KNO_3) the media no longer meets the nitrogen requirements of the callus. The different nitrogen levels had no significant effect on the morphogenesis of the callus, with equal amounts of roots and callus being produced on all four media. This appears to contradict GEIER's (1986) results where low levels of nitrogen were beneficial for regeneration of leaf segments and high levels accelerated root formation on callus.

Figure 3.41 and 3.42 graphically represent the culture response of callus to various temperature and light regimes. Temperature plays a major role in morphogenesis and development of tissue, with the optimum temperature varying from species to species (APPELGREN and HEIDE, 1972; STIMART, ASCHER and WILKEN, 1982). Growth of callus was highest at 25°C, the temperature normally associated with the growth of the plant (summer growing plant). In the experiment where culture tubes were grown outside (with resulting fluctuation in both temperature and light) callus growth was not as favoured as was the growth of callus in a constant 25°C. As mentioned previously, when temperatures rose to 35°C for ± 48 hours, growth was not adversely affected, but bacterial growth was triggered by the high temperature.

Growth of callus in differing light environments (callus grown 'outside' with day night fluctuations and callus transferred from dark to light) was inhibited more than those grown in a constant lighting regime. Callus grown in low light or light conditions grew better than when in total darkness contrary to the reports of KLEIN (1968) and YEOMAN and DAVIDSON (1971) but consistent with the observation of DE CAPITE (1955) and SEIBERT, WETHERBEE and JOB (1975).

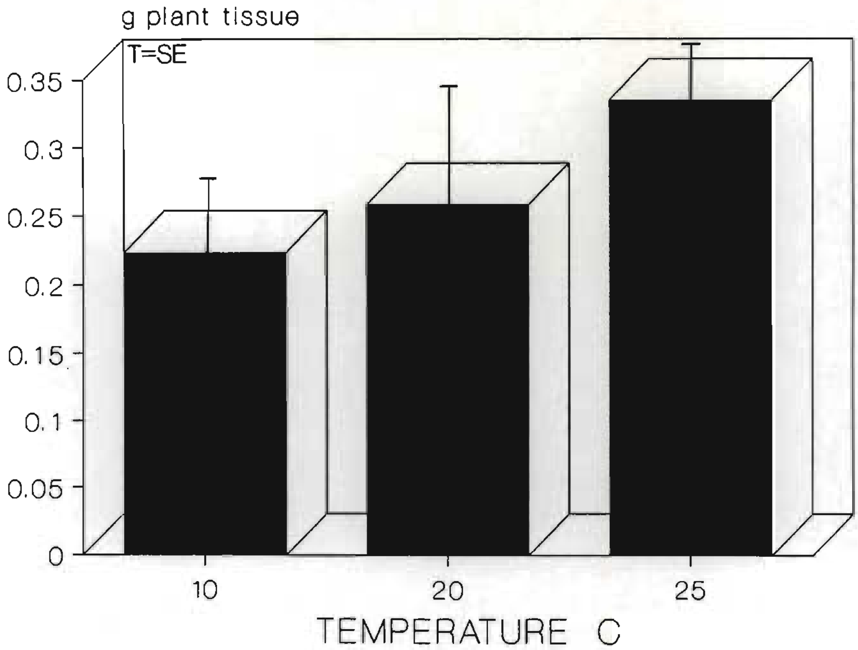


Figure 3.41 Temperature and growth of Gloriosa tissue in vitro.

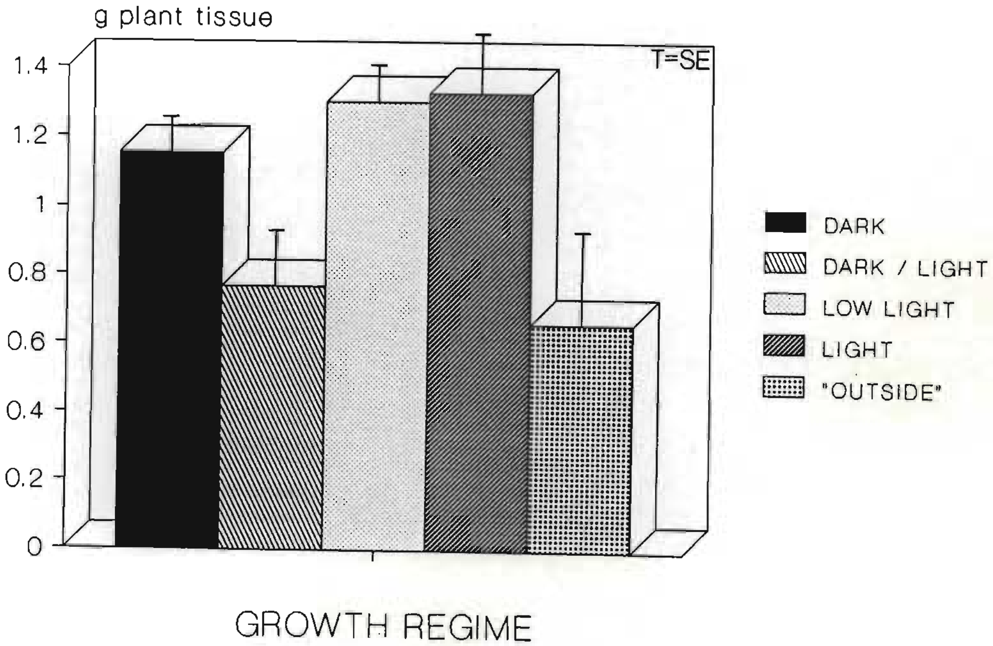


Figure 3.42 Growth response of Gloriosa tissue in various light regimes.

HAMMERSCHLAG (1978) found that light of differing intensities was both stimulatory and inhibitory with a cultivar specific response. LESHAM, LILIEN-KIPNIS and STEINITZ (1982) showed that light did not affect organogenesis or growth of cultures and variation can be ascribed to the use of different explant organs, a general observation that was also made during the morphogenetic experiments of Gloriosa and Sandersonia explants.

The suggestion of the use of daily and seasonally fluctuating temperature and light regimes would not appear to be ideal when one views the results of callus grown 'outside'. However, this suggestion may be applicable if the fluctuations are kept constant within limits (not as with the wide fluctuation found 'outside') and the light / temperature regime is designed for each species and explant.

3.3.17 Seasonality

An important factor to consider when culturing bulbous monocotyledons is the dormancy of the bulbs. Variation in temperature requirements exemplifies the significance of exploring and satisfying seasonal temperature requirements of plants (MURASHIGE, 1974). Corms taken as explants from the four seasons, all grew in culture at 25°C. The dormant corms required a slightly longer period to respond in vitro, indicating that the dormancy breaking requirements can be satisfied by exposing the corms to summer temperatures (25°C). It is important that the dormancy requirements of the plant should be met before attempting culture (SEABROOK, CUMMING and DIONNE, 1976; NARAYANASWAMY, 1977; NISHUICHI, 1980a). KUKULCZANKA, KLIMASZEWSKA and PLUTA (1977) found that explants taken in spring and summer were highly regenerative, probably due to the presence of endogenous growth regulators (MII, MORI and IWASE, 1974; ODANI, 1974; WRIGHT, 1975) or the mobilization of reserves from the storage organs. Table 3.26 summarizes the seasonal response of corm explants in culture.

Table 3.26 Seasonal response of Gloriosa corm explants in culture

Culture temperature °C	EXPLANT		
	Spring / Summer	Autumn / Winter	Actively growing *in vitro* cormlets
10°	no growth	no growth	no growth/senesced
25°	sprouting within 10-15 days	growth response only after 21 - 28 days	continued growth
10°/25°	growth only at 25°	growth only at 25°	variation in temperature from 25°/10°/25°/10° enhanced corm formation

No plantlet growth was observed at 10°C, however on transfer to 25°C plantlet growth occurred. Transfer of actively growing *in vitro* corms into the cold and back to 25°C resulted in enhanced production of corms. This indicated that the seasonal variation of the two species can be met *in vitro* by altering the temperature regime (the light regime did not appear to affect the seasonal response of corms *in vitro*). ZIMMER (1975) found that when freshly harvested corms of *Gloriosa* are placed in damp peat, 96,9% of the corms produced shoots at 30°C within six weeks, while it took 22 weeks for 67,2% of the corms to shoot when grown at 15°C. At 20°C and 25°C the corms entered a dormant condition. After storage at 10°C however, the shooting percentage of corms held at 20°C and 25°C was markedly increased, but not in corms held at 15°C and 30°C. CAROW (1980a) found that pre-sprouting storage of *Gloriosa* corms, at 10°C and 50 - 70% relative humidity for 60 days, resulted in 100% even sprouting in 9 - 23 days, showing that *in vivo* grown *Gloriosa* corms behave the same as those grown *in vitro* with regards to temperature requirements and shooting.

Reaction time and percentage response in culture for two-year-old corms was far better than for the older corms. However, use of young corms (one to two years) for explants would mean that these corms are derived from seeds. As flowering normally only occurs in three year or older corms (or provided they exceed a critical size (CAROW, 1977)), the nature of the young corms (flower colour and plant quality) is unknown. For selective in vitro breeding projects, the less reactive older corms would have to be used.

3.3.18 Anther culture

Results obtained from Gloriosa and Sandersonia anther cultures were the same as those for Clivia. Figure 3.43 shows the extremes in the size of the anther used for Gloriosa culture.

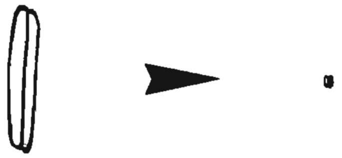


Figure 3.43 Actual sizes of Gloriosa anthers used for culture

Despite the use of all possible ages of anthers for tissue culture, the only in vitro response was the callusing of filaments when they were left on the anther. Filament callusing occurred for both Gloriosa and Sandersonia. Possible reasons for the failure of anther culture have been previously discussed in the Clivia section. KRIKORIAN and KANN (1985) advocated the use of inflorescence parts especially ovary bases and stamen filaments as an alternative source of regenerative material. Further study into utilization of filament callus is advocated, especially in the light of the success of floral organs as regenerative explants.

3.3.19 Protoplast culture

Results for protoplast culture of Gloriosa and Sandersonia were the same as those obtained for Clivia (easy isolation of viable protoplasts, wall regeneration and a small degree of "clumping"). The use of leaf material grown in vitro provided a better source of protoplasts, because the poorly developed cuticle allowed for penetration of the enzyme solution. The use of protoplasts for Gloriosa and Sandersonia tissue culture is recommended with further study aimed at the establishment of a suitable plating density. SIMMONDS, SIMMONDS and CUMMING (1979) felt that Lilium species (and Gloriosa and Sandersonia) are suitable for protoplast studies, as chromosomal and meiotic irregularities resulting from in vitro techniques could be more readily tolerated by vegetatively reproducing species, than those relying on sexual processes.

3.3.20 Hardening off

"Transplanting and re-establishing aseptic propagated plants under non-aseptic conditions is still one of the main problems in the tissue culture propagation of many plants" (ZIV, 1979). NOVAK and PETRU (1981) found that in most cases members of the Liliaceae, Iridaceae and Amaryllidaceae can be transferred directly into soil, but have a tendency to become dormant (HUSSEY, 1977a; 1977b; TAKAYAMA, MISAWA, TAKASHIGE, TSUMORI and OHKAWA, 1982). Hardening off of both Gloriosa and Sandersonia cormlets was achieved by simply removing the cormlets from culture, washing off of the excess agar, and potting out in a damp potting mixture. The dormancy requirements were met by short term storage at 10°C (10 days) and then transfer to 25°C (as indicated by the in vitro studies). These dormancy breaking requirements for Gloriosa and Sandersonia are similar to those found by ZIMMER (1975) and CAROW,(1980a). Between 80 and 95% of the in vitro corms sprouted after this treatment, without the need for cytokinins

(HUSSEY, 1977b) or leaves and roots (STEINITZ and YAHEL, 1982) for successful establishment of plants in the soil. Use of low temperatures for dormancy breaking of in vitro grown plantlets has been widely used in the monocotyledons (HILDERBRANDT, 1971; HUSSEY, 1977a; ZIV, 1979; TAKAYAMA, MISAWA, TAKASHIGE, TSUMORI and OHKAWA, 1982).

Hardening off of Gloriosa and Sandersonia plantlets did present difficulties as the culticle development on both species was poor, resulting in rapid water loss (SUTTER and LANGHANS, 1979). The use of mist sprays and plastic covers as advocated by ZIV (1979) was essential to prevent desiccation. Despite the long periods of acclimitization a success rate of less than 50% was achieved.

3.3.21 Conclusion

For maximum plantlet regeneration especially for commercial utilization the production of multiple cormlets in vitro via corm explants is recommended, especially due to the ease of the hardening off process. Using this technique callus formation is kept to a minimum avoiding problems such as chromosmal aberrations and loss of morphogenetic potential. Callus manipulation experiments revealed that there is a loss of morphogenetic potential when cultures are maintained for extended periods of time.

Although the culture requirements of different explants, and callus derived from different explants, varies, it is interesting to note that the explants taken from the same source on both Gloriosa and Sandersonia respond almost identically in culture (apart from the variations that could be ascribed to differences in age and seasonal growth).

3.4 Conclusions

Since HABERLANDT (1902) attempted the first plant tissue culture in order to develop a versatile tool to study morphogenesis and demonstrate totipotency, this technique has developed into a powerful research tool, and has found application in a wide range of plants. Today *in vitro* cultures have largely surpassed the mere stage of being a laboratory technique and large numbers of commercial laboratories actively use the technique of plant tissue culture for the rapid clonal multiplication of plants.

Methods that are theoretically available for the propagation of plants *in vitro* are numerous, but consist essentially of either multiplication of shoots from axillary buds, or the formation of adventitious shoots and or somatic embryos via a direct or indirect (callus) route. The aim of this research was to compare these various tissue culture methods in order to establish; the most suitable technique for the propagation of three indigenous monocotyledonous genera, to illucidate the various problems inherent in plant tissue culture and to establish basic guidelines for the successful establishment of *in vitro* cultures.

Perhaps the results from the study can best be summarized in the following two figures (Figures 3.44 and 3.45). For successful sterilization of explants an integrated approach is necessary. Firstly the "history" of the mother plant must be established, to ensure that it is disease free, genetically stable and desirable to the public. This is followed by the implementation of stage 0 (DEBERGH and MAENE, 1981) in which the plants are carefully removed from their natural habitat and transplanted into sterile soil, subjected to regular treatments with fungicides and pesticides, with particular attention being paid as to how the plant is watered (avoid excess wetting of the leaves). Implementation of stage 0 in *Gloriosa* and *Sander-*

Figure 3.44 Multiple corm production in Gloriosa

Figure 3.45 Clivia miniata var citrina in vitro

sonia plants improved explant sterilization by 60%. To establish aseptic cultures an optimum sterilization technique should be established for each species and explant, as a single sterilization technique may be insufficient or damaging to the specific explant. Hydrogen peroxide was ideal for fruit and flower explant sterilization and is easy to remove, however the percentage decontamination for corm explants was low. Mercuric chloride is a very efficient sterilant, but has a drawback in that if the chemical is not removed after sterilization explant vigour can be seriously affected. The best all-round sterilant is sodium hypochlorite which is cheap and readily available as a commercial bleach.

A number of features must be taken into account when choosing an explant, the physiological or ontogenetic age and explant size. The environment of the mother, the time of the year at which the explant is taken, the light regime under which the plant is grown and the water and nutritional status, are all important. The selection of an explant should be carried out systematically, BOXUS and DRUART (1980) feel that the selection of a suitable explant is very important as it not only conditions the success of the first phase of multiplication, but also the whole future of the culture. From the results obtained for Clivia, Gloriosa and Sandersonia it is apparent that the ideal explant origin varies from genus to genus. However, it must also be stated that similar plants (the same genus or closely related within a family) often show remarkably similar trends in culture when taken from the same organ at a comparable time of the year. The best source of explant for Clivia culture was floral and fruit tissue, a feature that was also found by MIN and JINSHENG (1984) for Clivia nobilis. In the case of Gloriosa and Sandersonia the best source of explants was corm tissue and the reaction in culture of the two species was almost identical, provided the explants were taken at the same stage of plant growth. The culture environment necessary for maximum growth was the same for Gloriosa and Sandersonia but different for Clivia. Although reference to previous publications gives an "idea" in which direction to aim ones research, they can also be misleading in that often the specific technique / explants used are not reported (a major advantage would be obtained if, when publishing data, more specific information should be reported, such as the "history" of the mother plant, its age, the season of the year in which the culture was performed, pretreatments, etc.). The success or failure of tissue culture is often determined in the greenhouse prior to the sterilization of the plant material.

Callus tissue can be derived from numerous sources depending on the status of the explant, and subsequent callus performance can be related to the origin of the callus (viz. the difference in callus growth, on the same media, at the same time of the year for <u>Gloriosa</u> and <u>Sandersonia</u>). Although callus provides an ideal system to investigate nutritional and environmental responses of plant tissue it may have major drawbacks. Morphogenetic potential of the callus may diminish over long periods in culture, and genetic aberrations may result in long term culture (the creation of somaclones is important in that it allows for the production of new clones for breeding purposes). Results from callus cultures of all three species were disappointing, and relatively few plants were derived during subculture experiments. However, the determination of a suitable growth medium, subculture times, light and temperature regimes as well as suitable hormone combinations could be established using callus (derived from the three species) as a form of bioassay.

Although the hormones were able to enhance explant growth reactions in culture, the reaction of the initial explant was primarily dependent on the physiological status of that explant. The hormone effects could be related to the different types of explant.

MURASHIGE and SKOOG's (1962) nutrient medium provides a good all-round nutrient medium for the successful establishment and maintenance of most tissue cultures. Although there is a lot to be said for the use of the easy measurable quantities of a Schenk and Hildebrandt medium, the availability of Murashige and Skoog medium as a premix, makes the medium a suitable choice for commercial institutions. Light and temperature can play a significant role in culture growth, however, use of the standard 25°C and a 16-hour light, 8-hour dark cycle or continuous light provides a more than adequate culture environment for the majority of plant tissue cultures. Light and temperature do however play a significant role in seasonal variation of explants, with better performing explants being derived during the spring and summer months. By manipulation of the culture environment a seasonal response can be induced in vitro to hasten the formation of storage organs in Gloriosa and Sandersonia cultures.

Unfortunately the techniques of protoplast and anther culture were not sucessfully applied to the three study plants, but the utilization and establishment of these types of culture will have a major impact on the selective breeding of these valuable petaloid monocotyledons.

DEBERGH (1987) stated that micropropagation is probably the tissue culture technique with the largest economical emanation; however, there are still a large number of problems which hamper the full economical expansion of this "powerful" technique, namely the problems of repeatability, explant origin, abuse of the adjective disease free and hardening off. "When selected mother plants are used their physiological and sanitary status still have considerable influence on the repeatability and reliability of a system" (DEBERGH, 1987). In some cases micropropagation systems have been developed based on an occasional success in Stage I. Often repeatability of culture systems from published papers meet with failure, largely due to the factors mentioned above. A great deal more success would be achieved if these reports were more specific, especially when discussing the origin of the mother plant. Too often the details of explant origins are ignored with the classic "Explants taken from the study species were sterilized in ... " etc. It is advocated that the explant origin should be better specified, taking into account the "history" of the mother plant, its size, the ambient temperature, time of year, the age and physiological status. Reports of this nature would lead to an improvement in the repeatability of a technique. There is one major drawback however, which boils down to the "attitude" of the researchers themselves. Although the following quotation was specifically aimed at the establishment and production of secondary metabolites in culture, it is very significant.

> "The problem of assessing causes for changes in the ability of plant cell cultures to synthesize and accumulate secondary metabolites is aggravated by the simple fact that one source material in the hands of two experts may give rise to two kinds of callus and cell suspension cultures, producing and non-producing callus even though the protocol for tissue isolation, callus formation and subculture appear to be rather similar" (KURZ and CONSTABEL, 1985).

For the establishment of a successful tissue culture procedure an integrated approach to all aspects of the tissue culture technique is necessary. Sterilization techniques should be empirical and specific for each species and each type of explant. Probably the most crucial factor in establishing a tissue culture procedure is the choice of the explant. Many researchers still do not pay enough attention to the origin of the explant material or the type of propagation method used to establish the culture so as to guarantee conformity of the culture. Further refinements to the procedure can then be attempted by establishing optimum hormone combinations, light and temperature regimes, subculture period and nutritional supplementation.

The highly sought after Clivia miniata var citrina can be successfully cultured using fruit and floral explants. Although this procedure may limit the number of plants produced in culture due to the seasonal nature of flowering, approximately 30 plantlets can be produced on a single "slice" of fruit wall material. The subsequent hardening off stage has a success rate of 80%, partially due to the unique nature of the Clivia cuticle. Gloriosa superba and Sandersonia aurantiaca can be rapidly propagated using corm explants as starting material. The in vitro stimulation of cormlets is the most successful technique (with an exponential nature in the number of plants / corms produced) with the subsequent hardening off being remarkably simple. Limited plant regeneration can be achieved using callus cultures, provided that the callus has not been subjected to extended periods of subculture. The potential of anther and protoplast culture can not be understated, however to establish this type of culture involves a long term research project.

CHAPTER 4

CHARACTERIZATION OF Clivia miniata AND Clivia miniata var citrina

4.1 Introduction

Clivia miniata bears an inflorescence with red to orange flowers, but the most valuable form of Clivia is the yellow flowering Clivia miniata var citrina. The history of the two types of Clivia miniata has been discussed in Chapter 2, with the colour difference being shown in Figure 2.1. POLE EVANS (1931) recorded that

> "... 'all plants raised from seeds' of Clivia miniata var citrina have yellow flowers indicating we are dealing with a pure strain but except for the colour of the flowers we have not been able to detect any tangible characters which would separate it from Clivia miniata and have therefore kept it as a yellow variety."

DUNCAN (1985) reported that many different forms of var citrina are in cultivation. The origin of these plants is uncertain, and whether these plants were collected in the wild or were a result of hybridisation in cultivation, is unknown. DUNCAN (1985) continues; "there are a number of reports of the forms of the var citrina which do come true to type from seed, while there are as many to the contrary." The breeding of Clivia is complicated by a long maturation period of five to seven years, poor flower quality and poor vegetative growth from selfed plants. The application of an in vitro technique not only allows for multiple plant production, but also facilitates the breeding of the species.

Plantlets of var citrina were produced in culture from mother tissue (Chapter 3). However, variability is often observed after plant propagation in vitro. It is now widely accepted that pre-existing genetic variability in non-meristematic explants and changes in genetics during callus growth, mainly accounts for the variability found in plant tissue cultures (somaclones) D'AMATO, 1978; LARKIN and SCOWCROFT, 1981). Cells may become polyploids due to endoreduplication, selective stimulation of polyploids, chromosomal aberrations and nuclear fragmentation (MURASHIGE and NAKANO, 1965; D'AMATO, 1978; KASHA, KOTT and SEGUIN-SWARTZ, 1982; THORPE, 1982). There are also however, examples where a high degree of uniformity in plant regenerated from callus has been recorded (KRIKORIAN, STAICU and KANN, 1981).

In the light of the possibility that genetic variation may have occurred during var citrina tissue culture, it was decided to take a brief look at the basic morphology and biochemistry of the two types of plants. This research was performed in order to determine whether there was any recordable differences that can be used to identify the two types of plants after passage in culture. Bearing in mind that Clivia has a long maturation period of five to seven years, the use of an easily identifiable feature to assist the grower would be advantageous especially when marketing "unknown" plants produced in culture.

"It is frequently difficult to distinguish between cultivars of ornamentals on the basis of morphological characters, especially when not flowering" (DE LOOSE, 1979). In order to assist identification,

> "... criteria based on chemical, biochemical and enzymatic methods have been introduced with the aim of 'fingerprinting' cultivars. Flavanoid chemical markers determined by the technique of high performance liquid chromatography and soluble protein and isoenzyme banding patterns are among the best known. Mention should also be made of the scanning electron microscope to determine the surface ultrastructure characteristics of pollen grains, bud scales and leaves." (DE LOOSE, 1979).

Based on the above quote a brief investigation was undertaken to determine whether there was a discernable difference between the two varieties of *Clivia miniata*.

4.2 Material and methods

4.2.1 Plant material

The collection and cultivation of *Clivia* plants is outlined in Section 3.2.1.

4.2.2 Electron microscopy

Electron microscopy of the plant material was performed as outlined in Section 3.2.13.

4.2.3 Peroxidase activity

Dr P. Brain (Botany Department, University of Natal, Pietermaritzburg) assisted in the testing of the peroxidase activity and its isoenzyme patterns in leaves of *Clivia miniata*.

4.2.5 High performance liquid chromatography and spectrophotometry.

Extracts were made from the leaves and flowers of both varieties of *Clivia*. The extraction techniques for anthocyanin and flavanoids was performed according to the procedures outlined by HARBORNE (1973). Extracts were subjected to high performance liquid chromatography (HPLC) using a Varian 5000 HPLC. The following conditions were used for identification.

column : Hypersil 5 ODS, 250 x 4 mm ID

solvent : methanol : 0,2 M acetic acid buffered with triethylamine pH 3.5.

0 : 100 to 100 : 0 over a 60 minute period, or
5 : 95 to 50 : 50 over a 90 minute period.

flow rate : 1 ml min^{-1}
pressure : ± 170 atmospheres
sensitivity : 0,05
detector : 254 nm.

Scans of the extracts were performed using a Varian UV 90 spectrophotometer using a scan range from 800 - 200 nm.

4.3 Results and discussion

Results from electron microscopy studies are presented in Figures 4.1 and 4.2. There appears to be very little variation in the surface ultrastructure of Clivia leaves (Figure 4.1). The use of stomata or trichome arrangement would provide an ideal system for plant identification as it does not require complex techniques. A standard check chart could be used by the plant breeder to determine the different plant varieties. Pollen studies of Clivia miniata (Figure 4.2) show small amounts of variation in exine patterning. In general the sculpturing on the exine of pollen taken from the "red" Clivia (Figure 4.2a and b) was larger than that found on the pollen of the "yellow" variety (Figure 4.2c and d). However, a large amount of variation in the "size" of exine sculpturing was observed when taking pollen from flowers of different ages. Thus the use of exine sculpturing cannot be used as an identification criteria. The use of pollen as an identification feature is useless, as the reason behind the research was to find an identification feature prior to flowering (preferably using the leaf when it is produced in culture).

Peroxidase activity in the leaves of Clivia was not recorded. DE LOOSE (1979) found that soluble proteins and peroxidases were not useful for characterisation of Rhododedron cultivars and that only esterase isoenzymes were sufficiently

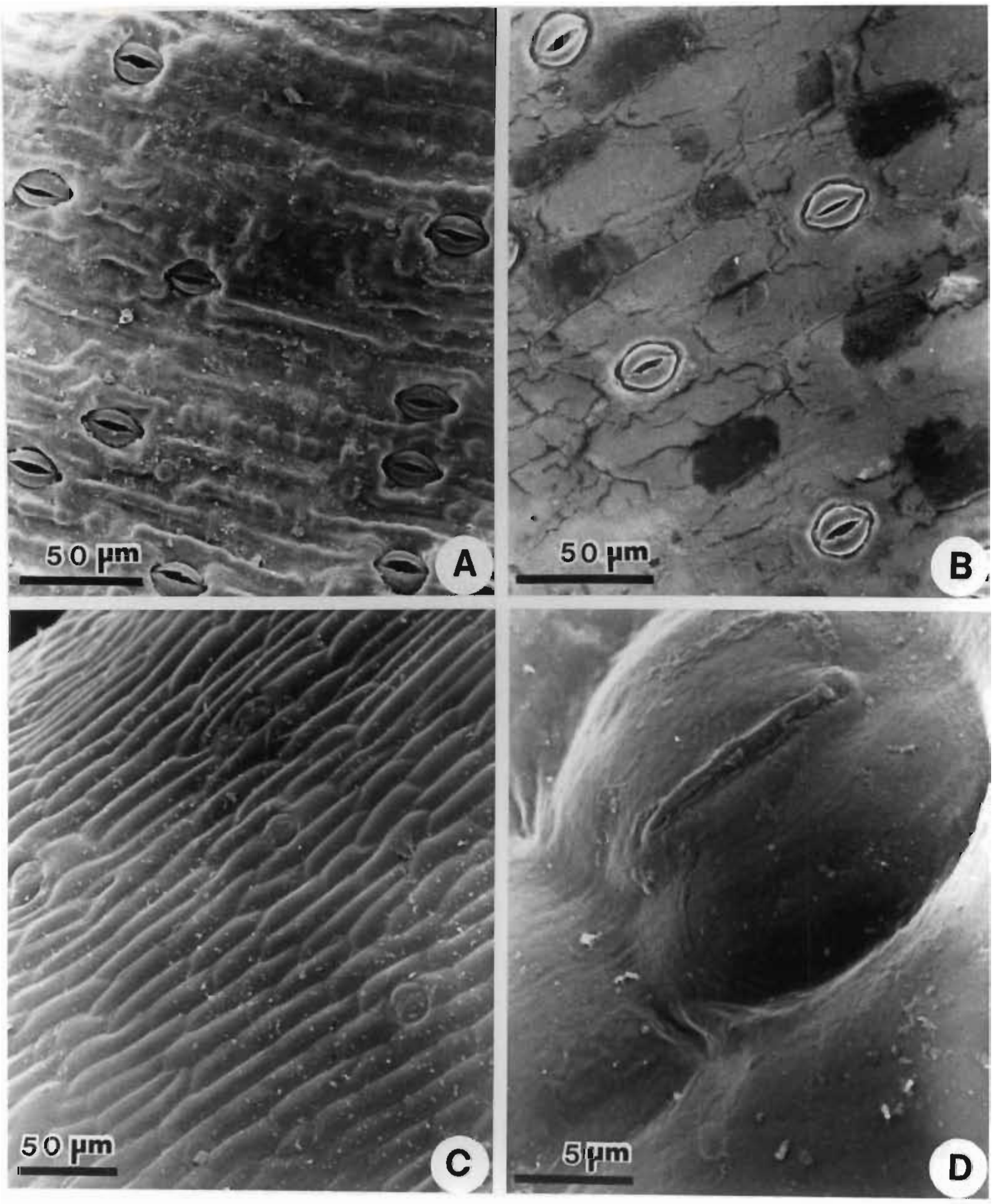

50 µm
A
50 µm
B
50 µm
C
5 µm
D

Figure 4.1 Surface structure of Clivia leaves

a) stomatal pattern of the "red" Clivia
b) stomatal pattern of the "yellow" Clivia
c) stomatal pattern of the "yellow" Clivia leaf produced in vitro
d) non functional stoma of Clivia leaf produced in vitro

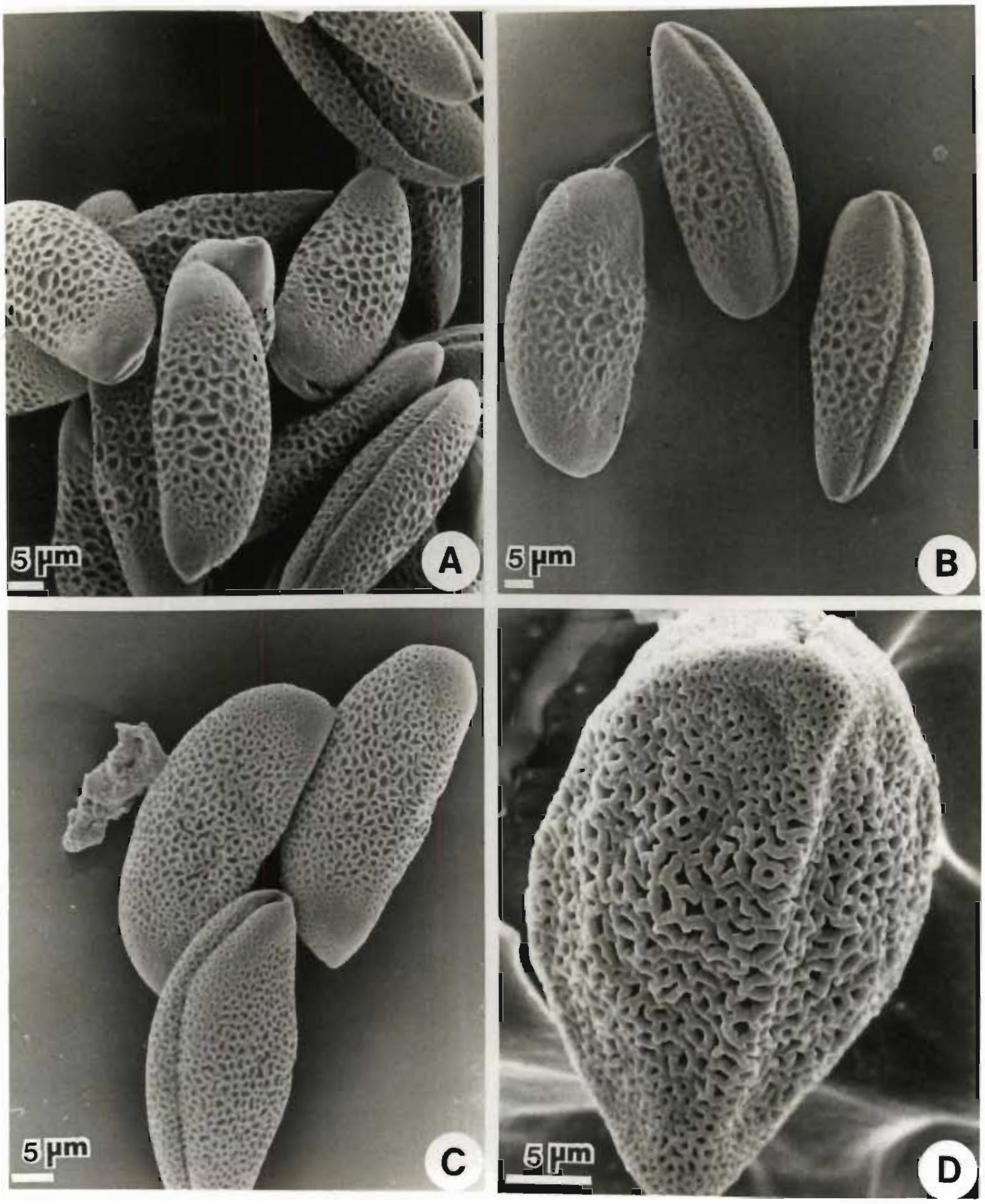

5 µm
A
5 µm
B
5 µm
C
5 µm
D

Figure 4.2 Clivia pollen

a and b) "red" Clivia pollen
c and d) "yellow" Clivia pollen

polymorphic to distinguish the cultivars. Peroxidase activity and its isoenzyme patterns alter with changes in plant development. Qualitative and quantitative differences in peroxidase isoenzymes in organised and unorganised tissues indicates their role as an indicator of differentiation (WOLTER and GORDON, 1975). Use of differing ages of Clivia leaves may give more positive results for peroxidase activity. PATIENCE and ALDERSON (1987) developed a system to study peroxidases during the rooting of lilac shoots produced in vitro. In Lilium, bulb scales show a high activity for general proteins, while leaves have a pronounced peroxidase activity (VAN TUYL, VAN DIJK and VAN RAAMSDONK, 1986).

Although peroxidase activity was not recorded other compounds can be analysed for (esterase, soluble proteins) (DE LOOSE, 1979). The use of isoelectric focusing has been used by VAN TUYL, VAN DIJK and VAN RAAMSDONK (1986) for the identification of wide interspecific hybrids in their early stages of development, and the characterisation of species to determine relationships within the genus Lilium.

There are drawbacks in the use of electrophoretic techniques in that specific equipment and trained technicians are required to perform the technique. However, although Clivia leaves used for identification did not show peroxidase activity, the utilization of this technique should not be ruled out. Isoenzyme analysis allows for the identification of nearly all genotypes and can be done for nearly all loci in all growth stages.

HPLC traces of Clivia petal and leaf extracts are shown in Figure 4.3 and 4.4. There is a difference in the quantitative aspect of the traces, but there does not appear to be any significant differences in the qualitative aspects of the HPLC traces. The extracts from both vegetative and reproductive tissue of the two

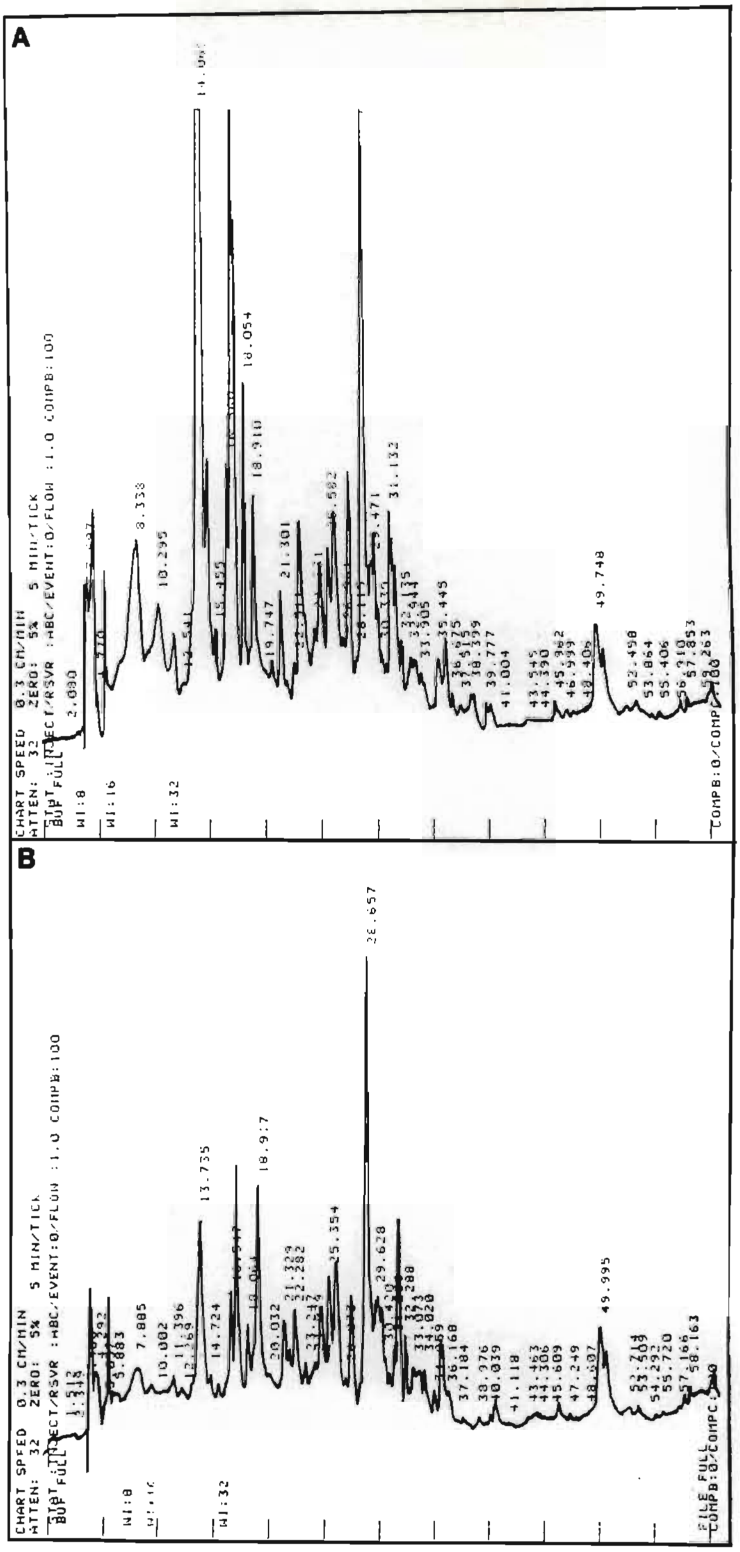


Figure 4.3 HPLC traces of extracted Clivia petals

a) "red" petal

b) "yellow" petal

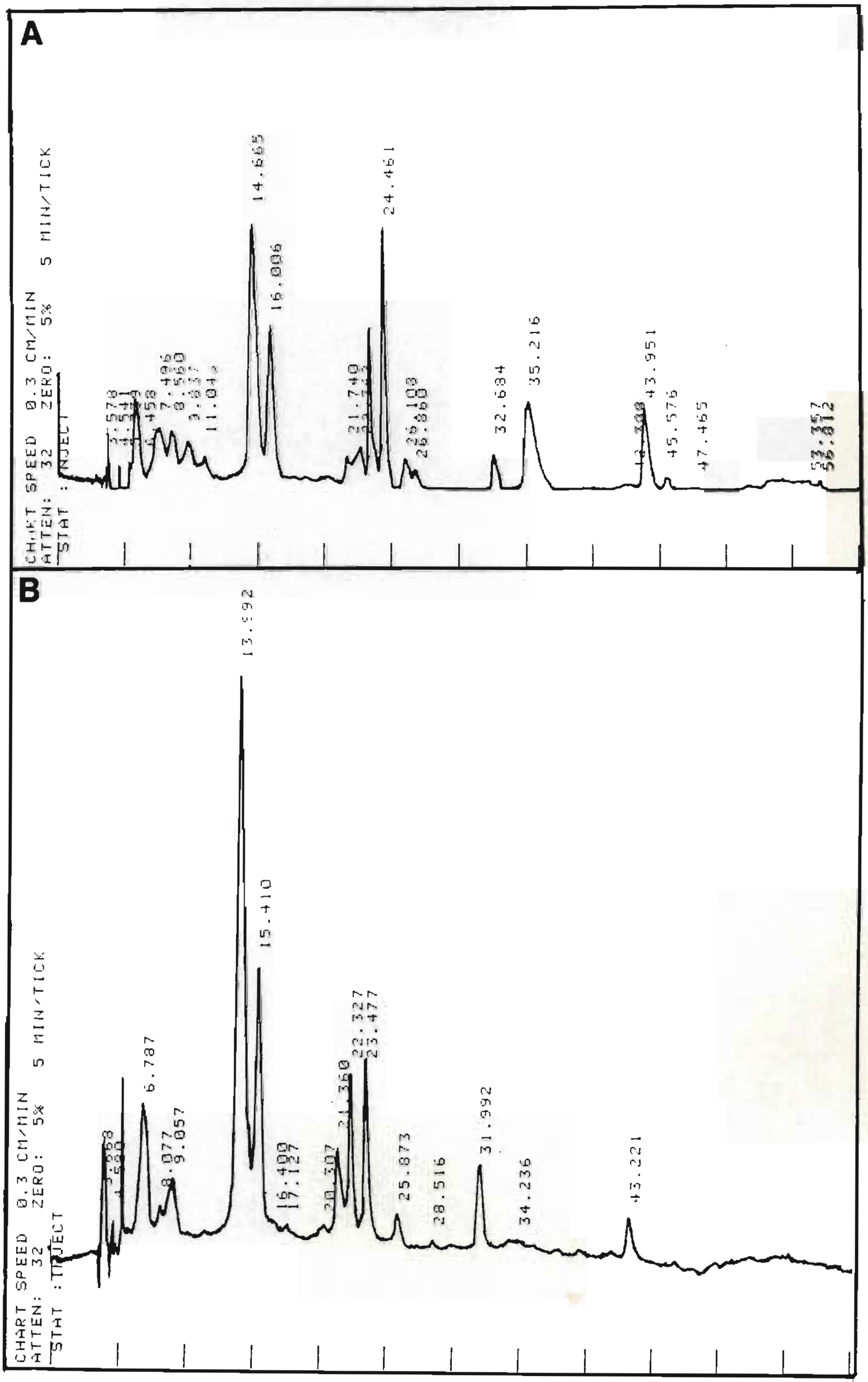


Figure 4.4 HPLC traces of extracted Clivia leaves

a) "red" leaf

b) "yellow" leaf

varieties of Clivia are remarkably similar when identified on the HPLC with the detector set at 254 nm. Scans of extracts made from vegetative tissue of the two varieties using the spectrophotometer, also show very little qualitative differences in the two traces.

Although very little success was achieved using the described techniques, the utilization of these techniques for plant identification cannot be understated. Further study into the characterisation of the two Clivia varieties is recommended.

4.4 Conclusions

Despite the lack of positive results, the use of these identification techniques should be pursued to investigate the difference between Clivia miniata and Clivia miniata var citrina. From personal experience and communication with growers of yellow clivias, it is apparent that there are no noticeable distinguishing characteristics (leaf colour, size, etc.) that discern the two types of plants from one another. Selfed plants of var citrina are generally slow growing with smaller amounts of chlorophyll in their leaves. However, the parents are normal vigorous plants. Variation in gross morphology of the plant can often be ascribed to the conditions under which they are grown (plants grown in shade flower better than plants grown in sun).

More detailed studies into the biochemistry and anatomy of the two varieties is advocated. The use of more specific extraction for flavanoids and anthocyanins, paper and thin layer chromatography (both one and two dimentional) are also recommended (VAN STADEN and BROWN, 1970). The best avenue however, to pursue is the technique of electrophoresis, with attention being paid to the soluble protein and esterase activity. Investigation and comparison into the varieties of alkaloids formed in Clivia could also be performed, e.g. cliviasindhine, a new

Lycorine alkaloid (GRUNDON, 1985) or clivonidine, clivojuline, cliviahaksine or cliviaaline (GRUNDON, 1984). DAHLGREN, CLIFFORD and YEO (1985) reported that the Amaryllidaceae are easily characterized chemically, with many alkaloids not known to occur in other plants being constantly found in this group of plants. These compounds are said to be produced due to the over production of tyrosine (HEGNAUER, 1963).

> "Except for the colour of the flower" and the ripe fruit "we have not been able to detect any tangible characters which would separate it from Clivia miniata and have therefore kept it as a yellow variety " (POLE EVANS, 1931).

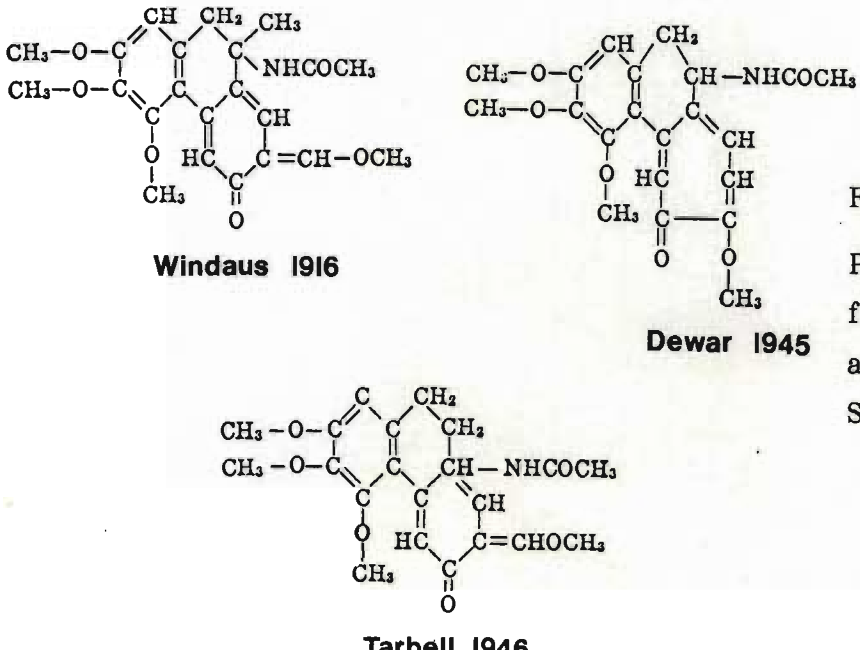
Preliminary results from the above experimentation would back up the statement by Pole Evans. However, subsequent research should be able to pinpoint a means of identifying the two varieties of Clivia. From this, one should be able to classify var citrina as a true variety, or merely a colour variant (hybrid).

CHAPTER 5

COLCHICINE, AND THE PRODUCTION OF SECONDARY PLANT METABOLITES IN TISSUE CULTURE

5.1 Colchicine

The extracts of Colchicum species were first referred to in 1550 B.C. by the Egyptians (EIGSTI and DUSTIN, 1955). In 1819 Pelletier and Caventou extracted a substance with basic properties from Colchicum autumnale (the meadow saffron) which they regarded as veratrine. Geiger and Hesse in 1833 undertook research on this alkaloid and recognised it as a new alkaloid which they named colchicine. This compound was not a homogeneous substance and Hübler (1864) was the first to isolate the compound in "a condition of purity" (PICTET, 1904). Colchicine was recorded in the bulbs at a concentration of 0,2%, and 0,4% in the seeds. Zeisel gave colchicine the formula $C_{22} H_{25} NO_6$, Hübler gave the composition $C_{17} H_{19} NO_5$ and Hertel and Bender $C_{17} H_{23} NO_6$ (PICTET, 1904). "The structural formula of colchicine $C_{22} H_{25} 0_6 N$ is not absolutely certain yet." (HAMERSLAG, 1950), with Figure 5.1 showing possible structural configurations.



Windaus 1916

Dewar 1945

Tarbell 1946

Figure 5.1

Possible structural configurations of colchicine, adapted from HAMERSLAG (1950).

The structural formula of colchicine is now known to be $C_{22}H_{25}O_6N$ with the following arrangement (Figure 5.2).

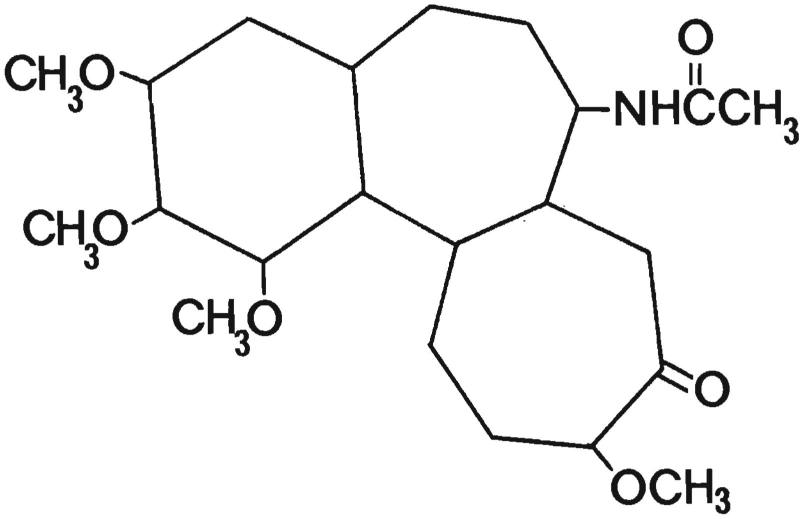


Figure 5.2 Structure of colchicine

CLEWER, GREEN and TUTIN (1915) reported that Warden in 1880 and 1881 made a study of the 'drug' present in the tuber Gloriosa superba and found that the tuber contained a "neutral", bitter principle (superbine), salicylic acid, a fluorescent principle and three resins. "Superbine" was thought to be related to the "bitter principle of squill", a fatal dose of superbine for a full grown cat was 0,0107 g. Determination of the content of superbine gave a suggested formula of $C_{52}H_{66}O_{17}N_2$ which was "put forward with reserve" (CLEWER, GREEN and TUTIN, 1915, WATT and BREYER-BRANDWIJK, 1962). CLEWER, GREEN and TUTIN (1915), using the dried tubers of Gloriosa superba collected in Ceylon, isolated an enzyme which readily hydrolysed amygdalin and a considerable amount of an alkaloid. The mixture of alkaloids consisted mainly of colchicine $C_{22}H_{25}O_6N$ with an amount, estimated using the method of the United States Pharmacopoeia, of 0,3%. SUBBARATNAM (1952; 1954) using Gloriosa tubers divided the alkaloid fraction into colchicine mp 151 - 152° and a new alkaloid Gloriosine ($C_{22}H_{25}O_6N$

mp 248 - 250°) with a concentration of 0,1%. SARIN, JAMWAL, GUPTA and ATAL, (1974) and THAKUR, POTESILOVA and SANTAVY (1975) reported the presence of colchicine from Gloriosa, the neutral phenolic fraction giving a total of 24 alkaloids and small amounts of basic alkaloids of a non-tropolone nature. Table 5.1 lists the chemicals isolated from Gloriosa by THAKUR, POTESILOVÁ and SANTAVY (1975).

Table 5.1 Neutral and phenolic alkaloids isolated from Gloriosa superba, adapted from THAKUR, POTESILOVÁ and SANTAVY (1975)

Alkaloid	Distribution in the plant		
	Corm	Seed	Leaves and flowers
β-lumicolchicine	*		*
N-Formyl-β lumicolchicine	*		
Y-lumicolchicine	*		*
N-Formyl·Y·lumidesacetylcolchicine	*	*	*
Substance X-1			*
Cornigerine			*
Colchicine (Superbine)	*	*	*
Substance G-1	*		
N-Formyl-N-deacetylcolchicine (Gloriosine)	*	*	*
3-Demethyl-β-lumicolchicine	*		*
3-Demethyl-N-formyl-N-desacetyl-β-lumicolchicine	*		
3 Demethyl-Y-lumicolchicine	*		
3-Demethylcolchicine	*	*	*
2-Demethyl-β-lumicolchicine	*		
2-Demethyl-N-formyl-N-desacetyl-β-lumicolchicine	*		
3-Demethyl-N-formyl-N-desacetylcolchicine	*		
Lumiderivative	*	*	
2-Demethycolchicine	*		
2,3-Demethyl-N-desacetylcolchicine	*		*
2,3 Demethylcolchicine	*		
2-Demethyl-N-formyl-N-desacetylcolchicine	*		
3-Demethylcolchiceine	*		
2-Demethylcolchiceine	*		

The toxic properties of Colchicum were reported to have been known to Dioscorides, (a physician of Nero), who advocated its use as a cure for tumours not yet spread. Colchicum extracts were used as treatment for gout in the sixteenth century (DALTON, 1979). Preparations of colchicine have been used to cure acute gout, but colchicine is also an extremely toxic substance which has killed a human adult in a single dose of 3 mg, "but the minimum fatal dose is probably nearer 8 mg" (WATT and BREYER-BRANDWIJK, 1962).

Table 5.2 summarizes the lethal dose of colchicine for various animals.

Table 5.2 Lethal doses of colchicine adapted from STEYN (1934)

Animal		Lethal dose
Frog	15 - 20°C	1,2 - 2,0 g
	30 - 32°C	0,002 - 0,004 g
White mice		0,003 - 0,01 g
Rabbit		0,003 - 0,005 g
Dogs		0,001 g
Cats		0,0005 - 0,001 g

Colchicine is less effective on cold blooded animals than warm blooded ones. Colchicine is also used in the induction of polypoidy in plants, however, the effect of colchicine on plants and plant growth will be discussed later in this review.

Warden in 1880 (STEYN, 1934) recorded that Gloriosa roots are used in India medicinally to "cure" blood diseases, swellings, wounds, abscesses and pains. The action of colchicine is said to resemble veratrine, but "does not provoke sneezing on being brought in contact with the mucous membrane" (PICTET, 1904). The main use of colchicine lies in the treatment of gout. Acute attacks can be shortened considerably and lessened in intensity by the administration of colchicine, until gastro-intestinal disturbances appear. It is, however, not a cure (HAMERSLAG, 1950). The action of the colchicine is on the activity of leucocytes. Overdoses of colchicine, after a three to six hour delay result in lowering of the body temperature, depression of the central nervous system, "strangling" nausea, burning and rawness of the mouth and throat, vomiting and colic which is a result of the alkaloids' paralysing influence on the spinal motor and vasomotor nerves. Colchicine also produces temporary leucopaenia followed by leucocytosis. The colchicine is discharged into the gastro-intestinal tract resulting in inflammation of the mucous membrane. At death the body temperature falls, the central nervous system undergoes functional changes and respiration fails (HAMERSLAG, 1950; WATT and BREYER-BRANDWIJK, 1962). Porcupines in South West Africa seek out the roots of Gloriosa and eat them with impunity (WATT and BREYER-BRANDWIJK, 1962).

Susceptibility of cancer cells to X-ray treatment is increased by colchicine treatment and carcinomas have been healed or stopped; however, "this connection of cancer and colchicine is inconclusive" (HAMERSLAG, 1950). Colchicine affects both normal and cancerous cells, but is not selective, cells with high rates of division and metabolism are affected first (WATT and BREYER-BRANDWIJK, 1962). The biological effects of colchicine on mitosis and inhibition of tumours result from a disruption of the microtubule system, the effects are related to the binding of colchicine to α tubulin (DUMONT, BROSSI and SILVERTON, 1986). The action of colchicine on human malignant tumours, and leukemia, has shown to be of minor value (COOK and LOUDON, 1952; FELL and RAMSDEN, 1967). At present there is renewed interest in the use of colchicine as a possible cure for cancer related diseases (although the high toxicity of the alkaloid is a major stumbling block in colchicine utilization in therapy) (EVANS, TANIS and HART, 1981).

A number of reviews on the biosynthesis of colchicine have been published (PICTET, 1904; HAMERSLAG, 1950, BENTLEY, 1957). The following review of the biosynthesis of colchicine is based on the review of WILDMAN and PURSEY (1968). Figure 5.3 gives four examples of biogenic schemes proposed prior to tracer investigations.

Colchicum autumnale and Colchicum byzantium have been used in most studies. The degradation schemes used for the identification of carbon atoms and groups is represented in Figure 5.4.

The phenyalanine-cinnamic pathway has been shown to be involved in the elaboration of ring A and carbon atom C-5, C-6, and C-7 (HERBERT and KNAGG, 1986), the genesis of the tropolone ring was 'problematic'. It has been shown that the aromatic ring and the benzyl carbon atom of tyrosine is involved in the genesis of the tropolone ring.

> "An important clue to the actual biosynthetic pathway was the discovery that androcymbine (Figure 5.5) occurs along with colchicine type compounds in Androcymbium melanthioides. The similarity of androcymbine to CXVI (Figure 5.5) was striking, as well as the fact that androcymbine has the same absolute stereochemistry as colchicine. This suggested that a 1-phenethyliso-quinoline structure (and the dieone) is involved in the biosynthesis of colchicine" (WILDMAN and PURSEY, 1968).

The details of colchicine biosynthesis is outlined in Figure 5.6. The origin of the colchicine type of molecule falls in line with other types of alkoloids, "with a fortuitous combination of molecular circumstances appearing to be responsible for the production of the unusual tropolone moiety from a more normal isoquinoline structure" (WILDMAN and PURSEY, 1968; DALTON, 1979; HERBERT, 1985).

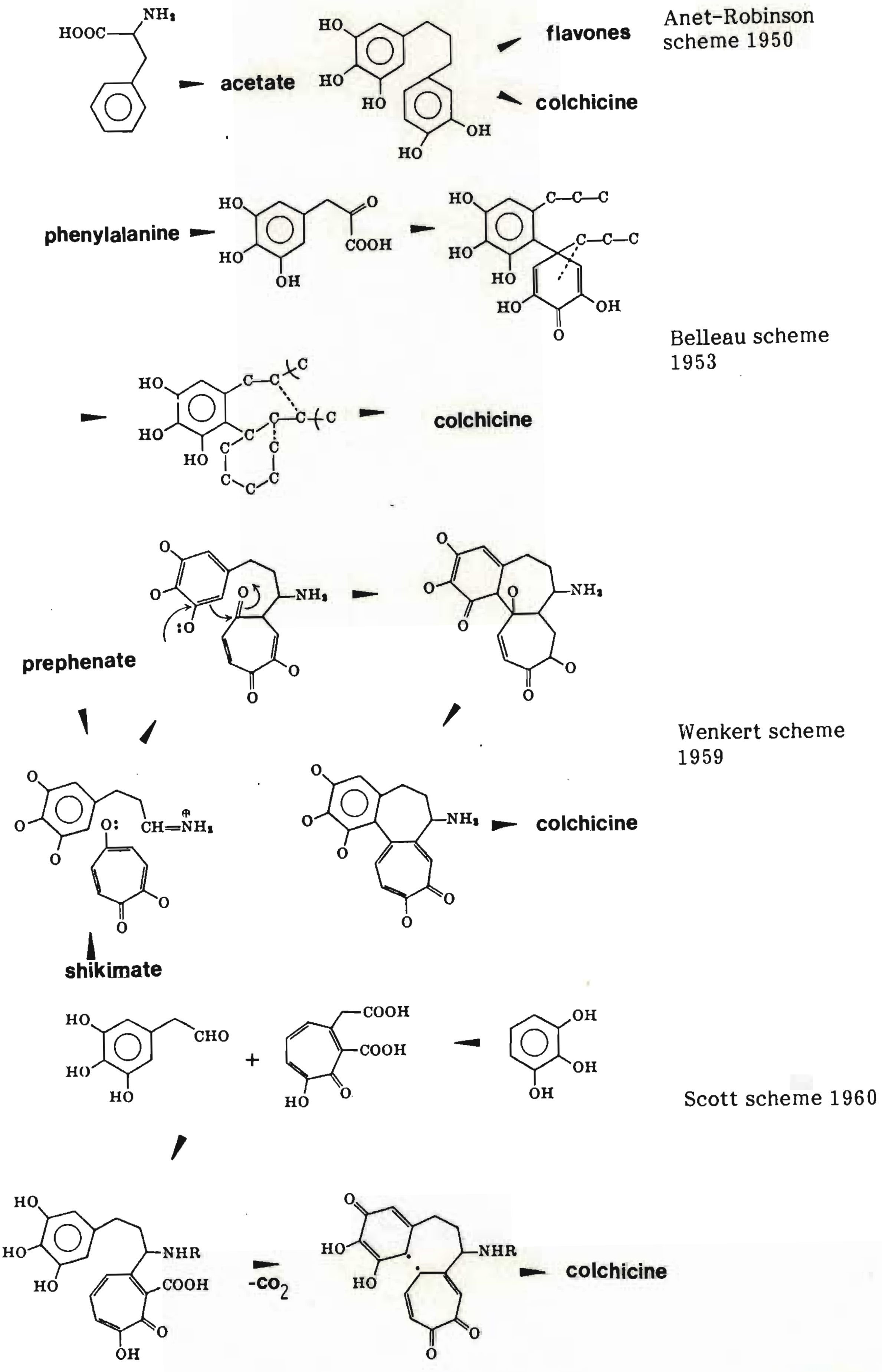


Figure 5.3 Biogenic schemes (adapted from WILDMAN AND PURSEY, 1968)

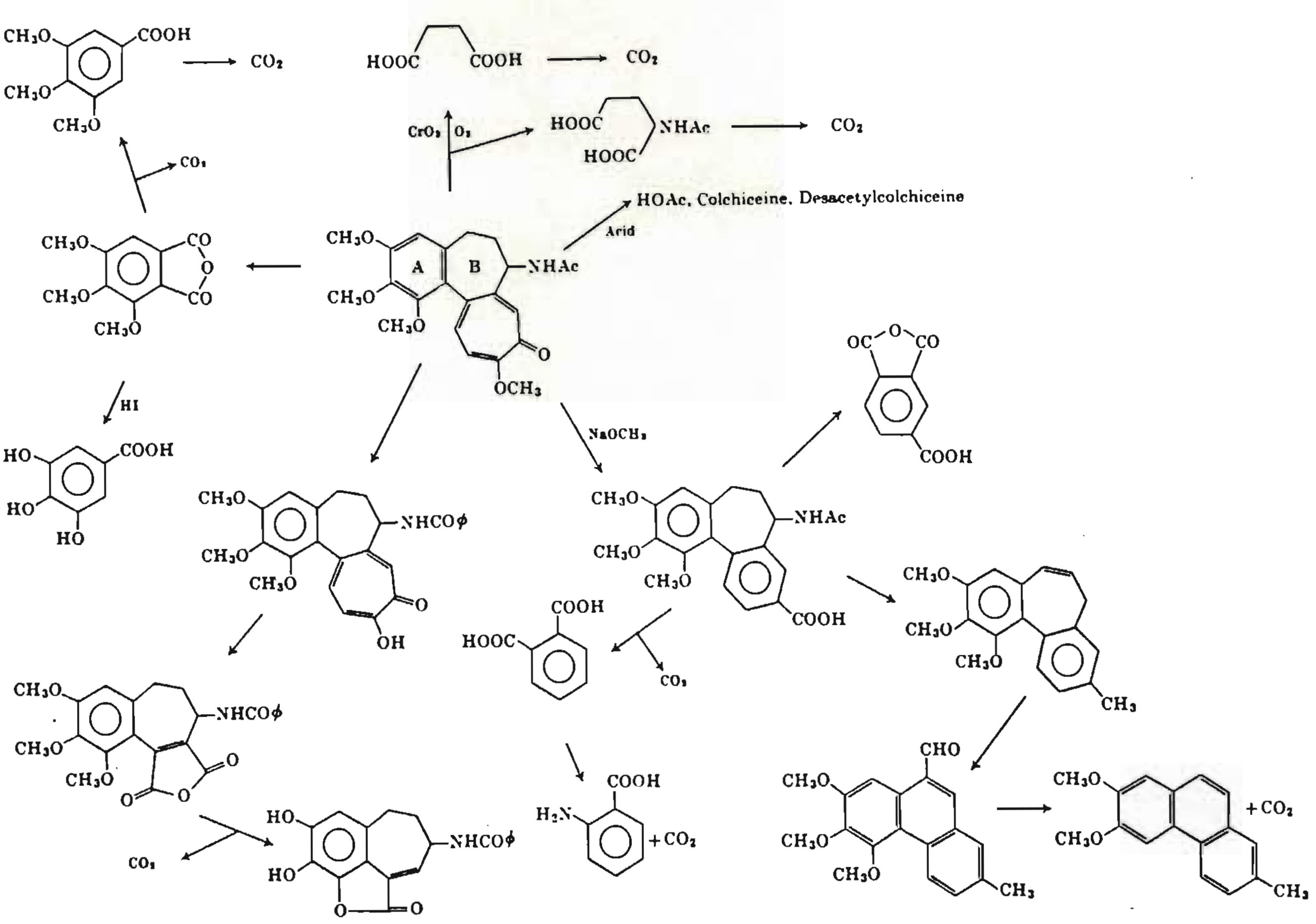
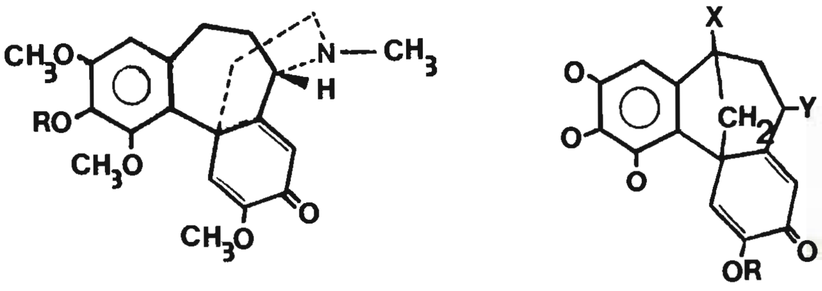


Figure 5.4 Degradation scheme of colchicine from WILDMAN and PURSEY (1968).



R=H

Figure 5.5 Androcymbine and CXVI

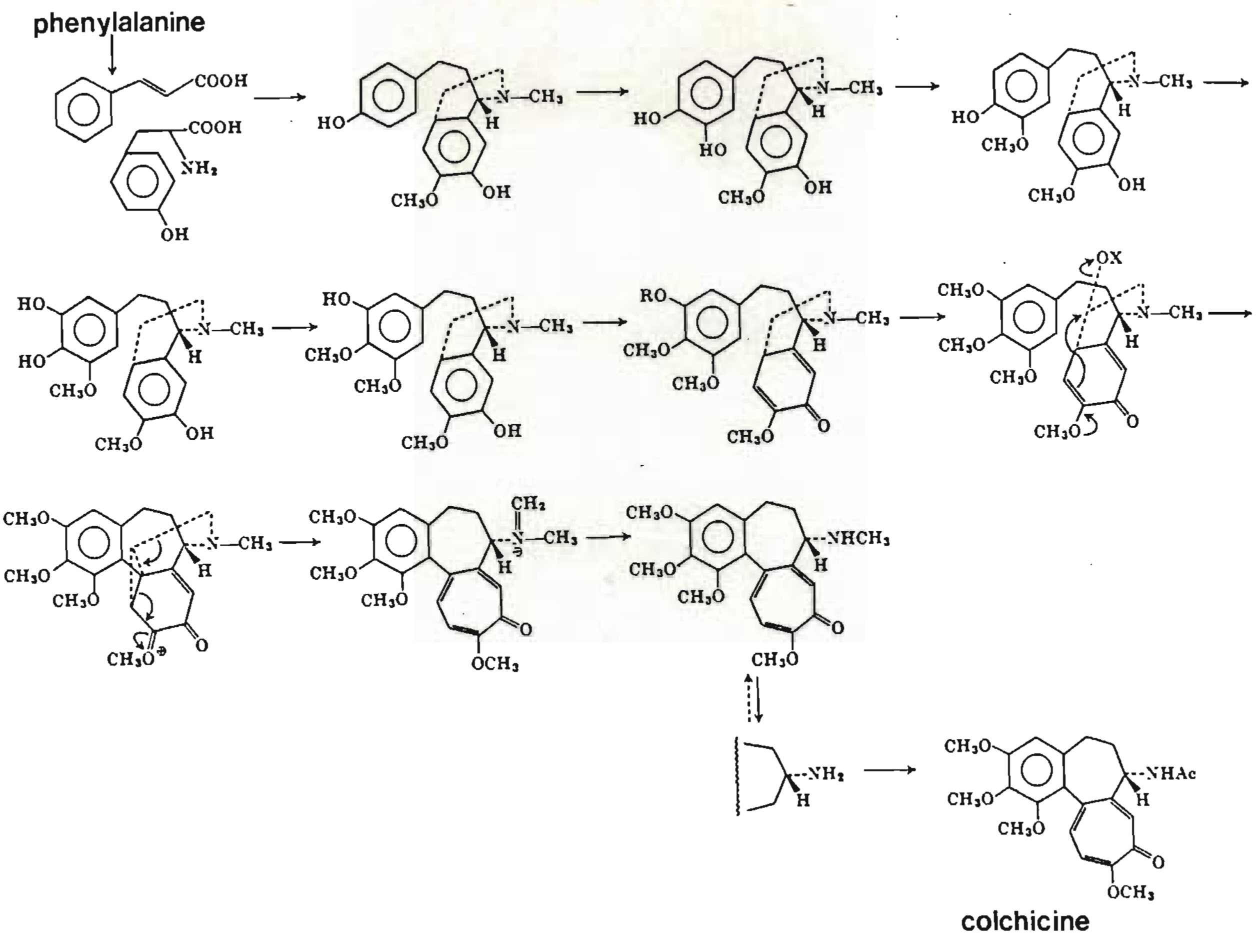


Figure 5.6 Biosynthesis of colchicine (adapted from WILDMAN and PURSEY, 1968).

Due to renewed interest in the pharmacology of colchicine, a number of researchers have published on chemical colchicine synthesis and the production of structural analogues, without using plant material as the source of colchicine (BLADE-FONT, 1977; EVANS, HART and KOELSCH, 1978; EVANS, TANIS and HART, 1981; BOGER and BROTHERTON, 1985; 1986). EVANS, HART and KOELSCH (1978) investigated the potential of tropolone synthesis and this resulted in an efficient synthesis of desacetamidoisocolchicine, a common intermediate in the synthesis of colchicine. EVANS, TANIS and HART (1981) reported that there had been ten total syntheses reported prior to their paper, "but in most cases the associated problems of colchicine synthesis were ignored". Conversion via allylic bromination of desacetamidoisocolchicine is inefficient and it normally only results in a 12% yield. In most cases synthesis proceeds through desacetamidocolchicine, and involves the intermediacy of the free tropolone colchiceine with "severe regiochemical problems" (EVANS, TANIS and HART, 1981). These authors' latest synthesis involves the utilization of cyclopropanated derivatives of a quinone monoketal as a tropolone dication equivalent. BOGER and BROTHERTON (1985) based a formal total synthesis of colchicine on the use of a

> "... thermal, four-carbon + three-carbon cycloadition of a α-pyrone with the cyclopropenone ketal in a process proceeding by way of the reversible thermal generation of a three carbon 1,3-dipole, best represented as a nucleophile and dilocalized singlet vinylcarbene".

BOGER and BROTHERTON (1986) using the above synthesis rationalised that the use of deacetamidocolchiceine en route to colchicine synthesis in initial studies, was based on the preparation of deacetamidocolchiceine and a deaetamidoisocolchicide, a compound with biological significance. BLADE-FONT (1977) and DUMONT, BROSSI and SILVERTON (1986), by racemization of natural colchicine derived a number of colchicimoids, the utilization of colchicimoids in treatment of disease, necessitates their production and preparation by efficient techniques which has been established by racemization of colchicine in refluxing acetic anhydride, after hydrolysis and chemical manipulation. Despite difficulties encountered in the synthesis of structurally related tropolones, chemical synthesis of colchicine has been achieved. At present commercial colchicine production relies on the extraction of the alkaloid from Colchicum.

It is pertinent to mention the significance of the congeners of colchicine. Initially all new colchicine derived substances were called after a letter of the alphabet depending on the chronological isolation of the compound (e.g. Substance B). CERNOCK, MALINSKY, TELUPILORA and SANTAVY (1954), tested natural and synthetic derivatives of colchicine for their toxicity. Colchicine is normally the alkaloid present in the highest concentration in the corm of Colchicum. Substance F (demecolcine, colcemid or colchamine) N-methyl desacetyl colchicine (Figure 5.7) is the next most abundant compound (FELL and RAMSDEN, 1967). Colchicoside is similar to colchicine with a glucose molecule attached (Figure 5.7). Lumicolchicine (Figure 5.7) is normally produced as a result of the conversion of colchicine by the action of light. Table 5.1 summarised the congeners found in Gloriosa.

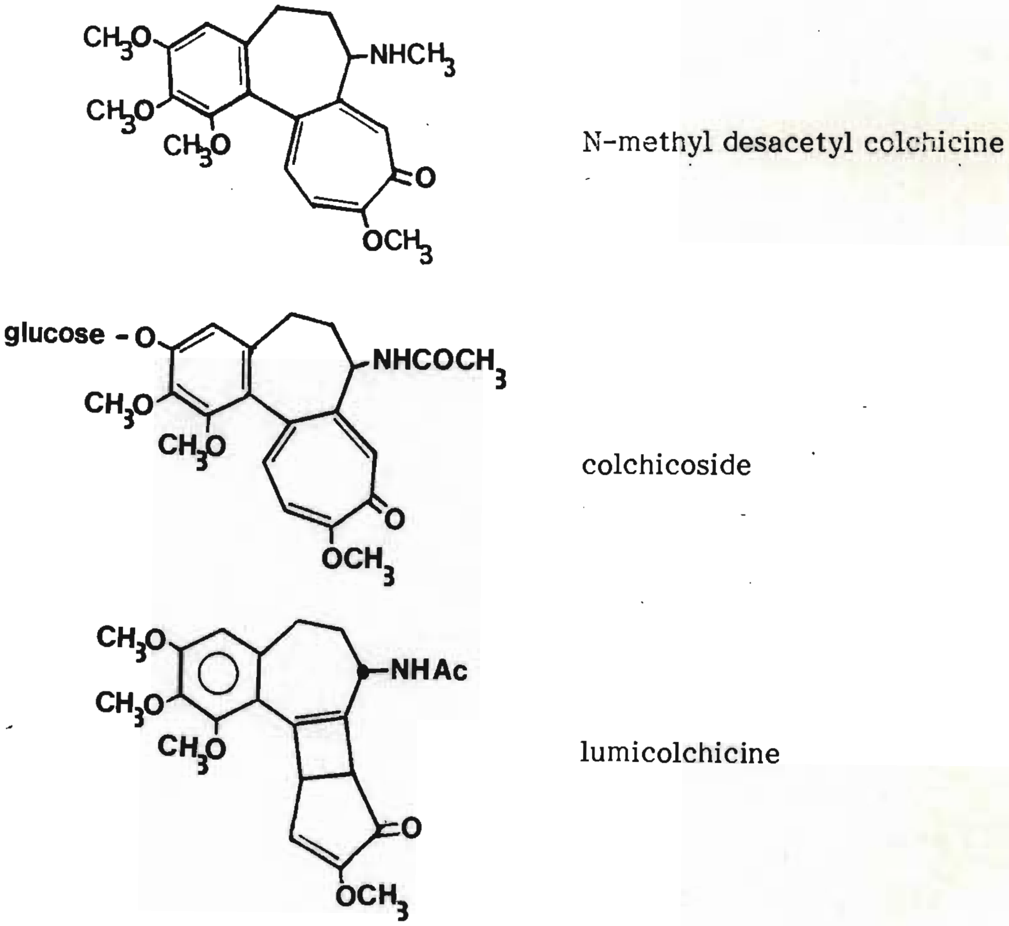


Fig.5.7 Chemical structures of N-methyl desacetyl colchicine, colchicoside and lumicolchicine

The following is a description of the manufacture of colchicine as detailed by HAMERSLAG (1950): Corms or seeds of Colchicum may be used, however, seeds are preferred because of their higher colchicine content. The plant material is ground and extracted in percolators. Denatured alcohol, methanol or dilute methanol is used for the initial extraction at ± 50°C, this is repeated four times, the solvent is evaporated off, water is added to the residue and allowed to cool. "Filteraid" is added to the solution which is then filtered. Lipid extraction is achieved by using petroleum ether. The "aqueous liquor" is extracted with chloroform or ethylene dichloride, the chloroform extract is retained and the aqueous fraction is extracted four or five times more. The chloroform/ethylene dichloride solution is dried by mixing with anhydrous sodium sulphate, and filtered, the clear filtrate is evaporated to a gummy residue. This solution is mixed with water and the water is removed under vacuum, ensuring all traces of chloroform are removed. The aqueous solution is placed in the cold and allowed to crystallize. Colchicine salicylate is made by mixing 74 parts colchicine and 26 parts salicylic acid. The National Formulary specifies a colchicine content of not less than 0,45% in seeds used for commercial manufacture, while the British Pharmacopoeia requires a minimum content of 0,3% in seeds and between 0,35% - 0,25% for corms. (Organic matter and ash is limited to 1% in commercial preparations of colchicine).

MALICHOVA, POTESILOVA, PREININGER and SANTAVY (1979), speculated that dried leaves and flowers could be used as a source for commercial colchicine production. MUNTEAN, SALONTAI, BOTEZ, CERNEA, CAREAN and TAMAS (1984), found that by planting 200 000 bulbs ha^{-1}, seed yields ranging from 22,5, 136,9 and 201,6 kg ha^{-1}, can be harvested over three years with an associated colchicine recovery of 56,2, 238,0 and 584,6 g ha^{-1}.

The meadow saffron or autumn crocus, <u>Colchicum autumnale</u> is the main commercial source of colchicine, the plant has an ovoid corm, one side is convex with the other side flattened. In autumn, lilac coloured flowers appear above the ground, later the flowers disappear and the leaves and stems with fruit appear, the seeds are derived from the capsule in large quantities (HAMERSLAG, 1950; CROMWELL, 1955; WILDMAN and PURSEY, 1968). Most <u>Colchicum</u> species contain colchicine, <u>Colchicum cornigerum</u> is the only plant shown to contain colchicine-like compounds without colchicine. Table 5.3 contains a summary of *Colchicum* species studied for their colchicine content and the plant part in which the alkaloid was isolated.

Table 5.3 Plants from which colchicine has been extracted (excepting *Gloriosa*, *Sandersonia* and *Littonia*) (* indicates traces only)

Species	Plant part	Colchicine % yield	Reference
Colchicum autumale		veratrine	Pelletier and Cavetou, in 1819
		colchicine	Geiger and Hesse, in 1833.
		colchicine	Hübler, 1864.
	corm	0,2%	PICTET, 1904.
	seed	0,4%	
	flower	0,8%	NIEMANN, 1933
	pericarps	0,16%	BLAZEK and SLOUF, 1949
	flowers	0,292%	SANTAVY, 1950
	corm	0,38 - 0,4%	CROMWELL, 1955
	seed	0,72 - 0,75%	
	corm	0,035%	WILDMAN and PURSEY, 1968
	seed	0,3%	
	flower	0,033%	
	corm	0,23 - 0,31%	GASIC, PETROVIC and CANAK, 1978
	flower	*	SEIFERT, 1979
	bud	*	
	corm	*	GLAVAC, KORNHAUSER and RAVNEK-GLAVAC, 1984
	seed		
	leaf	*	
	flower	*	

Table 5.3 (Continued)

Species	Plant part	Colchicine % yield	Reference
Colchicum alpinum		*	WILDMAN and PURSEY, 1968
Colchicum arenarium	corm	0,16%	GASIC and POPOVIC, 1980
	flower	0,29%	
Colchicum byzantium	corm	*	WILDMAN and PURSEY, 1968
	flower	*	
Colchicum cornigerum			WILDMAN and PURSEY, 1968.
Colchicum dörfleri	corms	*	GASIC and POPOVIC, 1977.
	flowers	*	
	stems	*	
Colchicum kesselringii	corms	*	WILDMAN and PURSEY, 1968.
Colchicum luteum	corms	0,21 - 0,25%	WILDMAN and PURSEY, 1968.
		0,26 - 0,27%	
	seeds	0,41 - 0,43%	
		0,40 - 0,44%	
	corm	0,4%	KOUL and THAKUR, 1977.
	aerial parts	0,2%	
	corm	*	MUNSHI and GOPA GHOSH, 1985.
Colchicum montanium	corm	*	WILDMAN and PURSEY, 1968.
Colchicum ritchii	corm	*	WILDMAN and PURSEY, 1968.
Colchicum turcicum	corm	*	BAYTOP, SUTLUPINAR and PHILLIPSON, 1980
	flower	*	

Other substances found in Colchicum species are: cornegerine, demecolcine, speciosamine, luteidine, luteinone, luteicine, kesselringine, colchiceine (WILDMAN and PURSEY, 1968; YUSUPOV and SADYKOV, 1976; MUKHAMED' YAROVA, YUSUPOV, LEVKOVICH, ASLANOV and SADYKOV, 1976; YUSUPOV, MUKHAMED' YAROVA and ASLANOV, 1976; CHOMMADOV and YUSUPOV, 1985; CHOMMADOV, YUSUPOV and ASLANOV, 1985).

Table 5.3 (Continued)

Species	Plant part	Colchicine % yield	Reference
Androcymbium melanthioides	corm	0,07%	WILDMAN and PURSEY, 1968.
	seed	0,12%	
	corm	0,07%	HRBEK and SANTAVY, 1962.
Bulbocodium	corm, leaves	*	WILDMAN and PURSEY, 1968.
	flowers	*	
Camptorrhiza	corms, seeds	0,023%	WILDMAN and PURSEY, 1968.
Iphigenia	corms	0,2%	WILDMAN and PURSEY, 1968.
	seeds	0,3%	
	aerial parts	0,006%	
Iphigenia indica	seeds	0,5 - 0,6%	KAPADIA, DEV, RAO and ANSARI, 1972.
Iphigenia pallida	seeds	0,5 - 0,6%	
Iphigenia stellata	seeds	1,2 - 1,9%	
Iphigenia		*	HEGDE and LUGADE, 1986.
Merendera		*	CROMWELL, 1955.
Merendera	whole plant	0,17%	WILDMAN and PURSEY, 1968.
Merendera persica	corms	0,02%	WILDMAN and PURSEY, 1968.
Ornithoglossum	corms	*	HRBEK and SANTAVY, 1962.

It was reported by CROMWELL (1955) that Klein and Pollauf, isolated colchicine from the genera Hemerocallis, Veratrum, Tulipa, Tofièldia and Ornithogalum?

Since the discovery of colchicine in Gloriosa by CLEWER, GREEN and TUTIN (1915), a number of researchers have isolated and quantified colchicine from Gloriosa and a number have put forward the idea that Gloriosa could serve as a commercial source of colchicine (EIGSTI and DUSTIN, 1955; WATT and BREYER-BRANDWIJK, 1962; NARAIN and KHOSHOO, 1967; SARIN, JAMWAL, GUPTA and ATAL, 1974; NARAIN and RAINA, 1975; SRIVASTAVA and CHANDRA, 1977; BELLET and GAIGNAULT, 1985). SARIN, JAMWAL, GUPTA and ATAL (1974) reported that seeds of Gloriosa contain 0,6% colchicine while Colchicum luteum contains only 0,25%. BELLET and GAIGNAULT (1985) compared the relative colchicine contents of Gloriosa and Colchicum (Table 5.4), as evidence for the commercial viability of Gloriosa as a source of colchicine.

Table 5.4 Relative colchicine content of Gloriosa and Colchicum, taken from BELLET and GAIGNAULT (1985)

	% Colchicine	Demethyl-3-colchicine	Colchicoside	Total
Colchicum	0,62	0,05	0,39	0,92
Gloriosa	0,9	0,19	0,82	1,64

It is evident from Tables 5.3 and 5.5 that colchicine concentration varies from organ to organ, it has also been shown that colchicine content may also be affected by plant age, seasonability and locality. One year old corms are recommended for use in commercial colchicine production, and the corms should be collected prior to flowering (HAMERSLAG, 1950). Seed size also may play a role in colchicine content (EIGSTI and DUSTIN, 1955). Bulbs and flowers of Colchicum were analysed for colchicine. It was found that the highest concentration of colchicine was found in fully opened flowers harvested in the early morning, leaf colchicine concentrations were highest in the morning or the evening (SEIFERT, 1979). WILDMAN (1960) reported that in spring when the leaves appear, the corm contains three times the colchicine content than does the corm collected in autumn, as the corm senesces the colchicine content decreases, while the colchicine content of the new daughter corm increases. GASIC, PETROVIC and CANAK (1978) found that soil Mn correlated with total alkaloid concentration, while Mn, Cu and Zn levels affect colchicine content of Colchicum. THAKUR, POTESILOVA and SANTAVY (1975) reported that the alkaloid content varies according to locality and time of collection, Gloriosa corms cultivated in hothouses in Europe contain 0,2% colchicine while dried corms from tropical Africa and India contain only 0,02% colchicine.

Table 5.5 Colchicine contents of Gloriosa, Sandersonia and Littonia. (* indicates no value given)

Species	Plant part	Colchicine % yield	Reference
Gloriosa superba	corm	0,3% 0,23 - 0,3%	CLEWER, GREEN and TUTIN, 1915. EIGSTI and DUSTIN, 1955.
	corm	0,03%	WATT and BREYER BRANDWIJK, 1962.
	corm	*	WILDMAN and PURSEY, 1968.
	seed	*	
	seed	0,60%	SARIN, JAMWAL, GUPTA and ATAL, 1974.
	corm	0,205%	
	corm, India, 1967	0,02	Review from THAKUR, POTESILOVA and SANTAVY, 1975
	corm, India, 1973	0,024%	
	corm, India, 1974	0,026%	
	corm, Africa, 1972	0,05%	
	leaf	0,05%	

Table 5.5 (Continued)

Species	Plant part	Colchicine % yield	Reference
	corm	*	MERCHANT and JOSHI, 1976.
	corm	0,54 - 0,92%	NTAHOMUUKIYE, HAKIZIMANA, NKILIZA and PUYVELDE, 1984.
	seed	0,37 - 0,46%	
	flower	1,18%	
	stem	0,33 - 0,41%	
	young leaves	2,36%	
	adult leaves	0,87	
	corm	0,9%	BELLET and GAIGNAULT, 1985.
Gloriosa simplex?	corm seed	*	HRBEK and SANTAVY, 1962
	corm seed	*	WILDMAN and PURSEY, 1968.
	corm	0,29%	THAKUR, POTESILOVA and SANTAVY, 1975.
Gloriosa superba subsp. virescence	corm, seed	*	HRBEK and SANTAVY, 1962
	corm, seed	*	WILDMAN and PURSEY, 1968.
	corm	*	THAKUR, POTESILOVA and SANTAVY, 1975.
Littonia modesta	corm	0,2 - 0,4%	HRBEK and SANTAVY, 1962; and WILDMAN and PURSEY, 1968.
	seed	0,5%	
	leaf	0,15%	
Sandersonia aurantiaca	corm	*	HRBEK and SANTAVY, 1962; and WILDMAN and PURSEY, 1968.

A number of tests have been developed for the identification of colchicine. Initially colour tests were used, colchicine gives a yellow colour in sulphuric acid and turns red when heated. Froehdes reagent gives a blue colour (HAMERSLAG, 1950).

Nitric acid gives colchicine a dirty violet colour which changes through brown to yellow. Acid hydrolysis of colchicine gives colchiceine that turns olive green in ferric chloride, ethanolic solutions of colchicine and ferric chloride gives a garnet red colour (CROMWELL, 1955). Colchicine yields were initially determined by gravimetric estimations of purified colchicine, using phosphotungstate precipitation. SEIFERT (1943) and KING (1951) based their colourmetric techniques on the colour reaction of acid treated colchicine (colchiceine) with ferric chloride, however, these techniques are only reproduceable if the alkaloid is in a pure form, or there is a high percentage of the alkaloid in the extract. Current official procedures involve the use of UV (ultra-violet) spectrophotometry (FORNI and MASSARANI, 1977; BRITISH PHARMACOPOEIA, 1980; US PHARMACOPOEIA, 1982; THOMPSON, 1985). Recently liquid chromatography has been used as a means of colchicine determination. The first HPLC separation was reported by FORNI and MASSARANI (1977) using a silanized silica gel column and acetonitrile and water as the solvent system. DAVIS and KLEIN (1980) separated six colchicine derivatives on a C_{18} reversed phase column with acetonitrile, methanol and pH6 phosphate buffer as the solvent system. THOMPSON (1985) used reversed phase liquid chromatography to identify colchicine using a 55% methanol, 45% phosphate buffer solvent system and an octylsilane bound spherical silica packed column, monitored at 254 nm (Figure 5.8). HAYASHI, YOSHIDA and SANO (1988) used a Lichrosorb RP-18 column and acetyl acetonitrile : methanol : water (7 : 1 : 12) to tentatively identify the alkaloid from Colchicum cells.

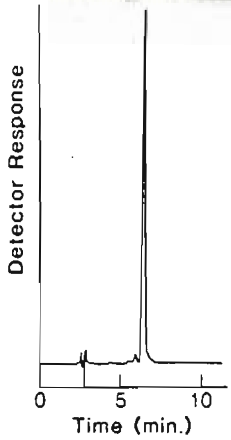
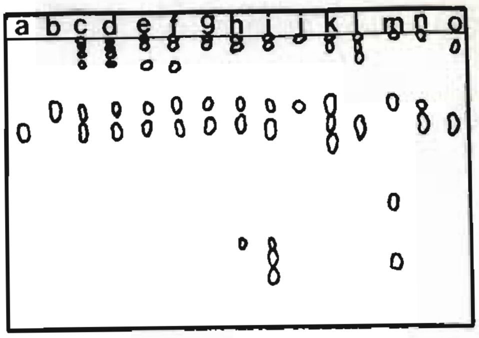


Figure 5.8 HPLC trace of colchicine, taken from THOMPSON (1985).

Both paper and thin layer chromatography can be used as a means of colchicine and colchicine derivative determination (Figure 5.9).



a colchicine
b substance b
c G. simplex seed
d corm
e G. virescens seed
f corm
g Littonia corm
h corm
i Littonia leaf
j seed
k Sandersonia corm
l Androcymbium corm
m leaf
n seed
o Ornithoglossum corm

Figure 5.9 TLC of Gloriosa, Sandersonia, Littonia and Androcymbium extracts from HRBEK and SANTAVY (1962).

Other identification methods include nuclear magnetic resonance (NMR), mass spectroscopy, optical rotation and optical rotatory dispersion (WILDMAN and PURSEY, 1968).

The amount of plant material required for colchicine extraction varies radically. CLEWER, GREEN and TUTIN (1915) used 42,52 kg of dried corms for extraction purposes, whereas today smaller amounts of plant material (20 -30 mg) (HAYASHI, YOSHIDA and SANO, 1988) can be used for the identification and quantification of alkaloids present in plant material.

Plant tissues are generally less sensitive to colchicine than are animal cells. Normally plants require 1 000 times higher concentrations of the alkaloid to arrest mitosis (EIGSTI and DUSTIN, 1955; ZRYD, 1979). Since Blakeslee discovered colchicine as a polyploid inducing agent in 1937 many researchers have tried to isolate chemicals with similar properties. "Colchicine is still the only alkaloid that fulfills the different requirements of an effective polyploidizing agent" (NARAIN and RAINA, 1975). Colchicine binds specifically to tubulin a subunit protein of microtubules, this interaction has been used to study the role of microtubules in mitosis, morphogenesis, intracellular transport and secretion (RAPAPORT, BERKLEY and BUCHER, 1975).

Reproduction of chromosomes, splitting into chromatids and condensation are not affected by colchicine. In normal mitosis chromosomes are pushed to the centre of the nucleus, the centromere becomes attached to the spindle and the chromosomes become arranged into an equatorial plate. With the colchicine this arrangement does not occur. Division of the centromere region is delayed for several hours, the region adjacent to the centromeres divide and daughter chromosomes part, the division is desynchronized in the telophase the chromosomes are drawn to one nucleus resulting in polyploidy. Colchicine binds to soluble dimeric tubulin at a 1 : 1 molar ratio to form a tubulin-colchcine complex. Addition of colchicine to tubulin solutions prevents the nucleation of new microtubules and causes rapid depolymerization of preformed microtubules (MOREJOHN and FOSKET, 1984).

To give a comprehensive review of the induction of polyploids by utilization of colchicine would be impossible, suffice to say that for polyploid production, buds or seeds are soaked in colchicine solutions, and the seeds / buds are removed and allowed to develop "normally". These techniques can be applied to both ornamentals (PRYOR and FRAZIER, 1968; ZAGORSKI and ASCHER, 1981; EL-MELIGY, 1985) and crop plants (ELLISON and TIANGCO, 1970; AHMAD, BRITTEN and BYTH, 1975; RAGHUVANSHI, PATHAK and SINGH, 1978); THIEBAUT, KASHA and TSAI, 1979; CURRAH and OCKENDON, 1987). The following are a few examples of the use of colchicine and the production of polyploids via tissue culture. HEINZ and MEE (1970) and BAYLISS (1976) found that polyploidy can be induced in suspension culture by the addition of colchicine. "Although there are not many references, it is known that application of colchicine to tissue or cell culture media has a polyploidizing effect" (ESPINO and VAZQUEZ, 1981). CHAVADEJ and BECKER (1984) reasoned that in certain cases polyploidy may cause an increase in the production of medicinal compounds (often as a result of loss of fertility or increase in the plant's susceptibility), and used colchicine to induce polyploidy in culture.

BRICOUT, GARCIA-RODRIGUEZ and PAUPARDIN (1978) reported an increase in essential oil production in in vitro grown plants treated with colchicine. GOLDY and LYRENE (1984) used colchicine and shoot cultures to produce 8x blueberrys from 4x plants. CURRAH and OCKENDON (1987) utilized anther cultures and colchicine to double haploid brussel sprout plants (see anther culture, Chapter 2). NOVAK (1983) used colchicine and shoot tip culture to establish diploid and tetraploid plants which had larger bulbs and were more vigorous than were the parent plants. SEMAN (1984) used meristem cultures and colchicine to study the usage of in vitro culture to shorten the selection process for Beta breeding. Ornamental plants have also been grown in culture together with colchicine. GURI, ZELCER and IZHAR (1984) studied the mitotic index in Petunia suspension cultures, ZAGORSKI and ASCHER (1981) measured the frequency of polyploid and mixoploid roots in Lilium cultures. CHEN and GOEDEN-KALLEMEYN (1979) found that over 50% of the plants initiated from Hemerocallis callus treated with colchicine were tetraploid.

Colchicine inhibits root elongation and may cause a swelling in the zone of elongation. It is speculated that this effect is due to a change in the deposition of the microfibrils in the cell wall, which affects the plant orientation for enlargement (UPADHYAYA and NOODEN, 1977, 1978; HOGETSU and SHIBAOKA, 1978). OPPENOORTH (1978) found that by treating bean leaves (induced to root with indoleacetic acid) with colchicine, dedifferentiation of the adventitious roots was inhibited, and growth was by cell enlargement only, if more than 30 meristematic cells were present the result was a recognisable primordium. Less than this number resulted in cells without a recognised structure. MACLEOD (1976) found that root primordia of Vicia treated leaves died if treated in the first 2,6 days of development; those between 2,6 - 3,6 days old had their growth inhibited; 3,6 - 4,9 day old treated roots grew as straight laterals, while older primordia formed c-tumours. BARLOW (1977) and HAMMERSLEY and McCULLY (1980) found that the number of protoxylem poles in treated pea roots increased from three to four or five, the new pattern originated distal to the c-tumour (swelling induced by colchicine). This vascular rearrangement is said to be due to the effect of colchicine on the quiescent centre.

"It was thought at one time that colchicine increases the rate of cell division in plants" (HAMERSLAG, 1950). SHARP and DAVIDSON (1969) induced callus and nodal tumours at the site of colchicine application in Brassica (it was not established whether this was a result of colchicine or decapitation). GOTTLIEB and GOLDSTEIN (1979) found that colchicine disrupts the normal colony development of Eudorina. GALATIS (1977) found that colchicine inhibited stomata generation and resulted in the formation of persistent stomal meristemoids. KORDAN (1980) found that in germinating lettuce seedlings treated with colchicine, that radical but not cotyledonary growth was inhibited. Hypocotyl extension growth was retarded with the production of large sized isodiametric parenchyma cells. SUGHA (1978) showed that pea seeds soaked in the extract of Gloriosa tubers had a retarded germination potential but subsequent growth was more rapid than in the control and seed weight and number were increased.

ZRYD (1979) isolated colchicine resistant cell strains from Acer and Daucus suspension cultures. The high resistance of plant cells to colchicine was ascribed to the small specific binding activity of the plant cells, and the highest resistance was ascribed to a nonspecific binding component in the cell surface or wall. "Loosening of the cell aggregates may be due to modification of the cell wall component and correlation with an increase in nonspecific binding" (ZRYD, 1979).

Various effects have been described between plant hormones and colchicine (MACLEOD and DAVIDSON, 1966). Leaves of pinto bean seedlings treated with colchicine resulted in a higher cytokinin content in the treated leaves over that in the control (ZATYKO, 1973). The relationship between colchicine, directional cell growth and gibberellic acid has been the subject of a number of research papers (SHIBAOKA, 1972; SAWHNEY and SRIVASTAVA, 1974; KEITH and SRIVASTAVA, 1978; DURNAM and JONES, 1982). The general conclusion from these papers is that colchicine eliminates gibberellic acid induced elongation growth, as colchicine causes a reorientation in the plane of cell division and colchicine was reported to be only effective in inducing reorientation in light, in darkness both radial and longitudinal cell expansion was inhibited. In oats, auxin and colchicine caused a mutually antagonistic interaction in cytoplasmic response of coleoptiles (THOMAS, DUNN and SEAGULL, 1977). Application of abscisic acid to cotton seedlings reduced colchicine and chilling injury (RIKIN, GITLER and ATSMON, 1981). Colchicine treatment of barley seedlings simultaneously with hormones (kinetin, benzyladenine, indoleacetic acid and 2,4-dichlorophenoxyacetic acid) gave a lower frequency of polyploidy, while hormone treatment after the colchicine treatment, increased the frequency of polyploidy (KUNAKH, KIFORAK and ALPATOVA, 1981).

The synthesis of colchicine in plant tissue has been successfully used in chemotaxonomy for the classification and identification of members of the Colchicaceae (Wurmbaeoideae) (SANTAVY, 1980, SANTAVY, 1982; HEGNAUER, 1986).

During final preparation of this thesis a series of papers by YOSHIDA, HAYASHI and SANO (1988a, 1988b) and HAYASHI, YOSHIDA and SANO (1988) was published in which they investigated the production of colchicine and colchicoside in suspension cultured <u>Colchicum</u> <u>autumnale</u>. It was claimed that these were the "first report on the production of colchicine alkaloid by plant tissue culture" (HAYASHI, YOSHIDA and SANO, 1988).

Colchicine is known to inhibit mitosis, interfere with the orientation of newly deposited fibrils in plant cell walls, induce polyploidy, relieve symptoms of gout and play a role in cancer therapy. These economically and culturally important phenomena together with the observation of BELLET and GAIGNAULT (1985) that Gloriosa (and Sandersonia) is valuable for the pharmaceutical industry, makes these plants ideal species for the study of colchicine biosynthesis and production using an in vitro system for secondary plant metabolite production.

5.2 The production of secondary plant metabolites in tissue culture

> "Historically the study of secondary metabolites has been the province of the organic chemist. Molecules that appear to be 'ballast' or to serve no essential metabolic function clearly deterred the biologist and until comparatively recently the involvement of plant physiologists and biochemists in this field was largely conspicuous by its absence" (HASLAM, 1986).

The alkaloids form a very diverse family of natural products. By definition an alkaloid "is a nitrogen containing compound beyond this prerequisite the definition becomes more difficult" (ROBINSON, 1974). The alkaloids are classed according to the heterocyclic ring occurring in their structure (WALLER and DERMER, 1981). To date only two percent of the planet's estimated 200 000 flowering plants have been screened for alkaloids, the two percent producing more than 1 000 different forms (MYER, 1976). MOTHES (1969) speculated that alkaloid production in plants is not advantageous to the plant, however, in certain cases it is evident that alkaloid production is advantageous for plant survival (ROBINSON, 1974). The speculated major role of plant alkaloids, is the production of poisons or repellants to competitors, parasites and predators. "Alkaloids can be toxic to plants in high concentrations, even toxic to the same plant that makes them. The powerful effects of colchicine are well known" (ROBINSON, 1974). Alkaloid biosynthesis also plays an important role in providing a system to negate the effects of overproduction of compounds both beneficial and antagonistic to normal plant metabolism, especially waste products from nitrogen metabolism.

There are several major incentives for studying alkaloids. They are the oldest of the known drugs and still have significant use in modern medicine (WALLER and DERMER, 1981). Studies into the biochemistry of plant alkaloids have been stimulated by "native folklore medicine" (KUTNEY, 1987). Despite advances in synthetic compound production, numerous problems arise from the large scale production of alkaloids via chemical synthesis; these include multistep processes, long-term research to elucidate biochemical pathways, and stereochemical difficulties. In spite of the preferential use of pure compounds manufactured synthetically in drug production, many important compounds are still isolated from plants for the manufacture of medicinal substances (ROBINSON, 1982).

In certain cases plants that produce important chemicals for commercial production have not been genetically manipulated to produce optimal amounts of compound. This feature is compounded by the fact that many of the third world countries that grow plants for chemical production are politically unstable resulting in the irregular supply of plant material (DODDS and ROBERTS, 1985). The supply of plants from their natural habitats is also diminishing due to the exploitation and destruction of these areas, and certain plants have a natural limited geographic distribution (DOUGALL, 1979; KURZ and CONSTABEL, 1979a). The survival of threatened species has immediate value, with the genetic reservoirs playing an important role in the fields of medicine and pharmaceutical production.

The utilization of plant cells that are cultured <u>in vitro</u> provides a viable alternative to the problems involved in the production of chemical, pharmaceutical, and medicinal compounds (BARZ, REINHARD and ZENK, 1977; KURZ and CONSTABEL, 1979a, 1979b; STABA, 1980; BARZ and ELLIS, 1981).

The advantages of plant cell cultures for secondary plant metabolite production include:

i) the production of natural products, if the cultures possess higher levels of chemicals, than those found in the normal plant, or if the plant material is unavailable.

ii) valuable chemicals can be produced in controlled environments

iii) the production of novel compounds with useful biological properties

iv) the production of "plant specific" compounds that can only be isolated from intact cells (e.g. enzymes)

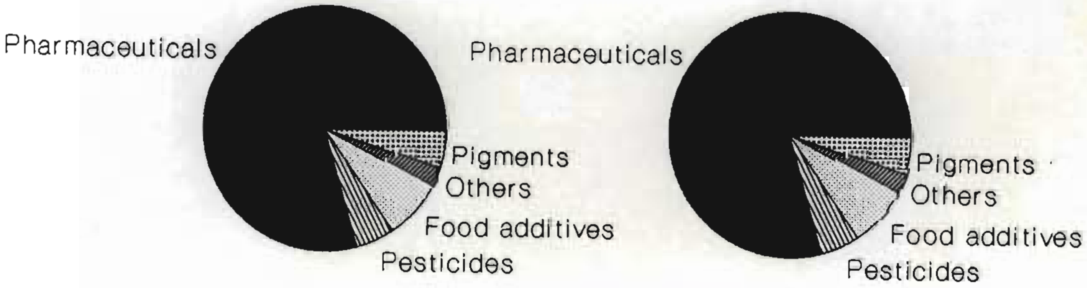
v) incorporation of precursors is easier to achieve in culture than in intact plants, plus chemical production by biotransformation from low cost precursors often cannot be achieved by chemical or microbial techniques.

vi) sterile cultures eliminate the problems associated with microbes and insects

vii) automated techniques allow for reduction in labour costs and an increase in productivity, plus large scale production is now feasible with available technology

viii) genetic modification of cells in culture to increase metabolite production (BUTCHER, 1977: TABATA, 1977; BARZ and ELLIS, 1981).

Figure 5.10 gives a summary of patents taken out in Japan which utilize plant tissue culture as a means of compound production.



1975-1979 (Total 20) **1980-1984 (Total 39)**

Figure 5.10 Patents related to secondary metabolites produced by tissue culture (FUJITA and TABATA, 1987).

The number of compounds shown to be present in culture is extensive (BUTCHER, 1977) and Table 5.6 lists the broad categories of plant metabolites that have been produced using *in vitro* techniques.

Table 5.6 Categories of plant metabolites produced in culture (adapted from NICKELL, 1980).

Alkaloids
Allergens
Amino acids
Anthraquinones
Antileukemic agents
Antimicrobial agents
Antitumor agents
Benzoic acid derivatives
Benzopyrones
Biotransformations
Carbohydrates
Cardiac glycosides
Chalcones
Condiments
Dianthrones
Emulsifiers, food
Enzymes
Enzyme inhibitors
Ethylene
Flavanoids
Flavors
Food
Fragrances
Furanochromones
Furanocoumarins
Growth regulators
Hormones
Immunochemicals
Insecticides
Insulin-like compounds
Latex
Lipids
Medicinals
Naphthaquinones
Nucleic acids
Oils commercial
Oils volatile
Opiates
Organic acids
Peptides
Perfumes
Phenolics
Pigments
Polysaccharides
Proteins
Spices
Steroids
Sterols
Saponins
Sapogenins
Sugar
Sweeteners
Tannins
Terpenes
Terpenoids
Virus inhibitors
Vitamins

KURZ and CONSTABEL (1979a) and NICKELL (1980), reported that one of the first classes of products to be investigated were the alkaloids with the first work probably appearing in 1957 showing the production of atropine by belladonna callus. There is a large volume of literature present outlining the production of alkaloids in tissue culture with the major research efforts directed towards the production of morphinans, codeine, norsanguinarine from *Papaver* cultures, indole alkaloids (cathranthine) from *Catharanthus* cultures, nicotine from tobacco, hyoscine, cephalotaxine, serpentine and voafrine A from *Datura*, *Cephalotaxus*, *Rauwolfia* and *Voacanga* cultures respectively (NICKELL, 1980; BERLIN and BODE, 1987; KUTNEY, 1987). Table 5.7 lists the alkaloids isolated in tissue culture.

Table 5.7 Plants utilized for the production of alkaloids *in vitro*

Plant	Alkaloid
Alstonia	Reserpine
Atropa	Atropine
Catharanthus	Ajmalicine, Akuammicine, Alstronine, Cathalanceine, Cathindine, Cavincidine, Cavincine, Desacetyl vidoline, Dihystrositsirikine, indole alkaloids, Lanceine, Lochneridine, Mitraphylline, Perivine, Perosine, Serpentine, Sitsirikine, Vinca alkaloids, Vindoline, Vindolinine
Cammellia	Caffeine
Camptotheca	Camptothecin
Cephalotaxus	Cephalotaxine, Harringtonine, Homoharringtonine, Homedeoxyharringtonine, Isoharringtonine
Cinchona	Qunindine, quinine
Coffea	Caffeine, purine alkaloids
Colchicum	Colchicine
Coptis	Berberine, Coptisine, Jatrorrhizine, Magnoflorien, Palmatine
Corydalis	Capaurimine, Norsanguinarine
Datura	Hyoscine, Hyorscyamine, Scopolamine, tropane alkaloids
Duboisia	Atropine, Hyoscine, Hyoscyarine, Scopolamine, Valtropine
Ephedra	Ephedrine
Hippeastrum	Amaryllidaceae alkaloids

Table 5.7 (continued)

Plant	Alkaloid
Lycopersicon	Tomatine
Hyocyamus	Cuscohygrine, Hyoscine, Hyoscyamine
Lupinus	
Medicago	Choline, Stachydrine
Macleaya	Norsanguinarine, Protopine, Sanguinarine, Scoulerine
Nicotiana	Anabasine, Anatabine, Nicotine, Nornicotine
Narcissus	Haemanthamine
Papaver	Acetonyldehydrosanguinanine, Aporphine, Benzophenanthredine, Codeine, Dihydrosanguinarine, Isothebaine, Morphine, Narceine, Narcotine, Norsanguinarine, Oxysanguinerine, Papaver alkaloids, Papaverine, Protopine, Sanguinarine, Stylopine, Thebaine
Peganum	β. Carboline alkaloids, Harmine
Phaseolus	Harman, Norharman, Indole alkaloids
Ruta	Eduline, Hydroxy-N-methoxy-N-methyl acridone, Hydroxy-N-methylacridone, Rutacridone
Rauwolfia	Reserpine
Scopolia	Apoatropine, Hyoscyamine, Scopolamine, Tropane alkaloids
Solanum	Glycoalkaloids, Solamargine, Solasodine, Solasonine
Stephania	Armorine
Tecoma	Actinidine, Boschniakine, Skytanthine, Tecomanine
Trichocereus	Candicine
Trigonella	Trigonelline
"Vinca"	Indole alkaloids
Voacanga	Voafrine

Cited by: BUTCHER (1977); STABA (1977); KURZ and CONSTABEL (1979a, 1979b); NICKELL (1980); BARZ and ELLIS (1981); SASSE, HECKENBERG and BERLIN (1982a, 1982b); STABA, ZITO and AMIN (1982); BANERJEE and SHARMA (1983); DODDS and ROBERTS (1985); DeLUCA, BALSEVICH, TYLER, EILERT, PANCHUK and KURZ (1986); SCRAGG, MORRIS and ALLAN (1986); BECKER (1987); KUTNEY (1987); HAYASHI, YOSHIDA and SANO (1988).

ARREGUIN and BONNER (1950) recorded that sterile cultures of Guayule (Parthenium argentatum) produced rubber in culture. ROUTIEN and NICKELL (1956) in their patent recorded ten types of plant tissue cultures that could be utilized for large-scale production of chemicals. TULECKE and NICKELL (1960), KLEIN (1960) and TULECKE (1961) all reported that secondary metabolites produced by plants could also be produced in cell cultures with potential for economic exploitation. NICKELL (1962) outlined the technology of large-scale submerged cell cultures. In general yields of secondary metabolites in culture were low, however, "the year of 1975 was regarded as a watershed in secondary product research" (COLLIN, 1987), as prior to 1975 the quantities found in culture were low or only a limited range of compounds were produced. After 1975 there began to appear a large number of reports where the secondary plant products were produced in higher concentrations than originally found in the parent (COLLIN, 1987). A large number of patents have been obtained for the production of compounds from cell culture (allergens, diosgenine, L. dopa, ginseng, saponin glycoides and glycyrrhizin) STABA, 1977), however, according to BERLIN, BEIER, FECKER, FORCHE, NOE, SASSE, SCHIEL, and WRAY (1985), and COLLIN (1987) the only compound to be commercially produced is shikonin from cultures of Lithospermum erythrorhiza.

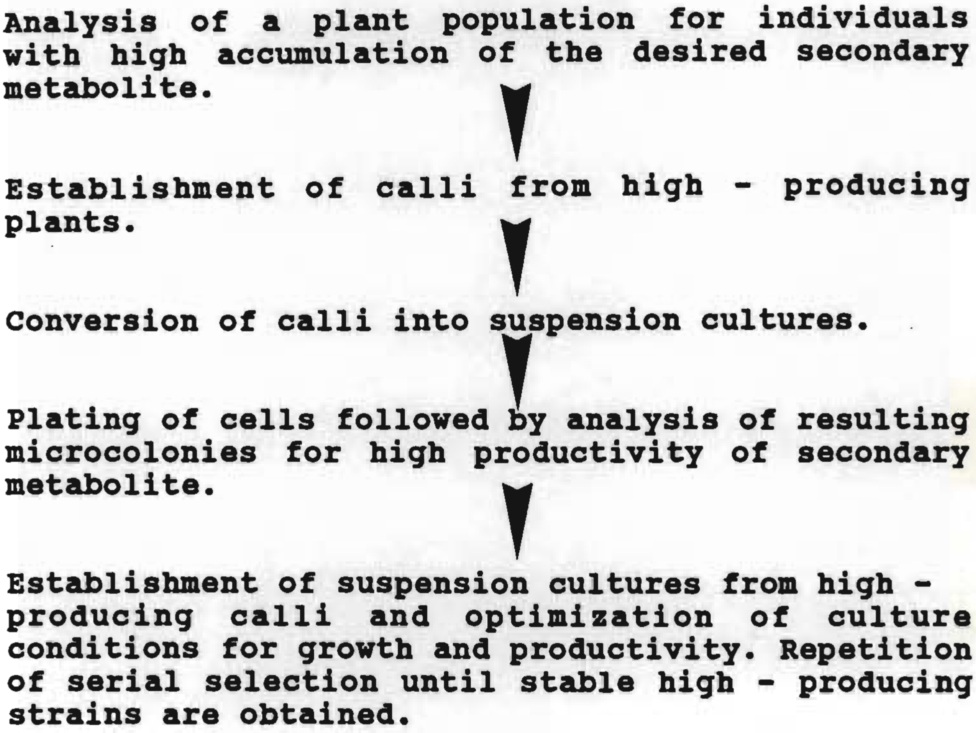
The following review briefly summarizes some of the tecnhiques used for the production of secondary plant products in culture and possible culture techniques that may improve the levels or range of products in culture.

"The species or cultivar to be selected for establishing a cell culture will be determined by the research project in question" (KURZ and CONSTABEL, 1979a). Species that are rich in the specific compound are expected to give cell cultures with equally high levels of compound! (COLLIN, 1987). However, this is not always the case. Plants vary in the production of metabolites from species to species and variety to variety, the selection of a high compound producing specimen is desirable before culture initiation e.g. Catharanthus roseus var Little Delicate was found to be superior to var Roseus in terms of the yield of catharanthine (KURZ and CONSTABEL, 1985). ZENK, EL-SHAGI, ARENS, STOCKIGT, WEILER and DEUS (1977) reported that there was a variation in alkaloid content in Catharanthus roseus cultures despite the fact that the cultures were derived from seedling from the same plant. Often a correlation between alkaloid producing plants and the resultant cultures cannot be drawn (KURZ and CONSTABEL, 1979a).

It is speculated that due to the techniques used for plant tissue culture and subculture, variation in the genetic complement of the plant may occur, resulting in variation in biosynthetic capacity. "The establishment of a high yielding cell culture, therefore, would still need to start with numerous explants from quality plants with subsequent selection of the most active strain" (KURZ and CONSTABEL, 1979a).

> "The origin of the explant may be vital to the success of secondary product production, however, cells grown in culture over extended periods of time indicate that explant origin is irrelevant, in terms of the cells' biosynthetic capacity. This adds extra weight to the theory of totipotency" (KURZ and CONSTABEL, 1985).

SASSE, HECKENBERG and BERLIN (1982a) found that Peganum cultures did not vary in the amount of β-carboline and serotonin produced despite the fact that the cultures were derived from different explants namely roots, shoots and leaves. On the other hand KINNERSLEY and DOUGALL (1980) showed variation in the ability of cultures to produce nicotine, depending on the position of the explant on the stem of the tobacco plant.



Analysis of a plant population for individuals with high accumulation of the desired secondary metabolite.

Establishment of calli from high - producing plants.

Conversion of calli into suspension cultures.

Plating of cells followed by analysis of resulting microcolonies for high productivity of secondary metabolite.

Establishment of suspension cultures from high - producing calli and optimization of culture conditions for growth and productivity. Repetition of serial selection until stable high - producing strains are obtained.

Figure 5.11 Strategy to select high metabolite producing lines for cell culture (BARZ and ELLIS, 1981)

Selection of high producing strains is essential for optimal production of the desired compounds, and by choosing these high producing strains:

> "... it is expected that the genetic or epigenetic factors which are responsible in living plants for the high productivity will be preserved and lead in the cell cultures to an enhanced level of secondary constitutent accumulation" (BARZ and ELLIS, 1981).

The proportion of high yields will depend on a number of cultural factors including, the culture environment, media composition and various stress related pressures (STABA, 1977; KURZ and CONSTABEL, 1979a).

An advantage of plant cell cultures over animal cell cultures lies in the fact that plant cells can be grown in synthetic media, "... unfortunately only a few studies have been devoted to a thorough examination on the effects of individual media components and their interaction on secondary metabolite production" (TABATA, 1977).

WESTCOTT and HENSHAW (1976), AMORIM, DOUGALL and SHARP (1977), MEHTA and SHAILAJA (1978) and DOUGALL (1980) all showed that by increasing the amount of nitrogen in the culture media there was a resultant decrease in phenolic and tannin production in Rosa and Acer cultures. MISAWA, TANAKA, CHIYO and MUKAI (1975) reported increases in plasmin inhibitor in Scopolia cultures with no effect on growth if there was a decrease in ammonium nitrate with an increase in potassium nitrate. FUJITA and TABATA (1987) found that shikonin production in White's media was high because of the lack of ammonia. This effect could be immitated in Linsmaier and Skoog's medium if the ammonia was omitted. MIZUKAMI, KONOSHIMA and TABATA (1977) reported an increase in total nitrogen resulted in an increase in shikonin production but only up to a specific point. This trend was also reported by COLLIN (1987) for both scopoletin and shikonin, but there was a decline in metabolite production at high levels of nitrogen. TABATA (1977) recorded that tannin productivity increases together with an increase in the carbon to nitrogen ratio, while SUZUKI, MATSUMOTO and MIKAMI (1984) found that a ratio of 1 : 1 ammonia to nitrate resulted in an increase of compound production over that of the control, this was also the case of diosgenin production while digitoxin production was favoured by a 1 : 2 ratio of ammonia to nitrate. Colchicine production from Colchicum suspension cultures was also stimulated using a ratio of 1 : 2 ammonia to nitrate (HAYASHI, YOSHIDA and SANO, 1988). Ubiquinone production was stimulated if ammonia is in a higher concentration than the nitrate, while nicotine production increased 24-fold when the ammonia was replaced by nitrate (COLLIN, 1987).

KURZ and CONSTABEL (1979a) and COLLIN (1987) all feel that ammonia and nitrate are inferior sources of nitrogen and better yields can be achieved with peptone, yeast extracts, glycine and casein hydrolysate. The general trend appears to be that nitrogen inhibits secondary metabolite production; this is explained by KURZ and CONSTABEL (1985): "... any levels of nitrogen sufficient to reinitiate growth in cells which have already entered the stationary phase would be detremental to product synthesis."

CONSTABEL (1968), DAVIES (1972), ZENK, EL-SHAGI and ULBRICH (1977) and COLLIN (1987) all reported that increasing the amount of carbohydrate resulted in an increase in the amount of secondary metabolite. The low levels of sucrose result in a lack of production because of a limited production of enzymes required for secondary metabolism (COLLIN, 1987). But, if low levels of sucrose do not reduce cell growth secondary metabolism may occur. BALAGUE and WILSON (1982) grew cell cultures in low levels of sucrose and secondary metabolism was inhibited. This inhibition persisted when sucrose levels were increased to 2%, however, alkaloid production occurred when the sucrose level was raised to 7%. DOUGALL (1980) reported an increase in shikonin production by increasing the sucrose concentration from 1 to 5%, shikonin production remained constant with a further increase in sucrose to 7% and 10%, however, there was a decrease in the fresh weight of the cultures.

AMORIM, DOUGALL and SHARP (1977) found that an increase in glucose resulted in an increase in phenolic production until nitrogen was added. TABATA (1977) reported that shikonin production is dependent on sucrose at high concentration, but there may be an antagonistic interaction between sugar and nitrogen.

ZENK, EL-SHAGI and SCHULTE (1975), MIZUKAMI, KONOSHIMA and TABATA (1977), DOUGALL (1980) and KURZ and CONSTABEL (1985) all found that sucrose was the superior form of carbohydrate. HAYASHI, YOSHIDA and SANO (1988) showed that sucrose was the only effective carbon source for colchicine formation in suspension cultured Colchicum cells. COLLIN (1987) reported that a change from sucrose to other forms (cheaper) of carbohydrate resulted in a decrease in secondary metabolite production. SUZUKI, MATSUMOTO and MIKAMI (1984) on the other hand showed that glucose, sucrose and galactose were more efficient forms of carbohydrate than were raffinose, lactose and rhamnose, but glucose was the best source overall. KURZ and CONSTABEL (1985) summarised the carbohydrate effect as, if low amounts are added to cell cultures the sucrose is used for cell growth and pathways for metabolite production are inoperative.

NETTLESHIP and SLAYTOR (1974), SASSE, HECKENBERG and BERLIN (1982a), BECKER (1987), COLLIN (1987) and FUJITA and TABATA (1987) reported that phosphate levels may or may not inhibit secondary metabolite production, however, in general low phosphate levels increase secondary product formation. It is speculated that these low levels result in enhanced enzyme activity (phenylalanine lyase, tryptophan decarboxylase and ornithine decarboxylase) which ultimately results in increased production. The decrease in phosphate results in an increase of enzymes responsible for diverting compounds (amino) from primary to secondary pathways. KURZ and CONSTABEL (1985) showed that phosphorous levels above 10 nM are inhibitory. ZENK, EL-SHAGI and SCHULTE (1975) and CAREW and KRUEGER (1977) showed that an increase in phosphorous resulted in an increase in the production of indole and anthraquinone compounds.

Sulphur, potassium, magnesium, iron, calcium, copper and vitamins have all been shown to affect cell growth in culture, with copper at high levels increasing shikonin production (FUJITA, HARA, SUGU and MORIMOTO, 1981). FUJITA and TABATA

(1987) summarised the effect of media on secondary metabolite production in the following words:

> "Direct use of one of the established media used to promote cell growth is usually unfavourable for secondary metabolite production, therefore, a suitable production media for each metabolite or plant species must be developed."

Plant growth regulators play an important role in the production of secondary metabolites in culture due to their ability to induce or repress certain biosynthetic pathways, hormones do not react with the intermediates of the biosynthetic pathway but shift cytological condition so as to favour / inhibit product formation (ZENK, EL-SHAGI, ARENS, STOCKIGT, WEILER and DEUS, 1977). TABATA (1977) and KURZ and CONSTABEL (1985) have stated that because production of secondary metabolites is a function of cell multiplication and differentiation, the addition of hormones to the culture media is of vital importance.

With respect to the auxins, the generalization made by most reviewers of the literature is that indoleacetic acid is the most favourable source of auxin for product synthesis and accumulation, while napthaleneacetic acid and 2,4-dichlorophenoxyacetic acid results in product inhibition (TABATA, 1977; KURZ and CONSTABEL, 1979a; 1985). HAYASHI, YOSHIDA and SANO (1988) however, found that indoleacetic acid was unstable in Colchicum cultures. CONSTABEL, SHYLUK and GAMBORG (1971), reported that anthocyanin production in Haplopappus cultures was suppressed by high concentrations of napthaleneacetic acid and 2,4-dichlorophenoxyacetic acid. NETTLESHIP and SLAYTOR (1974), ZENK, EL-SHAGI and SCHULTE (1975), PHILLIPS and HENSHAW (1977), SASSE, HECKENBERG and BERLIN (1982a) and HAYASHI, YOSHIDA and SANO (1988), all reported that 2,4-dichlorophenoxyacetic acid inhibited secondary metabolite production in culture. PINOL, PALAZON and SERRANO (1984) recorded that the absolute concentration of auxin controls the rate of nicotine synthesis, 11,5 µM napthaleneacetic acid inhibited alkaloid synthesis while 1 µM enhanced nicotine synthesis (PINOL, PALAZON, ALTABELLA, CUSIDO and SERRANO, 1985). ZENK, EL-SHAGI and SCHULTE (1975) showed that in Morinda cultures although anthraquinone production was inhibited by 2,4-dichlorophenoxyacetic acid it was stimulated by napthaleneacetic acid. In contrast, SUZUKI, MATSUMOTO and MIKAMI (1984) found that low concentration of napthaleneacetic acid enhanced anthraquinone formation but increased levels of the auxin resulted in a decrease. 2,4-Dichlorophenoxyacetic did not inhibit anthraquinone production. LIAU and IBRAHIM (1973) showed that high levels of auxins decreased the amount of p-coumaric acid and ferulic acid in culture but increased the amount of p-hydroxybenzoic acid and vanillic acid. Contrary to the above reports COLLIN (1987) recorded a number of examples where the presence of auxin (2,4-dichlorophenoxyacetic acid, indoleacetic acid and napthaleneacetic acid) resulted in an increase in scopoletin and saponin. KAUL, STOHS and STABA (1969) showed an increase in the production of diosgenin in culture using 2,4-dichlorophenoxyacetic acid, while SZOKE, DUNG, VERZÁR-PETRI and POTOCZKI (1982) showed similar results with Datura cultures. WETMORE and RIER (1963), JEFFS and NORTHCOTE (1967) found that auxin and sugar affects the amount of xylem formation and the degree of lignification. ALFERMANN and REINHARD (1971) could substitute the light requirement for anthocyanin production with auxin.

Explanations for the inhibitory role auxin plays on secondary metabolite production include; the fact that cells grown in 2,4-dichlorophenoxyacetic acid have low amounts of glutamine and aspartic acid, compounds necessary in alkaloid synthesis (KURZ and CONSTABEL, 1979a), 2,4-dichlorophenoxyacetic acid inhibits the metabolism of geranylhydroquinone an intermediate of shikonin production (KURZ and CONSTABEL, 1985), and auxin may inhibit the activity of putrescine N-methytransferase an intermediate of nicotine synthesis (TABATA, 1977).

COLLIN (1987) claimed that there was no clear role for cytokinins in secondary metabolite production in culture. LIAU and IBRAHIM (1973) found that kinetin increased the amount of p-courmarins and ferulic acid, KOBLITZ (1962) and BERGMAN (1964) also showed that kinetin can increase the amount of metabolites in Daucus and Nicotiana cultures. ZENK, EL-SHAGI, ARENS, STOCKIGT, WEILER and DEUS (1977) and KURZ and CONSTABEL (1985) recorded that benzyladenine at high concentration in the absence of auxin resulted in high yields of alkaloid, but the cytokinin alone did not stimulate growth. SCRAGG, MORRIS and ALLAN (1986) experimenting with cytokinins maintained that synthetic hormones inhibit quinidine production while natural cytokinins (zeatin) resulted in high levels of quinidine in culture. CONSTABEL, SHYLUK and GAMBORG (1971) found that cytokinins had a stimulatory effect on secondary metabolite production, but felt this increase may have been a function of time. Alternatively HIRAOKA (1976), SZOKE, DUNG, VERZAR-PETRI and POTOCZKI (1982) and HAYASHI, YOSHIDA and SANO (1988) reported that kinetin inhibited alkaloid production in Datura and Colchicum cultures.

Many researchers have studied the combined effects of cytokinin and auxin, FURUYA, KOJIMA and SYONO (1971) reported that secondary metabolite production is influenced by both cytokinin and auxin, low levels of 2,4-dichlorophenoxyacetic acid and kinetin increase diosgenin production (COLLIN, 1987). SCRAGG, MORRIS and ALLAN (1986) showed low yields of quinoline alkaloids, even though numerous hormone levels and combinations were tested, and concluded that hormones may not be effective in triggering or allowing accumulation of the alkaloid. KURZ and CONSTABEL (1985) recommended a two-step culture technique, step one utilizes auxin to establish a culture with high cell mass while step two uses cytokinin for the enhancement of metabolite synthesis.

Gibberellins have been shown to inhibit anthocyanin production (SCHMITZ and SEITZ, 1972), increase berberine production and inhibit shikonin production in the same culture (FUJITA and TABATA, 1987). Gibberellins may or may not inhibit secondary metabolite production in culture (COLLIN, 1987).

There appears to be no "unequivocal correlation" for the role of hormones on secondary metabolite production in culture, however, in general it is felt that auxin leads to dedifferentiation via stimulation of cell division resulting in diminished production of secondary metabolites (BECKER, 1987).

TABATA (1977) is of the opinion that there has been more information collected on the effect of light on secondary metabolite production in culture than any other physical factor shown to influence plant tissue culture. SEIBERT and KADKADE (1980) summarised their review saying that light can substantially improve the yield of certain metabolites in culture.

HAHLBROCK (1972), BRUNET and IBRAHIM (1973), MATSUMOTO, NISHIDA, NOGUSHI and TAMAKI (1973) all reported that metabolite synthesis is triggered by light. CORDUAN and REINHARD (1972) have stated that the synthesis of volatile oils is dependent on both the quantity and quality of the light. HAGIMORI, MATSUMOTO and MIKAMI (1984) found that the stimulatory effect of light on digitoxin biosynthesis was not due to any direct action of the light on the biosynthetic system, SEIBERT and KADKADE (1980) reported that maximum anthocyanin synthesis depended on the developmental stage of the culture at the time of light exposure. ALFERMANN and REINHARD (1971) replaced the light requirement of their cell cultures with auxin. REINERT, CLAUSS and VON ARDENNE (1962) and SEIBERT and KADKADE (1980) found that red light was ineffective in anthocyanin synthesis stimulation. TABATA, MIZUKAMI, HIRAOKA and KONOSHIMA

(1974) on the other hand showed that shikonin synthesis was inhibited by cool white light (white or blue) due to the inactivation of a co-enzyme. KURZ and CONSTABEL (1985) on reviewing the advantages of autotrophic and heterotrophic cultures found that lupanin and sparteine were partially synthesized in the chloroplast, while phenyl propanoids and flavanoids were primarily synthesized by heterotrophic tissue.

It appears that most secondary metabolites in culture are stimulated by light which is unfortunate as it would be more economically advantageous if the cell cultures could grow in the dark (TABATA, 1977).

Very little research has been produced regarding the temperature optima for cell culture growth and metabolite production (MARTIN, 1980a), however, cultures grown at low temperatures do experience a change in the pattern of metabolite production, and temperature may influence the kind of substance synthesized (KURZ and CONSTABEL, 1979a). Most plant cultures grow in a temperature range between 20 - 28°C. TULECKE and NICKELL (1960) recorded the temperature optima for the growth of a number of species with potential for commercial metabolite production, Lolium having an optimal range of 20 - 21°C, while Rosa grew best at 31 - 32°C. LOCKWOOD (1984) grew Papaver cultures at 36°C and then transferred them to 5°C, which resulted in the release of thebaine into the media, but 5°C inhibited further metabolism. COLLIN (1987) explained that the effect of low temperature on metabolite production is a result of chilling stress, which may cause inhibition of growth so that the intermediates are utilized in secondary pathways or alternatively cause membrane damage which results in the breakdown of compartmentation.

Precursor feeding may result in an increase in the production of secondary metabolites (especially when product formation is limited by the lack of a precursor) and may also result in the production of novel compounds not normally associated within the parent plant (KURZ and CONSTABEL, 1979a). A number of reports have been published showing that precursors can be successfully utilized in increasing secondary metabolites, as was the case in phenylalanine feeding to increase the levels of cinnamic acid, rosmarinic acid and hypoxoside (LIAU and IBRAHIM, 1973; ZENK, EL-SHAGI and ULBRICH, 1977; BAYLEY and VAN STADEN, 1988). Cannabidiol acts as a precursor for the increased production of cannabielsoin phenolics (LOH, HARTSEL and ROBERTSON, 1983). YOSHIDA, HAYASHI and SANO (1988a) found that phenylalanine, tyrosine and methionine did not effect the formation of colchicine in suspension cultures of Colchicum, p-coumaric acid, tyramine and demecolcine, however, did increase alkaloid formation, with optimal stimulation occurring when both p-coumaric acid and tyramine were added "because they act as triggers for colchicine formation." ZENK, EL-SHAGI and SCHULTE (1975) reported that anthraquinone production was not stimulated when indirect precursors were added to the medium but stimulation was achieved when more direct precursors were utilized, TABATA, MIZUKAMI, HIRAOKA and KONOSHIMA (1976) found that this was not always the case.

KURZ and CONSTABEL (1979a) are of the opinion that for successful use of precursors, the precursor should only be applied when the culture's physiological status favours the uptake and metabolism of the precursor, and that extracellular enzymes should not be allowed to convert the precursor to other potentially toxic compounds. TABATA (1977) stated that to utilize precursors for commercial ventures the substance must be cheap and non toxic.

A knowledge of the enzymes involved in metabolism is essential, as enzymes have always been correlated to secondary product formation and it is often enzymes that create a bottleneck in optimal productivity in culture (BECKER, 1987). SELBY, TURNBALL and COLLIN (1980), KNOBLOCH, HANSEN and BERLIN (1981) and

SASSE, HECKENBERG and BERLIN (1982b) all found that enzyme activity was decisive for the capacity of cell lines to synthesize metabolites. DeLUCA, BALSEVICH, TYLER, EILERT, PANCHUK and KURZ (1986) have recorded that factors which regulate the expression of biosynthetic enzymes should be studied, while LEE (1987) stated that alkaloid production is regulated by the action of enzymes which are genetically controlled.

The effects of oxygen and pH are also important factors that play a role in alkaloid formation in tissue culture (MARTIN 1980a).

The secondary metabolite pathways of higher plants are most often not well expressed in cell culture systems (SASSE, HECKENBERG and BERLIN, 1982b). TABATA (1977) has grouped the growth pattern of secondary metabolite production in culture into three types where:

i) secondary metabolite production occurs at the same time as cell growth,

ii) secondary metabolite production is delayed until cell growth stops or decreases, or

iii) secondary metabolite production is diphasic and lags behind the growth curve.

In general a meristematic cell will seldom exhibit secondary metabolite production whereas a mature parenchyma cell may contain the whole spectrum of secondary metabolites produced by the plant, which may result in an inverse relationship between cell division and metabolite production; this relationship is probably due to the effect of differential gene activity. The production of high concentrations of metabolites in culture has been explained by the fact that cell division and product accumulation occur simultaneously, or that cultures "sequester" a percentage of the biomass which allows these cells to mature and accumulate metabolites.

The reduction in metabolite production and accumulation may be due to a number of factors, these include nutritional and physical factors discussed already, as well as changes in cell differentiation, specialization and genetic mutation. KURZ and CONSTABEL (1985) stated that the problems of changes in metabolite accumulation may also be compounded by the fact that

> "... one source material in the hands of two experts may give rise to two kinds of callus and cell suspension cultures, producing and non-producing cells, even though the protocol for tissue isolation, callus formation and subculture appear to be rather similar",

and minute differences in the culture technique "that result in variation" impede discussion of biological factors in product formation. WINK and HARTMANN (1981) reasoned that reduction of product formation in culture is due to preferential accumulation of simple compounds by the cultured cells, rather than the structurally more involved compounds derived as a result of secondary metabolism.

Spatial orientation, compartmentalization of enzymes and substrates and a site for product accumulation are factors to be considered when studying secondary metabolism in specialized tissue (BUTCHER, 1977). HRAZDINA and WAGNER (1985) showed that compartmentation was vitally important in metabolite production. AMANN, WANNER and ZENK (1986) showed vesicle localisation in the enzyme system associated with isoquinoline alkaloids in Berberis. In this system the result from the reaction with the first vesicular enzyme must leave the vesicle, be modified in the cytoplasm and then it can serve as the substrate for the second vesicular enzyme. STABA (1977) reported that the activity of the mitochondria, microsomal and soluble fraction of Dioscorea cultures is actively involved in cholesterol metabolism, and vacuoles of young dividing cells may contain pigments and tannin. RENAUDIN, BROWN and GUERN (1985) concluded that, i) the ion trapping model is valid for indole alkaloids; ii) other forces (relative to alkaloid binding to cellular components) contribute to compartmentation, and iii) the distribution of alkaloids between cells and their culture medium depends on the above types of forces (which may vary between strains and culture components). (The ion trapping model as applied to alkaloids involves low molecular weight alkaloids which are lipophilic and diffuse through membranes when in neutral form, these alkaloids accumulate as non-diffusible cations in the acidic vacuoles of the cell. One can consider the extracellular media as a compartment which may result in an equilibrium in the concentration of alkaloids between the cells and the medium). HRAZDINA and WAGNER (1985) and DEUS-NEUMANN and ZENK (1986) on the other hand felt that the uptake of metabolites into vesicles involves an active process thus contradicting the ion trap theory.

KURZ and CONSTABEL (1979a) regarded the maturity of a cell culture as being directly related to the ability of the cultures to synthesize metabolites, they regarded a "well growing" suspension as one that consists of 50% meristematic, quiescent cells and senescing cells, however, PHILLIPS and HENSHAW (1977) on the other hand stated that the relationship between the cell cycle and secondary metabolite production is not certain. In some cases cells can both synthesize and accumulate products while in other instances this function is divided among the cells. In a number of plants particular compounds are only synthesized or accumulated in particular organs or tissues such as glands, laticifers and secretory ducts (TABATA, 1977). WIERMANN (1981) reviewed secondary plant products and cell and tissue differentiation in plants, however, in the case of plant tissue cultures, TABATA (1977) stated that differentiation may induce the production of desirable compounds, while often product synthesis normally found in specialized tissue can be associated with unorganised tissue cultures.

Differentiation has been shown to increase product formation (HIRAOKA and TABATA, 1974; VERZAR-PETRI, LADOCSY and OROSZLAN, 1982). HAGIMORI, MATSUMOTO and KISAKA (1980) found that root forming callus produced no more digitoxin or digoxin than did dedifferentiated callus, however, shoot forming callus produced higher amounts of metabolite than that normally found in Digitalis plants. This ability of shooting callus to increase metabolite production was also shown by TABATA, YAMAMOTO, HIRAOKA, MARUMOTO and KONOSHIMA (1971). FREEMAN, WHENHAM, MACKENZIE and DAVEY (1974) and TABATA, YAMAMOTO, HIRAOKA and KONOSHIMA (1972) found that when callus produced roots there was an increase in the amount of metabolite produced in culture. UOMORI and TOMITA (1974) showed that callus cells were unable to produce saikosaponins, but on induction of roots in culture high concentrations were recorded, indicating that the induction of organogenesis can be industrially applied to drug production using the large number of cells obtained from suspension culture. TABATA, MIZUKAMI, HIRAOKA and KONOSHIMA (1974) found that morphogenesis was not necessary for product synthesis, while YEOMAN, MIEDZYBRODZKA, LINDSEY and McLAUCHLAN (1980) showed that differentiation was detrimental to product synthesis.

DOUGALL (1979) and KURZ and CONSTABEL (1985) concluded that the assumption that specific structures should be present for metabolite production and that the gene required for accumulation is linked to morphological development, is unlikely due to gene specificity. It is the cultural conditions that enhance structural specialization that coincide with metabolite formation. It is thought that it should be possible to find cultural conditions where metabolite formation is enhanced without stimulating morphogenesis, viz. KAMO, KIMOTO, HSU, MAHLBERG and BILLS (1982) showed that laticifers were not required for metabolite production in Papaver cultures. KURZ and CONSTABEL (1985) go on to say that "products normally found in specialized cells may be synthesised and accumulated in callus cells and detection may depend on improved analytical techniques.

In plant suspension cultures the growth cycle exhibits a typical lag, exponential and stationary phase, the synthesis and accumulation of metabolites is normally associated with the late exponential and stationary phases. The inoculation density of suspension cultures has already been discussed in Chapter 2. The production of metabolites per unit volume is a commercially important factor. FUJITA and TABATA (1987) estimated a maximum density at ± 30 - 120 g dry weight of cells per one litre of tank space, and thus one must take into account problems associated with this high number of cells. To ensure adequate growth, the cells must be agitated without damaging the cells and there must be a sufficient supply of oxygen and nutrients.

There is a certain amount of debate as to the correlations that can be drawn between cultures grown on a solid substrate as opposed to a suspension culture. BARZ and ELLIS (1981) have recorded that surface grown cultures are of little value for commercial metabolite production due to; slow growth, the heterogenous nature of the callus, the degree of differentiation and the lack of correlation between high production of metabolites in solid culture to those in liquid culture. However, callus cultures are of scientific interest as they provide excellent systems to investigate the biochemistry and commercial potential of the desired compound.

In callus cultures variation in polyploidy and karyotype often occur, resulting in heterogenous cultures. These changes are probably due to endoreduplication, endomitosis or a selective advantage obtained by the changed genome. In general there is a loss in the production potential due to genetic instability (KURZ and CONSTABEL, 1985). Variation can regulate secondary metabolism which may also lead to an increase in the production capacity of the cells. Variation leads to instability of the cultures and ultimately variation in metabolite production with slow growing callus appearing to be more stable than fast growing callus.

FUJITA and TABATA (1987) recorded a case where the productivity of protoplast derived culture lines was equal to that found in the plant. Why the protoplast selection method is better than the normal selection method is unknown. KURZ and CONSTABEL (1985) reported a case where a non-metabolite producing line was changed to a new media with a resulting increase in the number and amount of alkaloids produced, however, two years later they innoculated the original callus line onto this "different" medium and got no response. This raises the question whether the competence for restoration of synthesis and accumulation has been lost over the two year period. Table 5.8 gives an example where there was a progressive decrease in product synthesis in a cell line over a period of eight years. (On the other hand some cell lines may be stable).

Table 5.8 Production behaviour of one Catharanthus roseus strain during an eight year period after one initial clonal selection (derived from COLLIN, 1987)

Year	Dry wt ($g\ l^{-1}$)	Serpentine ($mg\ l^{-1}$)	Ajmalicine ($mg\ l^{-1}$)
1976	15.8	290	60
1977	18.6	159	12
1978	17.3	54	56
1979	18.0	14	22
1980	16.7	11	19
1981	17.0	10	19
1982	16.8	8	12
1983	15.2	6	10
1984	17.2	4	10

The degradation of alkaloids is common and may be an important factor in determining the alkaloid content of a plant; unfortunately "our knowledge of the control of alkaloid breakdown is virtually nonexistent" (BECKER, 1987).

EILERT, KURZ and CONSTABEL (1987) are of the opinion that the use of elicitors for stimulation of secondary product production will have a great impact on plant tissue culture. When Papaver cultures were subjected to a number of biological elicitors from a range of microorganisms, it was found that there was an increase in sanguinarine in response to the elicitors (EILERT, KURZ and CONSTABEL, 1985). Papers have now been published in which both advantages and disadvantages of these elicitors are described. It is advocated that the elicitor approach should be closely studied as it offers an interesting alternative to media and environmental manipulation.

Inhibitors may also play a role in secondary metabolite research in culture. Inhibitors can be applied to cultures to increase the desired product or alternatively to "reroute" the biosynthetic pathway of the cell towards the production of the desired metabolite. Feedback inhibition also plays a role in product formation with the accumulation of the product in the cell resulting in inhibition of metabolite synthesis because specific cell structures are not present. Artificial absorbants (charcoal, silica gel) may be utilized to absorb metabolites and change the balance of the regulation of biosynthesis (BECKER, 1987).

Methods of mass culture for plant suspensions have been reviewed by KURZ and CONSTABEL (1979b) and MARTIN (1980b). The following figure represents three types of culture systems which can be employed for mass culture, including the Kurz system, which utilizes compressed air for agitation and aeration, the V fermenter by Veliky and Martin which uses a magnetic stirrer for agitation, while the phytostat of MILLER, SHYLUK, GAMBORG and KIRKPATRICK (1968) can be utilized for batch and continuous culture methods.

There are three basic forms of plant suspension culture:

i) batch culture, in which growth takes place in a closed system with a limited amount of media, which during culture may lead to variation in culture conditions;

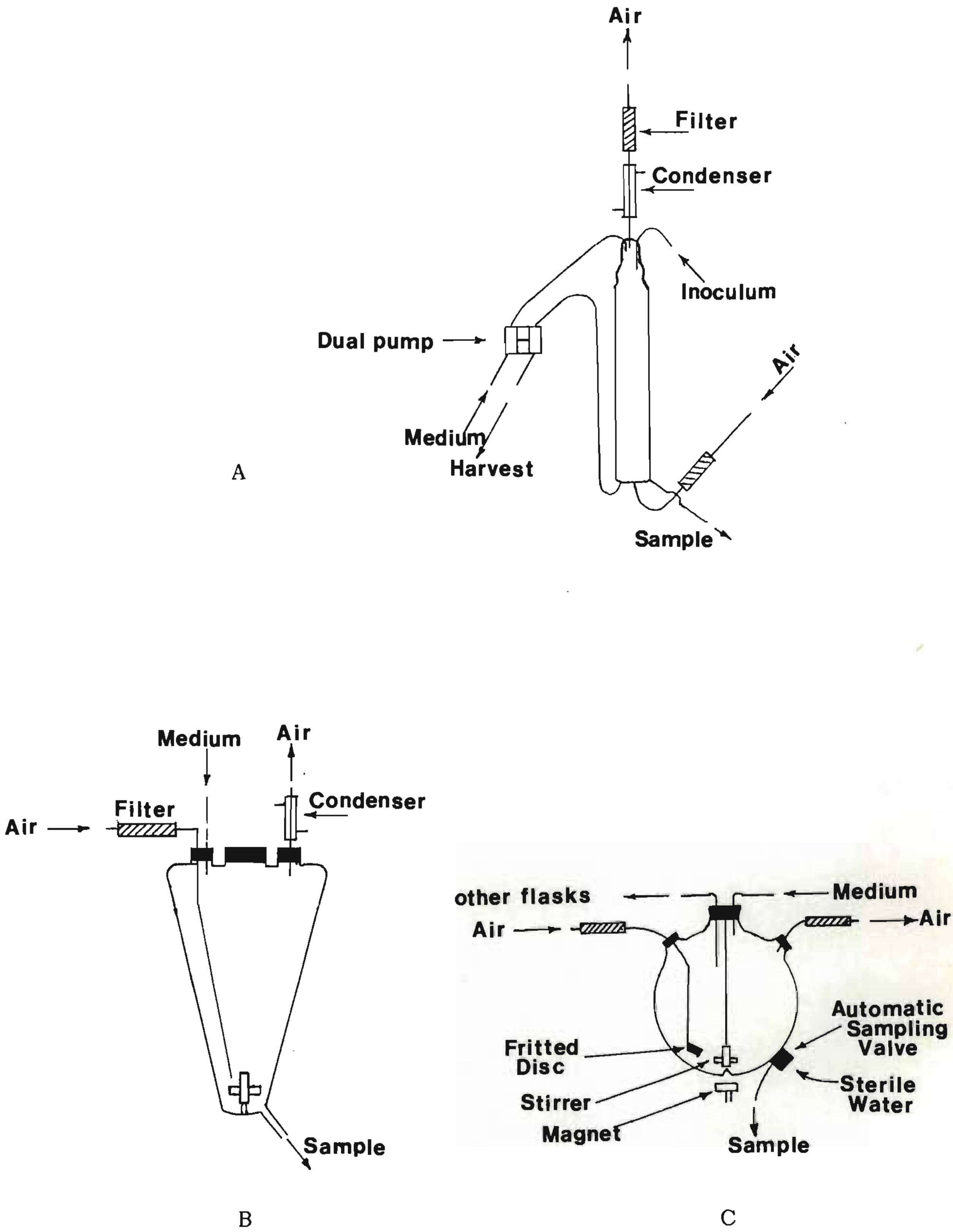


Figure 5.12 Three culture systems used for mass culture; a) Kurz system; b) V fermenter; c) Miller phytostat.

ii) continuous culture; this "open system" allows for continuous addition of media resulting in a steady state for growth, the system also allows for controlled nutrient feeding (especially precursors);

iii) continuous synchronous culture; this is also an open system but the cells are in the same stage of physiological development; this has the advantage of isolating compounds that only occur at certain stages of the growth cycle (MARTIN, 1980b).

TABATA (1977) concluded that for successful commercial application of plant cell cultures the following four points should serve as the minimum requirement:

i) the rate of cell growth should be high in order to give a high yield as soon as possible;

ii) the cells should be genetically stable;

iii) the metabolite should preferably be released into the medium without product degradation; and

iv) the cost of media, precursors and the chemical extraction technique should be low.

BARZ and ELLIS (1981) have recorded that plant tissue cultures are only economically feasible when those products selected for, are costly and plant specific. On the other hand BERLIN and BODE (1987) have stated that the feasibility of commercial product formation via tissue culture is related too closely with cost comparisons of field cultivation, and they feel that at our present state of knowledge of metabolite production in culture, cost discussion is not yet meaningful.

Despite these negative reports FUJITA and TABATA (1987) reported that 39 patents on useful secondary metabolites in culture were taken out in Japan between 1980 and 1984 and conclude that this large increase in the number of patents applied for gives an indication of the commercial interest in the production of metabolites from tissue culture. MISAWA (1977) lists a number of Japanese patents which involve the production of natural substances by plant cell culture; however, at present there is still only one compound that is commercially produced, shikonin from Lithospermum cultures (COLLIN, 1987). ALFERMANN and REINHARD (1978) have stated that quick success, as far as commercial application of plant cultures is concerned, is unlikely, whereas FUJITA and TABATA (1987) are far more positive and in concluding their paper it is stated,

> "many of us here are engaged in the search for speedier and more wide ranging methods for plant cell and tissue cultures that can be used to ensure the efficient production of useful plant metabolites. It is my belief that the end of that search will come in the foreseeable future."

HAYASHI, YOSHIDA and SANO (1988) on the assumption that secondary metabolites can be obtained in large amounts from tissue culture, felt that Colchicum cultures should be able to produce colchicine in a fermentor tank. The rationale behind the following research is similar, to investigate the ability of Gloriosa and Sandersonia tissue cultures to produce the alkaloid colchicine, and to control the production of colchicine in vitro by means of tissue, media and environmental manipulation.

CHAPTER 6

PRODUCTION OF COLCHI

6.1 Introduction

The present trend in biochemical production is via artificial synthesis, howe many desirable compounds still have to be extracted from plant material for the production of medicines, flavouring agents, essential oils, pesticides and herbicides. Thus plants remain a vital source of biochemicals as well as a source of new natural products.

Since the discovery of colchicine in *Gloriosa* by CLEWER, GREEN and TUTIN (1915) a number of researchers have isolated and quantified colchicine from *Gloriosa*. A number of researchers have put forward the idea that *Gloriosa* could serve as a commercial source of colchicine (EIGSTI and DUSTIN, 1955; WATT and BREYER-BRANDWIJK, 1962; NARAIN and KHOSHOO, 1967; SARIN, JAMWAL, GUPTA and ATAL, 1974; NARAIN and RAINA, 1975; SRIVASTAVA and CHANDRA, 1977; BELLET and GAIGNAULT, 1985).

Colchicine is known to inhibit mitosis, interfere with the orientation of fibrils, induce polyploidy, relieve symptoms of gout and play a role in cancer therapy. These phenomena together with the fact that *Gloriosa* and *Sandersonia* are valuable to the pharmaceutical industry makes these plants ideal species for the study of colchicine biosynthesis and production using an *in vitro* system.

6.2 Materials and methods

6.2.1 Plant material

In this study, material used included both mature and immature *Gloriosa* and *Sandersonia* plants grown *in vivo* as well as cultured tissue produced using the techniques outlined in Chapter 3. The collection and cultivation of *Gloriosa* and *Sandersonia* plant material is reported in Sections 3.2.1 and 3.2.2. *Littonia modesta* and *Androcymbium melanthioides*, members of the Colchicaceae indigenous to Southern Africa were also collected to determine colchicine content and suitability as a commercial source of colchicine.

6.2.2 Extraction technique

Two techniques for the extraction of colchicine from plant material were used to compare the efficiency of the techniques. The first technique was based on the extraction procedure used by SANTAVY (1950) (Table 6.1), the second was a technique based on the extraction of colchicine for commercial manufacture, as described by HAMERSLAG (1950), Chapter 5.1 and the techniques of WALASZEK, KELSEY and GEILING (1952) and HOFMAN (1978) (Table 6.2).

Using the technique outlined in Table 6.2 the petroleum ether, diethyl ether and aqueous extracts were analysed to determine whether colchicine loss occurred at these various stages of extraction. Using soybean callus impregnated with a known amount of colchicine the extraction procedure was performed to determine the efficiency of the extraction technique. All subsequent calculations of colchicine content were corrected using the factor for extraction efficiency. All extracts were stored at 10°C in the dark to prevent the degradation of the colchicine to lumicolchicine (WILDMAN and PURSEY, 1968).

Table 6.1 Extraction technique for colchicine based on the technique described by SANTAVY (1950)

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i) Freeze dried and powdered plant material was percolated with methanol (± 10 mls per g plant material);

ii) Methanol evaporated off using a rotary evaporator;

iii) Residue dissolved in 100 ml dH_2O;

iv) Aqueous solution partitioned three times with 100 ml ether and five times with 100 ml chloroform;

v) Ether and chloroform extracts washed twice with 30 ml dH_2O;

vi) Solutions dried with anhydrous sodium sulphate and the solvent removed by distillation;

vii) Chloroform extract dissolved in 45 ml benzene and filtered in a column with 15 g $A\ell_2O_3$ (column filled with petroleum ether bp 40 - 60°);

viii) Elution was performed using the following solvents (45 ml per solvent);

1	benzene
2	benzene ether 1 : 1
3	ether
4	ether chloroform 4 : 1
5 - 7	ether chloroform 2 : 1
8 - 11	ether chloroform 1 : 1
12 - 14	chloroform
15	chloroform
16	chloroform
17	chloroform methanol 98 : 2
18 - 20	chloroform methanol 96 : 4
21	chloroform methanol 92 : 8
22	chloroform methanol 84 : 16
23	chloroform methanol 70 : 30
24	chloroform methanol 60 : 40

fraction 12 - 14 yields colchicine.

(As this technique was time consuming the extraction procedure outlined in Table 6.2 was used for all subsequent extraction)

Table 6.2 Extraction procedure for colchicine, based on the techniques of HAMERSLAG (1950); WALASZEK, KELSEY AND GEILING (1952); HOFMAN (1978). (Figures in brackets indicate volumes used for 1 g of plant material)

---

i) 20 g Freeze dried material extracted using 200 mℓ (100 mℓ) methanol in a cold room at 10°C for two hours;

ii) Extract centrifuged at 4000 rpm for five minutes (remove plant material);

iii) Methanol extract evaporated off in a rotary evaporator;

iv) Residue redissolved in 50 mℓ dH_2O;

v) Aqueous extract centrifuged at 10 000 rpm for five minutes;

vi) Supernatent shaken twice in a separating funnel with 100 mℓ (50 mℓ) petroleum ether bp 40 - 60°;

vii) Aqueous extract shaken once in 100 mℓ (50 mℓ) diethyl ether;

viii) Aqueous extract washed five times in 50 mℓ chloroform, which is evaporated to dryness;

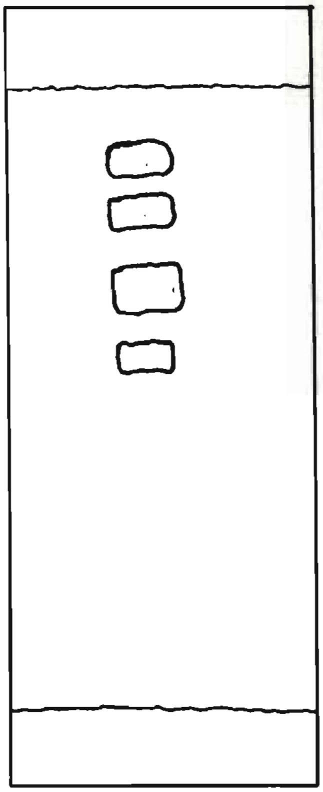
ix) Residue redissolved in 3 mℓ HPLC grade methanol (95%);

x) Extract filtered through a 0,45 µm millipore filter.

Extract can be used for high performance liquid chromatography or thin layer chromatography).

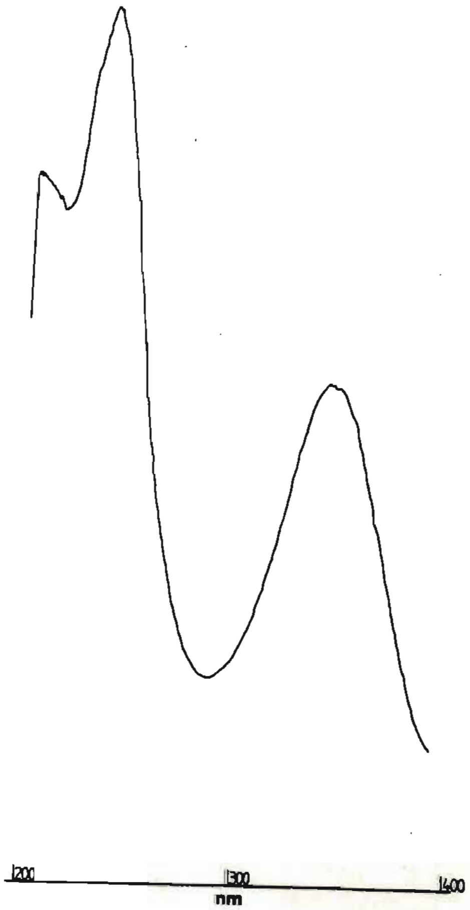
6.3.2 Thin layer chromatography (TLC)

Extracts were applied to Merck Kieselgel 60 F254 thin layer chromatography (TLC) plates and partitioned using a solvent system of chloroform : acetone : diethylamine (5 : 4 : 1) (ERTEL and WALLACE, 1970; HOFMAN, 1978) (Figure 6.1). Use of this solvent system has a drawback in that after ± 12 hours the solvent solution turns yellow, and therefore the solvent system must be made regularly. Using the silica gel plates colchicine spots show up under ultra violet light (245 nm) (ERTEL and WALLACE, 1970). The various Rf's were recorded and the desired bands were re-



Rf		Spot no.
0,87		4
0,79		3
0,67	= colchicine	2
0,56		1

Figure 6.1 Typical separation of colchicine using thin layer chromatography (TLC)



moved from the TLC plate and eluted using methanol. The eluant was filtered through Whatmans no 44 filter paper using six mℓ of methanol and taken to dryness in vacuo at 35°C. The residue was redissolved in one mℓ of methanol and subjected to spectrophotometry for quantification. (For quantification of colchicine contents, care was taken to use standard amounts of extract and silica gel from the TLC plate). A factor calculated as the degree of recovery of colchicine from the TLC plate was determined and included in calculations for colchicine content. "Purification" of the extract using TLC was necessary for quantification experiments as other compounds absorbing at 245 nm were also found in the extracts (see HPLC graphs of crude extracts).

6.2.4 Ultra violet spectrophotometry

Current official procedures for colchicine quantification involves the use of ultra violet (UV) spectophotometry. The spectrum for colchicine standards and various extracts were determined using a Varian DMS 90 UV spectrophotometer using a scan from 400 to 200 nm (Figure 6.2). Characteristic response curves for colchicine were determined with a maximum absorbance at 245 nm. For optimum quantification of the colchicine the absorbance of the extracts were read at 245 nm. WILSON and FRIEDKIN (1966) showed that colchicine concentrations could be determined using UV light. Standard curves for colchicine dissolved in both methanol and water were constructed at 245 nm for the quantification of colchicine in the extracts (Figures 6.3 and 6.4) (FORNI and MASSARANI, 1977; BRITISH PHARMACOPOEIA, 1980; US PHARMACOPOEIA, 1982; THOMPSON, 1985).

The technique of KING (1951) outlined in Table 6.3 based on the colour reaction of acid treated colchicine and ferric chloride, was also utilized for colchicine quantification. However, this technique is only reproducable if the alkaloid is in a pure form or if there is a high percentage of the alkaloid in the extract.

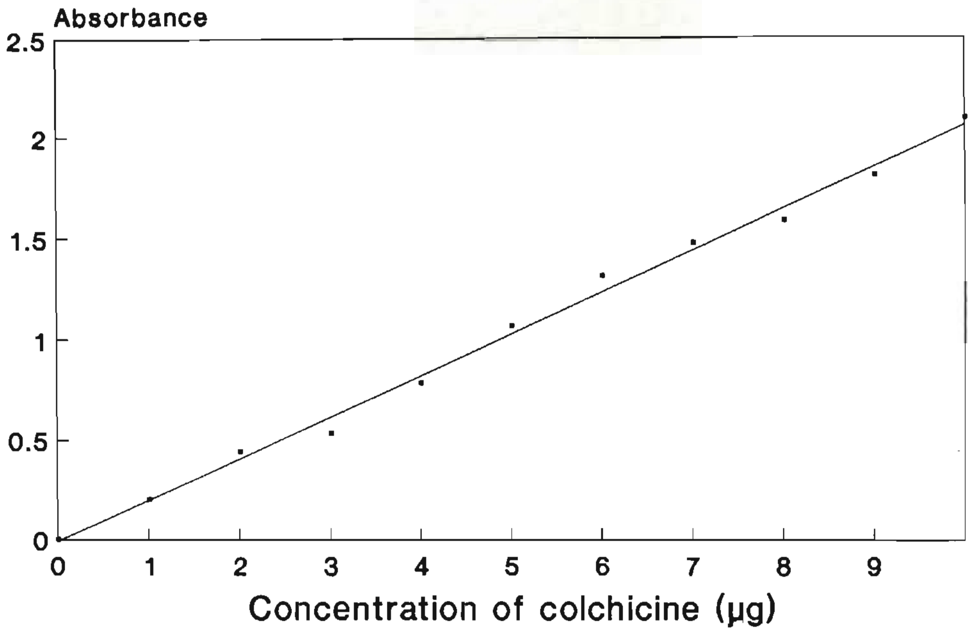


Figure 6.3 Calibration curve for colchicine, using methanol as the solvent.

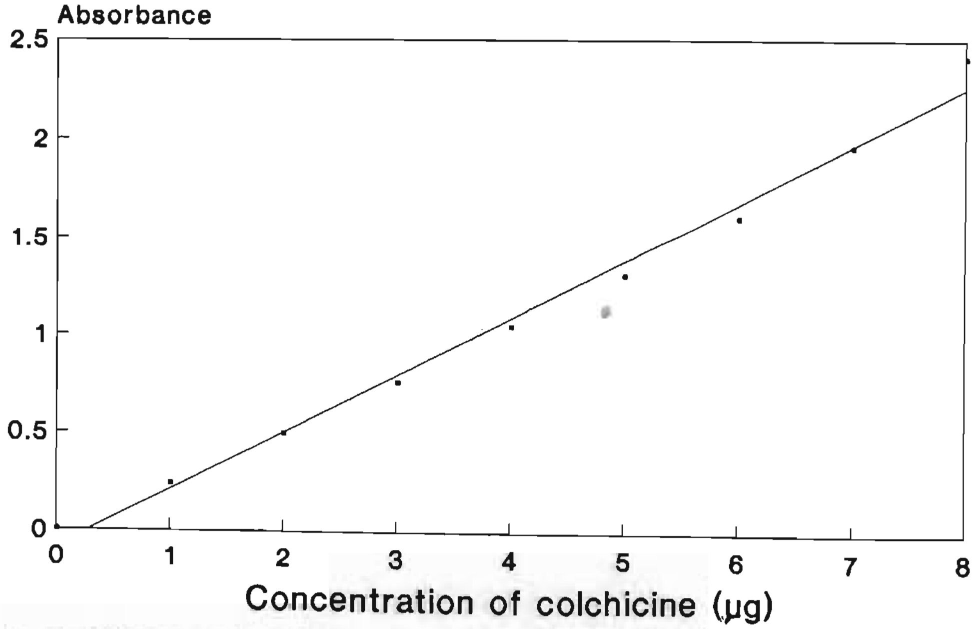


Figure 6.4 Calibration curve for colchicine, using water as the solvent.

Table 6.3 Colorimetric technique for colchicine determination after KING (1951)

---

i) Pure colchicine containing 2,5 mg was treated with 25 mℓ; nHCl in a Erlenmeyer flask by warming on a steam bath for one hour and then made up to 25 mℓ; with nHCl;

ii) Aliquots containing up to 0,5 mg of colchicine, were made up to 5 mℓ with nHCl and 0,1 mℓ of 5% ferric chloride, and the resultant colour read at 470 nm to construct a calibration curve;

iii) Extracts were treated as above.

6.2.5 High performance liquid chromatography (HPLC)

Known amounts of colchicine standards, crude extracts and extracts derived from TLC were subjected to high performance liquid chromatography (HPLC) using a Varian 5000 HPLC. After various combinations of solvent compounds and ratios, columns and detector conditions, the following was found to be successful for colchicine determination:

column : Hypersil 5 ODS, 250 x 4 mm ID;
solvent : Methanol, 0,2 M Acetic acid buffered with triethylamine at pH 3,5.
50 : 50 to 80 : 20 over a 30 minute period;
flow rate : 1 mℓ min^{-1};
pressure : 196 - 202 atmospheres;
sensitivity : 0,05;
detector : 254 nm;

(A more detailed representation of the programme used for HPLC is presented in Appendix 1).

In order to determine colchicine concentrations a standard curve was produced using known amounts of colchicine (Figure 6.5). Use of the HPLC for colchicine quantification as advocated by THOMPSON (1985) and tentative identification can be very precise, however, quantification using this technique can be time consuming and expensive (with regards the expense of pure chemicals used for HPLC). Figure 6.6 represents typical HPLC traces found for colchicine standards. Figure 6.6b represents the colchicine standard after 12 months in storage, with a large peak at ± 13 minutes. This peak is presumably the photoconverted compound (lumicolchicine). For this reason all extracts were processed within five days, to prevent breakdown of the colchicine compound.

6.2.6 Gas chromatography mass spectroscopy (GCMS)

Extracts from cultured material of Gloriosa and Sandersonia and corm and leaf extracts of Littonia and Androcymbium were chromatographed using TLC. The "spots" corresponding to colchicine were eluted and run on the HPLC, the fraction that co-chromatographed with colchicine was collected, dried and identified using gas chromatography mass spectroscopy (GCMS). Samples were identified using a Hewlett Packard 5988a GCMS. The column used was an HP1 crosslinked dimethyl silicone column with a helium flow rate of 30 cm. Initial temperature was 150°C for three minutes and then increased to 290°C with 30° increases and the injection port temperature was 290°C. GCMS analysis was performed with the assistance of Professor S.E. Drewes and Mr M. Watson of the Chemistry Department, University of Natal, Pietermaritzburg.

6.2.7 *In vivo* plant experiments

Plant material grown in pots in the garden (Section 3.2.1) were harvested, the soil was removed by washing in water, and the various organs were cut into sections and freeze dried in the dark. Initial extraction was performed using material

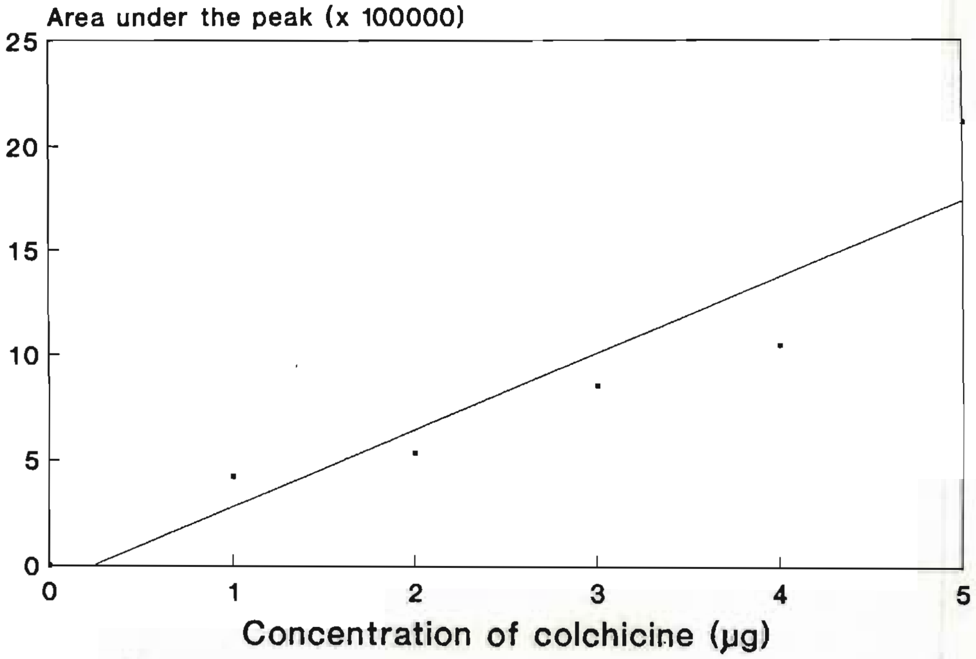


Figure 6.5 Calibration curve for colchicine using the HPLC.

Figure 6.6 High performance liquid chromatograph of colchicine standards

a) colchicine standard

b) colchicine 12 months after the solution was made up (note lumicolchicine peak at 12,7 minutes)

c) residue colchicine in the aqueous fraction after extraction from Gloriosa to determine whether there was a quantitative difference in the colchicine content of; corm, leaves, flowers, ovaries and seeds. The colchicine contents were quantified and identified using GCMS, HPLC and UV spectrophotometry. Following these intial extractions, isolation of the colchicine was performed using Sandersonia, Littonia and Androcymbium leaf and corm tissue.

Seasonal variation in Gloriosa plant material was also determined using plant material harvested as;

new seedlings;
mid-season plantlets;
mid-winter dormant corms, and
mid-winter non-dormant plantlets.

The relative concentration of colchicine and composition of the extracts were determined using HPLC and spectrophotometry. In all the experiments three replicates were used for each experiment.

6.2.8 In vitro investigation

Using cultured Gloriosa and Sandersonia tissue (as described in Chapter 3) extracts were made and quantified, to determine whether colchicine production can be achieved in vitro and whether the technique can be made economically viable.

i) Determination of the ability of callus tissue and root tissue to produce colchicine in vitro. Extracts were made from callus tissue grown on 2,4-dichlorophenoxyacetic acid and kinetin and from malformed roots which were produced when callus was removed from the source of 2,4-dichlorophenoxyacetic acid (Chapter 3). Both Gloriosa and Sandersonia tissue was tested. Fifteen individual replicate cultures were grown for each experiment, these replicates being randomly divided into three lots and extracted and quantified (this procedure was followed for all in vitro experiments).

ii) The effect of hormones on colchicine production *in vitro*. Synthetic (napthaleneacetic acid, kinetin) hormones, and natural (indoleacetic acid, zeatin) hormones, were utilized to determine whether the concentration or type of hormone could inhibit or stimulate colchicine production. A 4 by 4 grid (0, 0,1, 1, 5 mg ℓ^{-1}) of synthetic hormones was tested for both *Gloriosa* and *Sandersonia* tissue, while a 3 by 3 grid (0, 0,1, 1 mg ℓ^{-1}) was used for *Gloriosa* callus to determine whether natural hormones affect colchicine production *in vitro*.

iii) Old and new tissue. Old and new callus was extracted to determine whether relative age affects tissue ability to produce colchicine *in vitro*. new callus (three months old) and old callus (30 months old) was grown in
0 mg ℓ^{-1} BA, 5 mg ℓ^{-1} NAA
1 mg ℓ^{-1} BA, 0 mg ℓ^{-1} NAA
1 mg ℓ^{-1} BA, 1 mg ℓ^{-1} NAA
1 mg ℓ^{-1} BA, 5 mg ℓ^{-1} NAA
5 mg ℓ^{-1} BA, 0 mg ℓ^{-1} NAA,
and extracted for colchicine;

iv) Colchicine content and time in culture. Plant tissue was harvested at various time intervals (20, 30, 40, 60 and 100 days) to test the amount of colchicine in the plant tissue as a function of time after a subculture.

v) The effect of environmental stress. Plant tissue was grown in light,low light, dark, "outside" (see Section 3.2.9) and in fluctuating dark - light conditions. Extractions were made to determine which lighting regimes affected the ability of the plant tissue to produce colchicine.

vi) Temperature effect on colchicine production. Callus tissue was grown at 10°, 20° and 25°C and extracted to determine the relative amounts of colchicine.

vii) Carbohydrate effect. Various levels of sucrose (2%, 4% and 8%) were added to the culture medium to determine the effect of a carbon source on colchicine production.

viii) Nitrogen availability. Callus tissue was grown on Murashige and Skoog medium with variations in the amount of available nitrogen (both NH_4NO_3 and KNO_3 were reduced). Nitrogen was available at full, half, quarter and eighth strength of the normal concentration of nitrogen.

ix) Liquid and suspension cultures. Gloriosa and Sandersonia roots were grown in a liquid medium and extracted to determine whether colchicine is produced in a liquid culture environment. Suspension cultures were extracted for colchicine (both cell material and media) to determine whether the technique of plant suspension culture can be applied, for commercial colchicine production. The hormones used to supplement the media were as follows:

0,1 mg ℓ^{-1} kinetin	0,1 mg ℓ^{-1} NAA
0,1 mg ℓ^{-1} kinetin	1 mg ℓ^{-1} NAA
1 mg ℓ^{-1} kinetin	0,1 mg ℓ^{-1} NAA
1 mg ℓ^{-1} kinetin	1 mg ℓ^{-1} NAA

x) Callus tissue was fed with precursors to determine if precursor feeding can increase secondary metabolite production. Phenylalanine, cinnamic acid and tyrosine were applied to the culture media at concentrations of 0, 10^{-4}M, 10^{-6}M, 10^{-8}M and 10^{-10}M. Both plant tissue and the solid media were extracted and quantified. WILDMAN and PURSEY (1968) showed that phenylalanine is a precursor for colchicine. Carbon labelled (^{14}C) phenylalanine (Amersham) was added to a liquid medium and callus tissue was incubated for 72 hours. Callus and the media were extracted and the radioactivity from HPLC separation was determined using a Beckman LS 3800 scintillation counter. Fractions collected after HPLC were dried and suspended in Beckman Readysolv scintillator cocktail to facilitate detection (the ^{14}C phenylalanine was added at a concentration of ± 14 400 DPM per mℓ).

xi) Production of colchicine in whole plants grown in vitro. Plantlets grown in vitro were divided into original explant, corm and leaf material and each component was extracted for colchicine, to determine whether differentiation of plant organs is necessary for the production of colchicine in vitro.

The specific details of tissue types, hormones, light regimes, temperature and nutrient media, are fully detailed in Chapter 3. Three replicates were made for each treatment and using the factors for extraction efficiency and efficiency of compound removal from TLC an equation was constructed to calculate colchicine content. All concentrations are expressed as a percentage of the tissue dry weight.

6.3 Results and discussion

6.3.1 Extraction quantification and identification

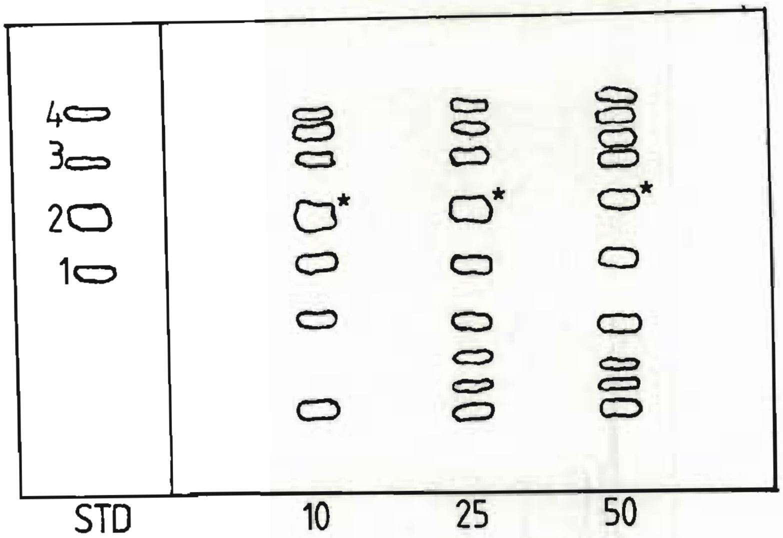
The extraction technique of SANTAVY (1950) was effective for colchicine removal from plant tissue, however, it was time consuming. Use of the extraction technique adapted from HAMERSLAG (1950), WALASZEK, KELSEY and GEILING (1952) and HOFMAN (1978) is recommended as it is relatively simple. It is important not to include portions of the aqueous extract together with the chloroform fraction as it is difficult to remove from the chloroform extract, and affects the final solution of the extract when resuspended in methanol. The extraction technique advocated is 87,5% effective assuming that 100% extraction is achieved during the methanol phase. Loss of colchicine in the petroleum ether and diethyl ether fractions was negligible. The percentage of colchicine remaining in the aqueous extract was ± 0,5% after five washes in chloroform. This leads to the conclusion that the remainder of the spiked colchicine (12%) was lost during the resuspension of the residues after evaporation.

Figure 6.7 gives a representation of the standard and <u>Sandersonia</u> corm extracts applied to TLC at different concentrations. The HPLC traces represented inFigure 6.8 represents the HPLC profiles of the various spots (1 - 4) after elution from the TLC plate. Figure 6.9 represents the UV scan characteristics of the various spots using a scan from 400 - 200 nm. All ten zones represented in Figure 6.9a were scanned but only "spot 2" gave a typical colchicine curve using the spectrophotometer. The HPLC trace for "spot 2" co-chromatographed with authentic colchicine. The second most prominent "spot" from the commercially available colchicine was "spot 1" which had a mass ion of 281. In order to positively identify "spots 1 and 2", they were subjected to GCMS with the results and the fragmentation data displayed inFigures 6.10 and 6.11.

For all further extraction quantification and identification experiments, the extraction technique outlined in Table 6.2 was used, and "spot 2" was scraped off and eluted from the TLC plate. Quantification was performed using the HPLC and the spectrophotometer, using methanol as the solvent. Although the use of water as a solvent gave similar results (Table 6.4), use was made of methanol because it is more volatile and easier to remove, especially when spotting plates and evaporating the eluant.

Table 6.4 A comparison of the levels of colchicine obtained using water and methanol as solvent systems

Extract	Amount of colchicine in µg from 1 mℓ of extract	
	Methanol	Water
root	50 306	43 474
leaf	1 932?	3 864
seed	33 123	31 053
ovary	4 140	5 520
root in liquid culture	717	634



STD = Standard

10 ; 25 ; 50 = Amount of <u>Sandersonia</u> extract applied per spot (µl)

Rf's

	10	25	50
	0.88	0.90	0.92
	0.84	0.84	0.86
			0.81
	0.77	0.77	0.77
colchicine	0.62*	0.64*	0.66*
	0.50	0.50	0.51
	0.35	0.35	0.33
		0.25	0.25
		0.18	0.18
	0.11	0.11	0.11

Figure 6.7 TLC of the standard and a <u>Sandersonia</u> corm extract applied at different concentrations

Figure 6.8 HPLC traces of the various "spots" obtained using TLC (see Figure 6.1)

a) colchicine standard
b) TLC plate
c) "spot 1"
d) "spot 2" (colchicine)
e) "spot 3"
f) "spot 4"

Figure 6.9 UV scan of the "spots" derived after separation of a corm extract on TLC (see Figure 6.7). NB. Only "spot 7" = "spot 2" shows the typical colchicine curve

```
Colchicine
Average of 13.141 to 13.388 min. from DATA:88GC362.D
Colchicine Std,Finnie,          150(3)-290@30.IP290.
    m/z       abund.      m/z      abund.      m/z      abund.       m/z      abund.

   51.00         15     131.00        17     196.00        57     256.00         41
   52.00         17     133.00       305     197.00        88     265.00        119
   55.00         27     139.00       387     198.00        29     266.00        303
   57.00         17     140.00       258     199.00        40     267.00        344
   58.00         20     141.00       113 ►207.00       1885     268.00        263

   60.00         53     142.00        47     208.00       603     269.00        374
   63.00         32     147.00       232     209.00       451     270.00        178
   69.00         18     148.00        71     210.00        66     271.00         62
   73.00        721     150.00        22     211.00       246     280.00         26
   74.00         45     151.00        95     212.00        59 ►281.00       1908

   75.00        127     152.00       451     213.00        51     282.00        750
   76.00         94     153.00       148     221.00       133     283.00        288
   77.00        160     154.00       114     222.00        51     284.00        106
   78.00         38     155.00       132     223.00        62     294.00         95
   81.00         20     156.00        64     224.00        44     296.00         45

   82.00         20     163.00        24     225.00        25 ►297.00       1317
   87.00         24     165.00       108     226.00        24     298.00        557
   89.00         28     166.00        20     227.00        57     299.00        207
   91.00        114     167.00       137     228.00        18     300.00        308
   96.00        390     168.00       114     236.00        45     311.00        417

   97.00         33     169.00        33     237.00        64 ►312.00       2282
  102.00         53     177.00        47     238.00       183     313.00        442
  103.00         46     178.00        27     239.00       293     314.00         92
  104.00         16     180.00        52     240.00        48     325.00         23
  113.00         20     181.00       135     241.00        23     327.00         16

  114.00         74     183.00       172     242.00        18     328.00        129
  115.00        280     184.00        31     249.00        26     340.00         59
  117.00         54     185.00        18     251.00       195     341.00         22
  119.00         42     191.00       193     252.00        79 ►356.00         60
  126.00        101     192.00        23     253.00        86     371.00        484

  127.00        314     193.00        95     254.00       570 ►399.00        912
  128.00        209     194.00        17     255.00       109     400.00        154
  129.00         33     195.00        92
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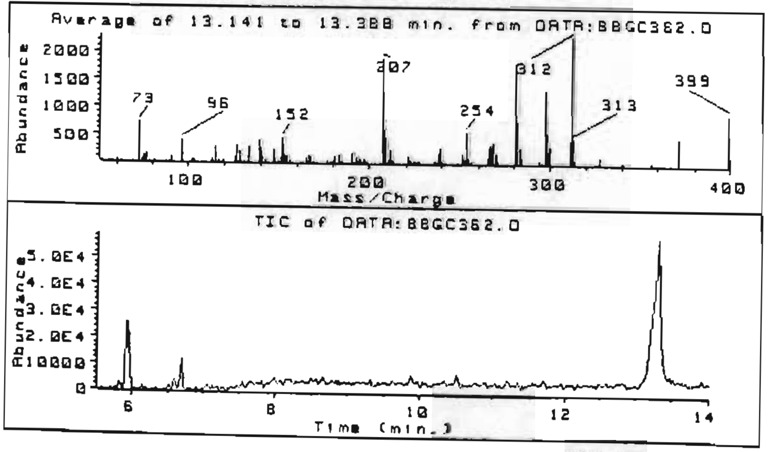

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T: Scan 312 (13.285 min) of D
Z: TIC of DATA:88GC362.D
Y: Scan 311 (13.260 min) of D
X: Average of 13.141 to 13.38
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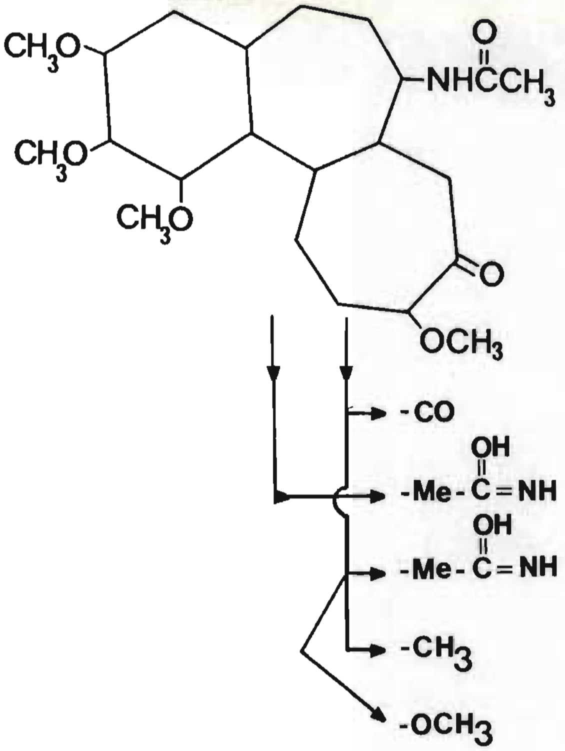
Figure 6.10 GCMS of the colchicine standard (arrows indicate diagnostic fragmentation peaks of colchicine)

Colchicine

MASS



399

371

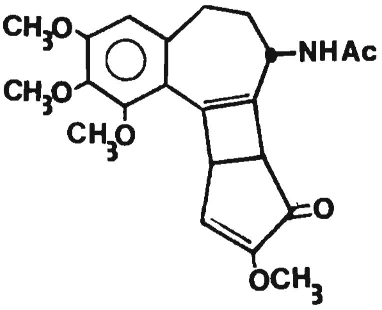
340

312 base peak

297

281

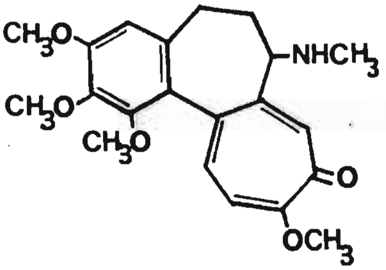
Lumicolchicine



no intense peak at 371

356 base peak (M-43)
(Wildman and Pursey, 1968)

Demecolcine



has a base peak at m/e 207
(M-164)

Figure 6.11 Fragmentation of colchicine and colchicine congeners during mass spectrometry.

A comparison of the results obtained using the spectrophotometric technique and the HPLC technique for colchicine quantification is displayed in Table 6.5. Due to the cost and time factor involved using the HPLC technique, use of the spectrophotometric technique is advocated as the results were very similar to those found for HPLC.

Table 6.5 Quantification of colchicine from various extracts using HPLC and spectrophotometric techniques (values given as % dry weight)

Extract	Spectrophotometer	HPLC
Gloriosa root	0,9075	0,9817
Gloriosa leaf	0,0687	0,0522
Gloriosa seed	0,6050	0,7150
Gloriosa ovary	0,0825	0,1375

For the tentative identification of colchicine and quantification of colchicine at relatively high levels the use of HPLC is advocated. The advantages of using the HPLC technique include the fact that the extract did not have to be "purified" extensively, and the quantification can be made by using the area under the peak for colchicine to determine its concentration. The spectrophotometric technique is accurate and reliable and is still used for official procedures (FORNI and MASSARANI, 1977; BRITISH PHARMACOPOEIA, 1980; US PHARMACOPOEIA, 1982; THOMPSON, 1985).

CLEWER, GREEN and TUTIN (1915) used 42,52 kg of dried corms for extraction. For identification and quantification of alkaloids present in this study 1 g of plant material could be extracted to give reproducable results.

6.3.2 In vivo plant experiments

Colchicine from the various organs of Gloriosa was found to be at levels similar to that found by NTAHOMUUKIYE, HAKIZIMANA, NKILIZA and PUYVELDE (1984) and BELLET and GAIGNAULT (1985) and not at levels reported by CLEWER, GREEN and TUTIN (1915), EIGSTI and DUSTIN (1955) and THAKUR, POTĚSILOVA and SANTAVY (1975). See Table 5.5.

Levels of colchicine found in the various organs of Gloriosa are summarized in Table 6.6.

Table 6.6 Comparison of colchicine levels found in the various organs of Gloriosa superba (value given as % dry weight ± indicates standard error)

Explant	Percentage colchicine expressed as dry weight old plant (3 years old)	new plant (1 year old)
corm	0,9074 ± 0,2	0,9200 ± 0,25
leaf	0,0680 ± 0,03	0,7800 ± 0,35
seed	0,6050 ± 0,25	
ovary	0,0825 ± 0,01	
flower	0,0959 ± 0,03	

It is evident that colchicine levels in Gloriosa corms ranges around ± 0,9%, and if the levels of colchicine in Colchicum is 0,62% as reported by BELLET and GAIGNAULT (1985) then Gloriosa would be a much better source of colchicine for commercial exploitation. Colchicine levels in corms are constant around 0,9% and levels of 0,2% as often recorded was only found in dead or senescing corms. Colchicine levels in old leaves of Gloriosa was exceptionally low and not comparable with the levels found by NTAHOMUUKIYE, HAKIZIMANA, NKILIZA and PUYVELDE

(1984), however, the level of colchicine in new leaves was comparable at levels ± 0,7%. CROMWELL (1955) also reported that new leaves (of Colchicum) had higher levels of colchicine than did older leaves. Colchicine levels in seeds is comparable with that found by SARIN, JAMWAL, GUPTA and ATAL (1974). Levels of colchicine in ovaries and flowers was low, in the order of 0,09%. A comparative study of the crude extracts of the various plant organs was performed on the spectrophotometer and the HPLC, with the results graphically represented in Figures 6.12 and 6.13.

The spectrophotometric traces support the relative levels of colchicine (Figure 6.12) found using HPLC on the various organs, extracts from each organ shows the typical colchicine curve. The breakdown products of the various organs are clearly displayed inFigure 6.13 with the major breakdown product corresponding with "spot 1" (M 281) and a product with a retention time of ± 11 minutes (a retension time of ± 13 minutes is equivalent to lumicolchicine).

The GCMS traces of Gloriosa corms grown in culture corresponds with that of authentic colchicine. Unfortunately, however, a large percentage of the extract prepared for GCMS was photoconverted to lumicolchicine (β and y) (WILDMAN and PURSEY, 1968; KEITH and SRIVASTAVA, 1978), which has a major base peak at M 356 (WILDMAN and PURSEY, 1968). Using the programme described for GCMS colchicine retention time was ± 13,1 minutes while lumicolchicine has a retention of 11,6 minutes. Lumicolchicine is a structural isomer of colchicine which is formed when colchicine is exposed to light in the absence of air (WILDMAN and PURSEY, 1968). Lumicolchicine is said to have properties similar to colchicine except that it does not affect mitosis, nor does it disrupt microtubules or bind to tubulin subunits (KEITH and SRIVASTAVA, 1978). The GCMS trace (Figure 6.14) for Gloriosa corms appears to be a mixture of colchicine and its congeners (lumi-

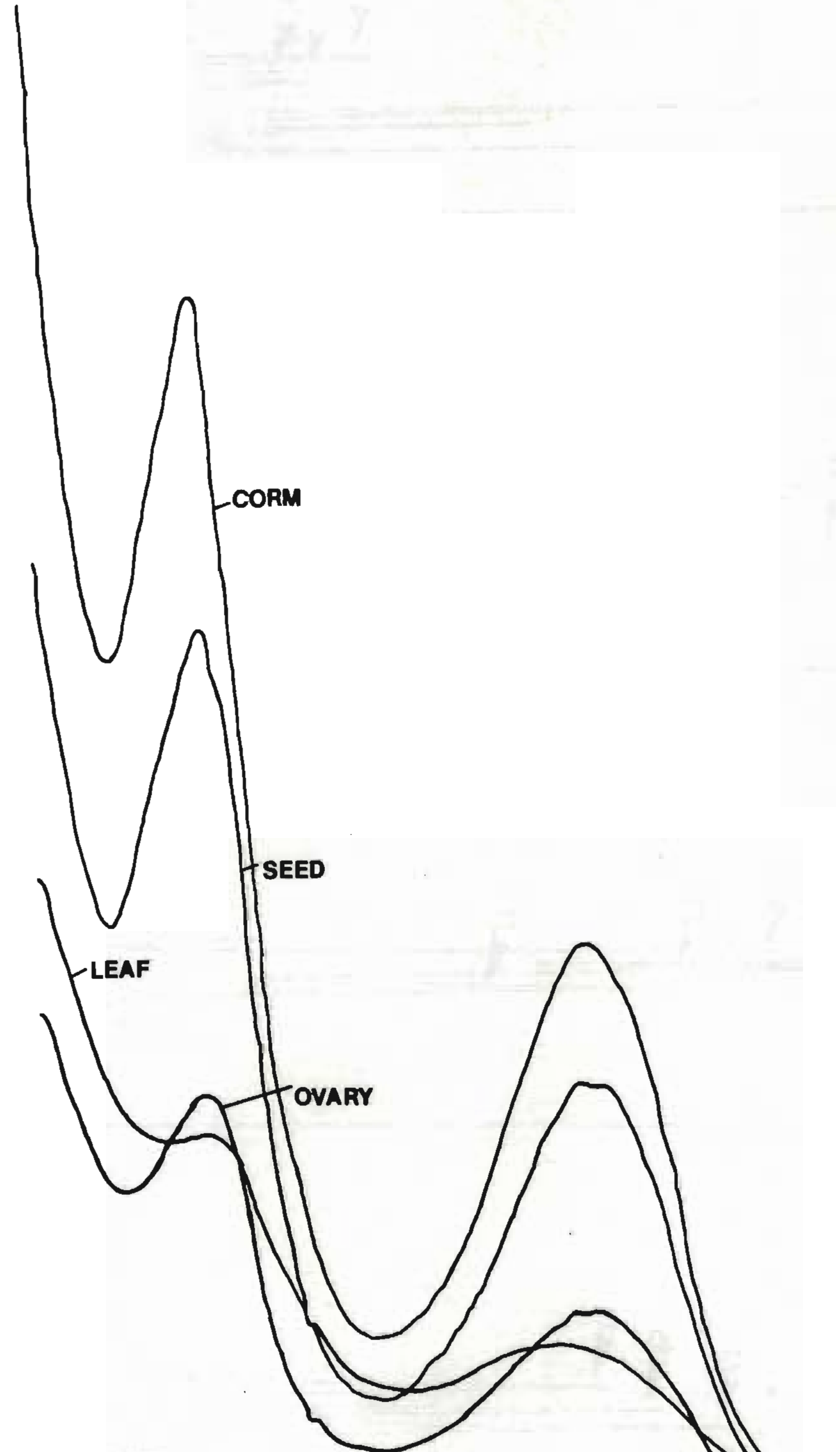
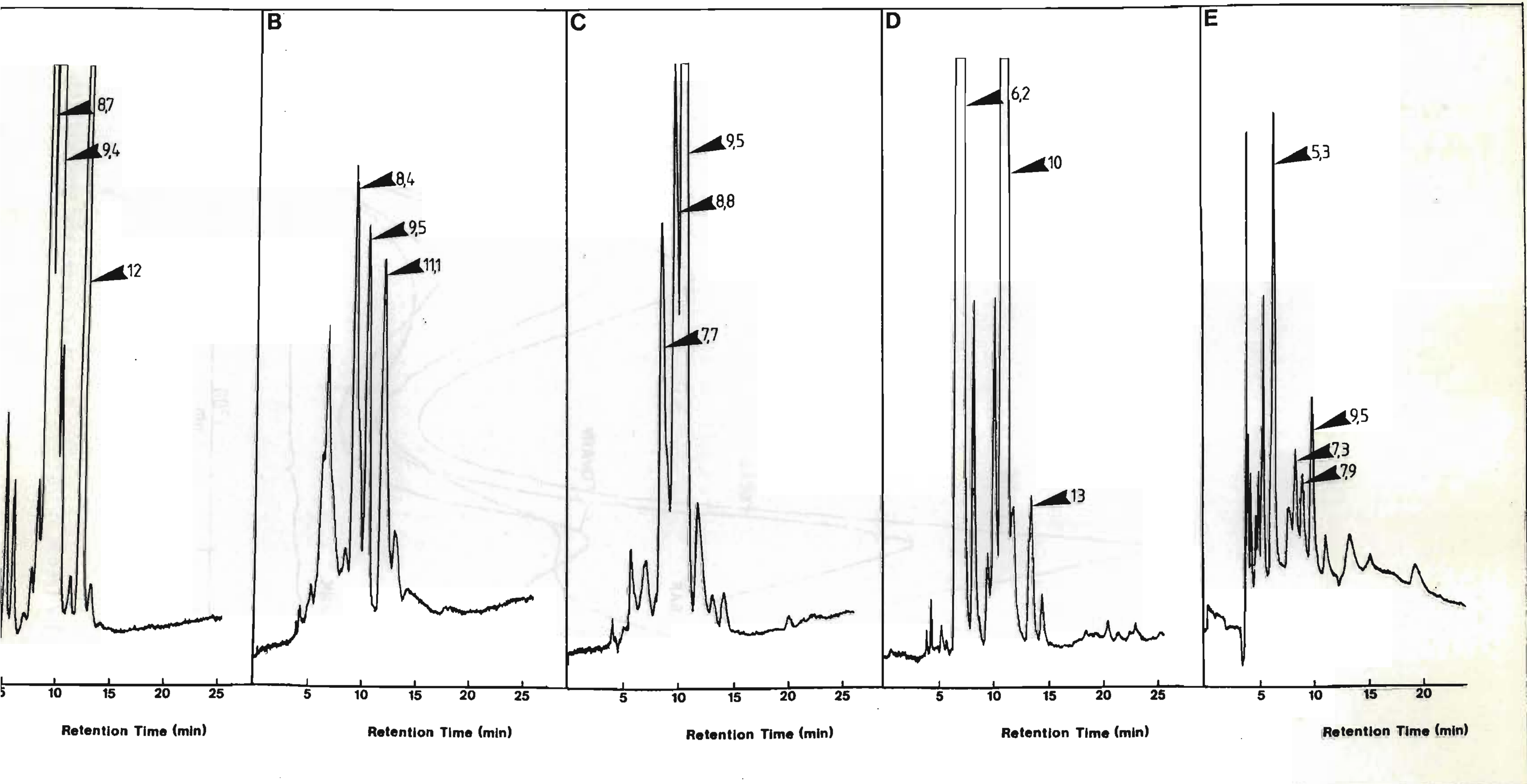


Figure 6.12 UV traces of Gloriosa corm, leaf, ovary and seed extracts


8,7
9,4
12
5
10
15
20
25
Retention Time (min)
B
8,4
9,5
11,1
5
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20
25
Retention Time (min)
C
9,5
8,8
7,7
5
10
15
20
25
Retention Time (min)
D
6,2
10
13
5
10
15
20
25
Retention Time (min)
E
5,3
9,5
7,3
7,9
5
10
15
20
Retention Time (min)

Figure 6.13 HPLC traces of the various organs of Gloriosa

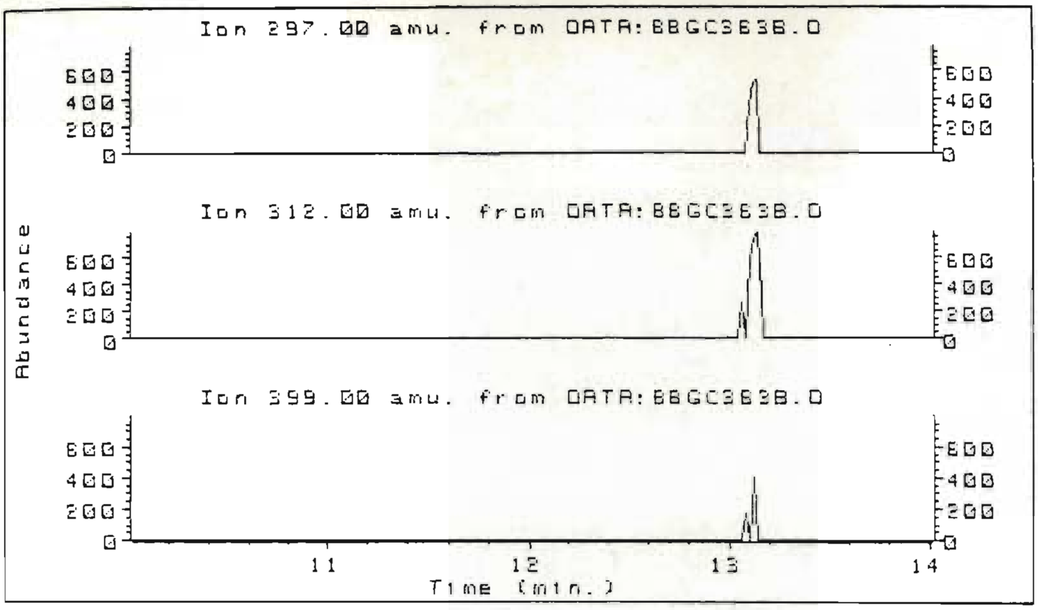
a) corm

b) leaf

c) ovary

d) seed

e) flower (note they all contain colchicine at various concentrations)



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Z: Set of 3 GCMS
Y: null.
X: Scan 158 (13.118 min) of D

Gloriosa

```
Scan 158 (13.118 min) of DATA:88GC3638.D
Gloriosa Rt.,Finnie,           150(3)-290@30.IP290.
     m/z      abund.     m/z     abund.      m/z       abund.     m/z      abund.

    191.25       674   267.05       408   298.30        248   341.20        898
    193.25       356►281.15      2592   311.30        228   342.20        420
 ►207.15      5459   282.15       847►312.20        741   355.30        422
    207.95      1275   283.15       523   325.20        539►399.35        402
    209.05       777►297.20       515   327.10        350   429.05        508
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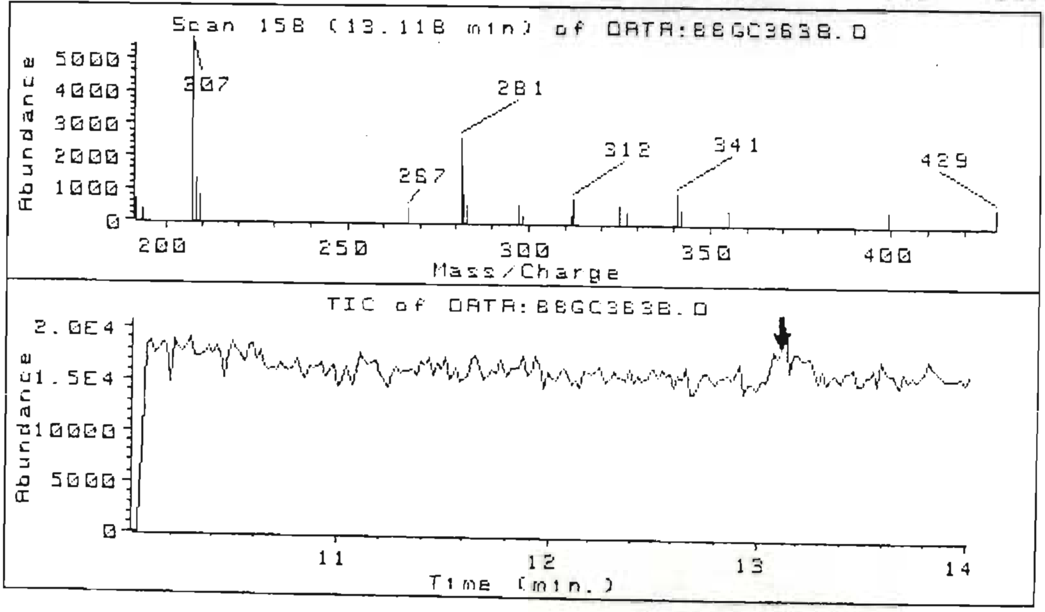


T: Scan 156 (13.078 min) of D
Z: TIC of DATA:88GC3638.D
Y: Scan 159 (13.137 min) of D
X: Scan 158 (13.118 min) of D

Figure 6.14 GCMS of Gloriosa, (see also Figure 6.10 and 6.11). Gloriosa tissue grown in vitro has the same fragmentation pattern as does authentic colchicine

colchicine, demecolcine) (Figure 6.11) (WILDMAN and PURSEY, 1968).

The seasonal variation in the colchicine content of *Gloriosa* plantlets is displayed in Table 6.7.

Table 6.7 Variation in colchicine levels as a result of seasonal growth in *Gloriosa* plants (± indicates standard error)

		(% dry weight)	
		Corm	Leaf
Spring	New seedling	0,9299 ± 0,151	0,7873 ± 0,167
|	Mid-season	0,4830 ± 0,25	0,036?
|	Senesecing	0,5923 ± 0,153	0,1664 ± 0,04
▼	Dormant	0,7440 ± 0,220	-
Winter	Mid-winter non dormant	0,6700 ± 0,190	-
	Dead	0,2489 ± 0,09	0,1455 ± 0,1

It has been shown previously that colchicine content may be affected by plant age and seasonality. One-year-old corms of *Colchicum* are recommended for commercial colchicine production, and these plants should be collected prior to flowering (HAMERSLAG, 1950). *Gloriosa* corms of all ages have approximately the same colchicine content, but colchicine levels are highest in the beginning of the growth process. WILDMAN (1960) reported that in *Colchicum*, the colchicine concentration was three times higher in spring when leaves appear, than in corms collected in autumn. As the corm senesces the colchicine content decreases while the concentration increases in the daughter corm. This trend was also shown for *Gloriosa* with the highest concentrations being recorded early in spring. As the growth of the plant proceeded the concentration decreased to a level of ± 0,7%. Corms growing in winter respond similarly to those growing in summer. Breakdown of the colchicine occurs on the death of the plant organ. Figure 6.15 shows the

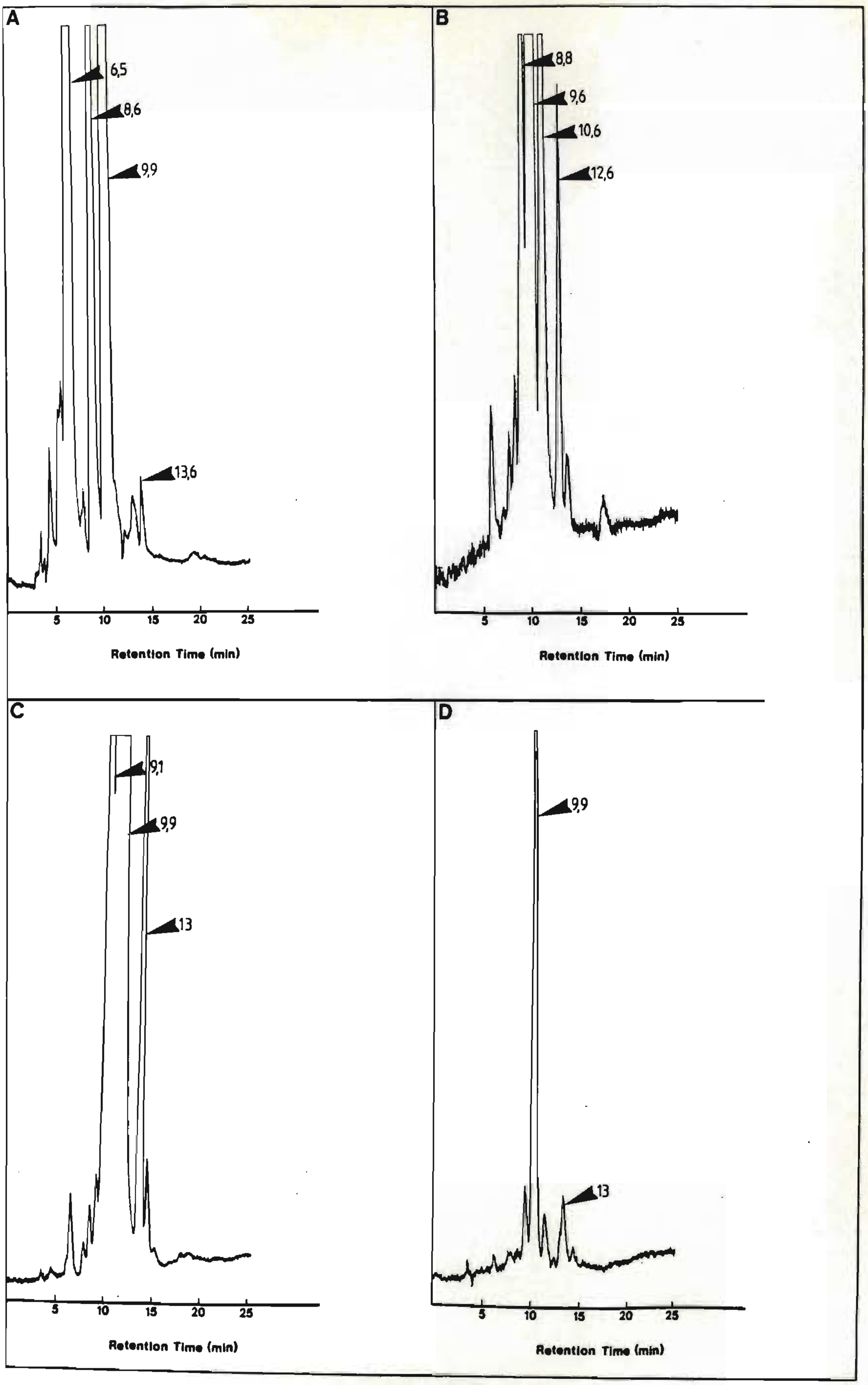


Figure 6.15 HPLC trace of Gloriosa ccrms harvested at different times of the year

a) dead
b) senescing
c) dormant

HPLC traces of a dead corm, a senescing corm and a dormant corm. The trace for the dormant corm indicates that the extract comprises predominantly of colchicine, while in the other two cases a large number of breakdown products and metabolites are present in the extract (see also Figure 6.8).

Comparisons of alkaloid concentrations with previous reports are made difficult by the fact reported by THAKUR, POTESILOVA and SANTAVY (1975) that alkaloid content also varies with locality.

The colchicine content in corm extracts of Gloriosa, Sandersonia, Littonia and Androcymbium is given in Table 6.8 with the respective HPLC traces shown in Figure 6.16. GCMS traces for Sandersonia, Littonia and Androcymbium were the same as that found for Gloriosa with a large amount of lumicolchicine in the extract. The GCMS trace for Sandersonia is shown in Figure 6.17.

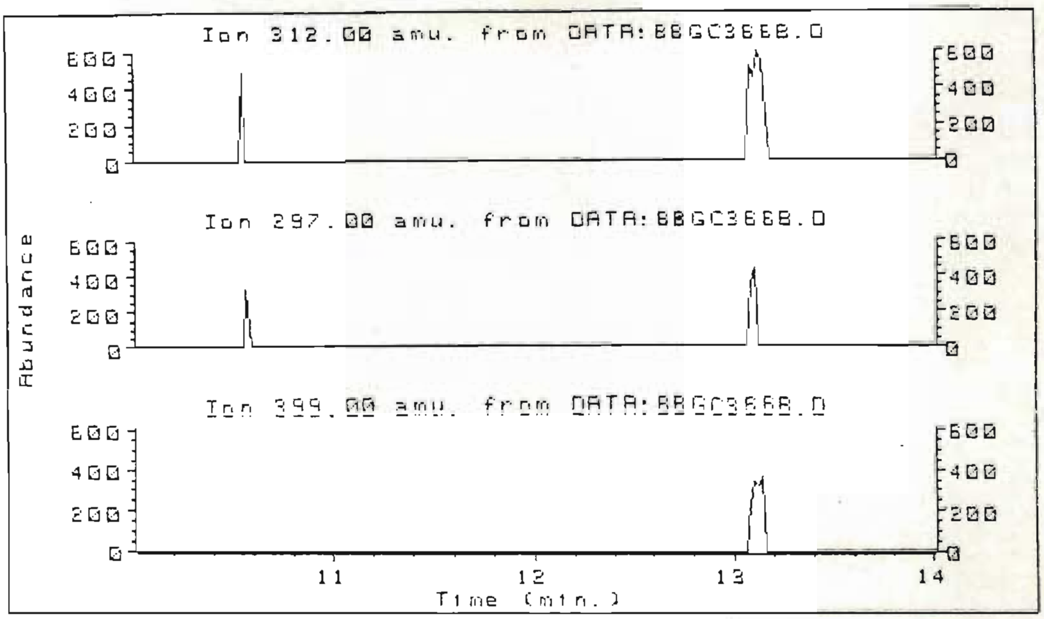
Table 6.8 Comparative table of colchicine levels extracted from corms from four members of the Colchicaceae (% dry weight ± indicates standard error)

Plant	Colchicine concentration
Gloriosa	0,9075 ± 0,2
Sandersonia	0,8 - 1,1
Littonia	0,45 - 0,65
Androcymbium	0,2 - 0,3

Levels of colchicine in Littonia correspond with those reported by HRBEK and SANTAVY (1962) and WILDMAN and PURSEY (1968). Levels of colchicine in Sandersonia are comparable with those found in Gloriosa. Although the GCMS

Figure 6.16 HPLC traces of a) Sandersonia
b) Littonia
c) Androcymbuim (all contain the colchicine peak)

Sandersonia



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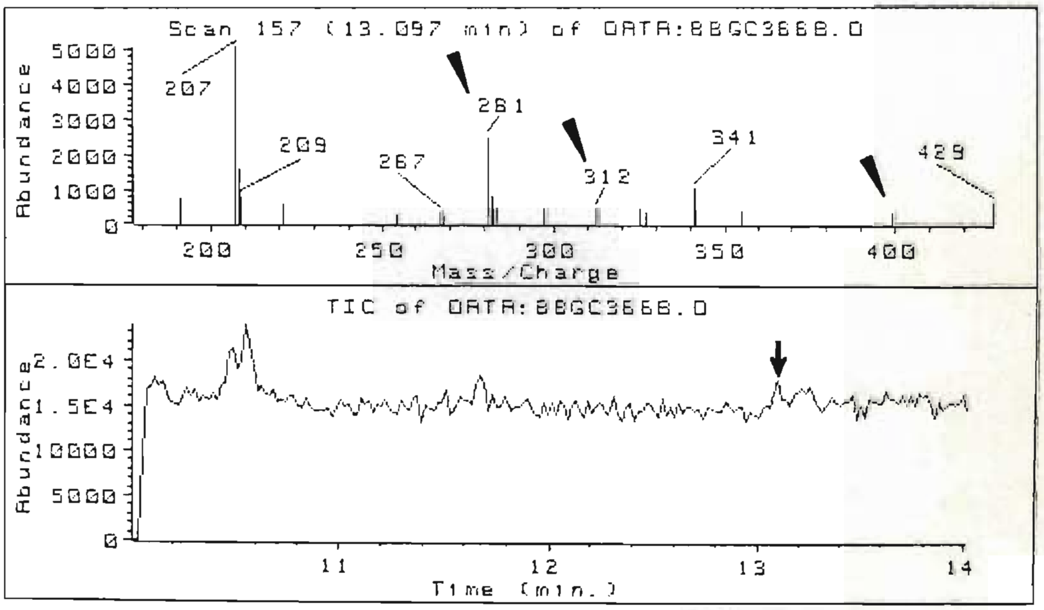

T: null.
Z: TIC of DATA:88GC366B.D
Y: Scan 157 (13.097 min) of D
X: Scan 157 (13.097 min) of D

Figure 6.17 GCMS of Sandersonia. Sandersonia extracts have the same fragmentation pattern as does colchicine (see Figure 6.10 and 6.11) for Sandersonia showed a high percentage of lumicolchicine, it does indicate that colchicine was the compound initially present in the extract. The presence of colchicine at high levels also makes Sandersonia an ideal alternative for commercial colchicine production with levels far in excess of that recorded for Colchicum by BELLET and GAIGNAULT (1985).

6.3.3 In vitro investigations

The utilization of plant cells that are cultured in vitro provides a viable alternative to the problems involved in the production of chemical, pharmaceutical and medicinal compounds (BARZ, REINHARD and ZENK, 1977). HAYASHI, YOSHIDA and SANO (1988) reasoned that Colchicum cultures should be able to produce colchicine in a fermentor tank. They described the production of alkaloids in suspension cultured Colchicum cells and examined the effects of growth substances and nutritional factors on the formation of colchicine. HAYASHI, YOSHIDA and SANO (1988) have also claimed that their report was the first on the production of colchicine alkaloids by plant tissue culture.

The following data records the production of colchicine in tissue cultures of Gloriosa and Sandersonia. The absolute values of the colchicine levels vary between experiments, as the experiments were performed over a three year period. In general the levels recorded in tissue cultures of Gloriosa and Sandersonia are ± 10 - 25 times lower than those found in plants grown in vivo. Variation in the levels of colchicine between experiments can be ascribed to differences in the time of the year in which the cultures were made, the levels of hormones in the media, the age of the callus and the status of the callus at the time of culture. (Variation within experiments are however comparable).

Table 6.9 shows the levels of colchicine in callus maintained on media supplemented with 2,4-dichlorophenoxyacetic acid and malformed roots grown on a medium without 2,4-dichlorophenoxyacetic acid.

Table 6.9 Levels of colchicine in callus and malformed root cultures of Gloriosa and Sandersonia (% dry weight, ± indicates standard error)

Treatment	% colchicine
Gloriosa callus grown in light	0,027 ± 0,003
Gloriosa callus grown in dark	0,03 ± 0,005
Sandersonia callus grown in dark	0,037 ± 0,003
Malformed Gloriosa roots grown in light	0,041 ± 0,003
Malformed Sandersonia roots grown in light	0,034 ± 0,004

The level of colchicine in callus maintained on 2,4-dichlorophenoxyacetic acid is slightly lower than in the tissue grown on a medium devoid of 2,4-dichlorophenoxyacetic acid. This difference in the level of colchicine may be a result of the presence of 2,4-dichlorophenoxyacetic acid or may be due to the morphogenetic change from callus to malformed roots. NETTLESHIP and SLAYTOR (1974), ZENK, EL-SHAGI and SHULTE (1975), PHILLIPS and HENSHAW (1977), SASSE, HECKENBERG and BERLIN (1982a) and HAYASHI, YOSHIDA and SANO (1988) all have reported that the presence of 2,4-dichlorophenoxyacetic acid inhibits secondary metabolite production in culture. KURZ and CONSTABEL (1979a) recorded that cells grown in 2,4-dichlorophenoxyacetic acid have low amounts of glutamine and aspartic acid, compounds necessary in alkaloid synthesis. In a number of plants particular compounds are only synthesized or accumulated in particular organs and tissue. HIRAOKA and TABATA (1974), VERZAR-PETRI, LADOCSY and OROSZLAN (1982) found that differentiation could increase product formation, however HAGIMORI, MATSUMOTO and KISAKA (1980) showed that root forming callus produced no more digitoxin or digoxin than did undifferentiated callus. FREEMAN, WHENHAM, MACKENZIE and DAVEY (1974) and TABATA, YAMAMOTO, HIRAOKA and KONOSHIMA (1972) all showed that when callus produced roots there was an increase in the amount of metabolite produced.

Although the levels of colchicine are not that much more elevated for roots in culture, there is an obvious difference between callus and root cultures. Unfortunately one cannot separate the effect of 2,4-dichlorophenoxyacetic acid and root production on the production of colchicine in vitro, as on removal of the callus from the presence of 2,4-dichlorophenoxyacetic acid root production automatically occurs.

UOMORI and TOMITA (1974) found that on induction of roots, high levels of secondary metabolites were recorded. This may indicate that the induction of organogenesis can be industrially applied to drug production using the large number of cells obtained from suspension cultures.

Figure 6.18 shows the presence of colchicine in in vitro cultured malformed roots. These lower levels of metabolites found in cultured material may be due to a number of factors:

the rate of degradation is higher than the rate of synthesis;
the necessary intermediates / enzymes are not present and
subculture may result in the loss of the cells' ability to produce colchicine.

Plant growth regulators play an important role in the production of secondary metabolites in culture due to their ability to induce or repress certain biosynthetic pathways. Figures 6.19 and 6.20 demonstrates the effect various synthetic hormones have on the colchicine content of Gloriosa and Sandersonia callus and root cultures.

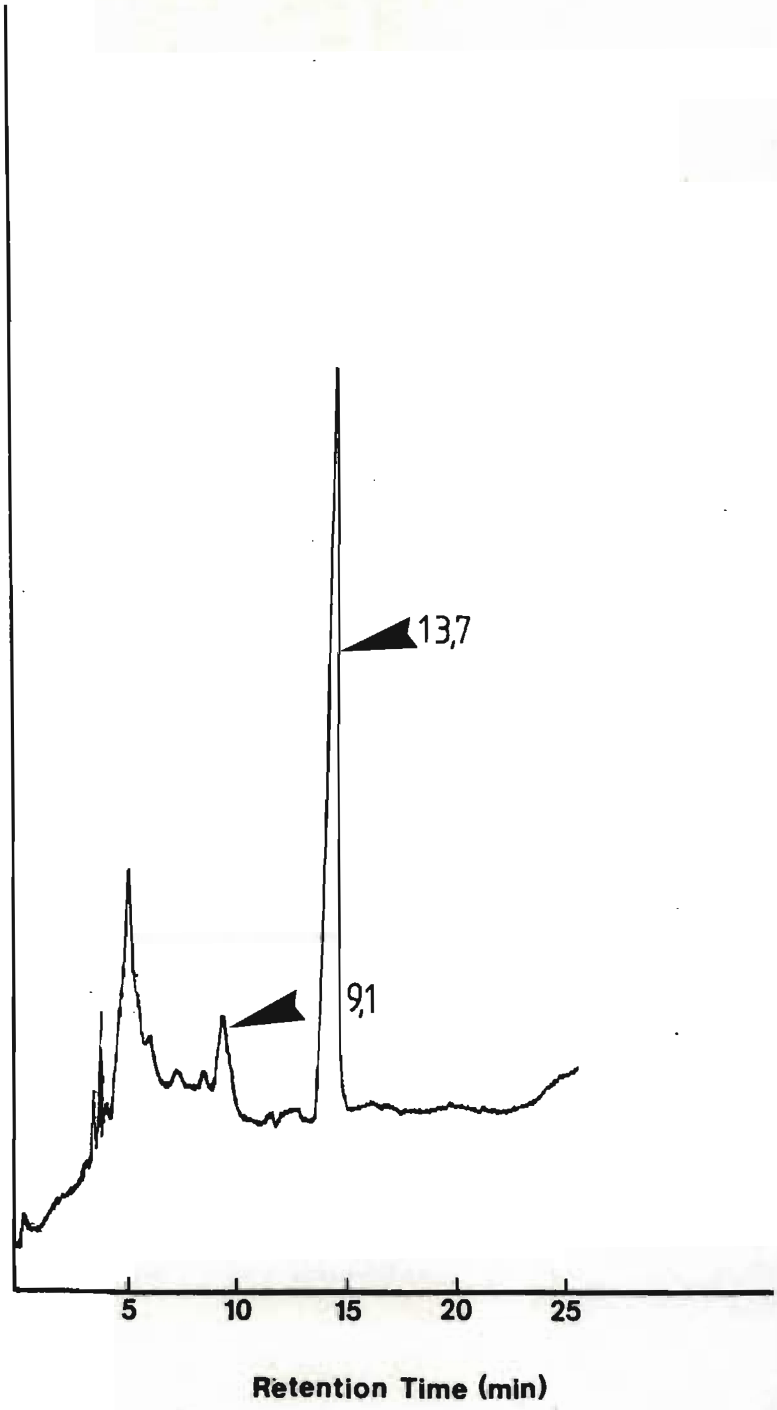

Figure 6.18 HPLC trace of malformed Gloriosa roots in vitro (contains a peak co-chromatographing with colchicine)

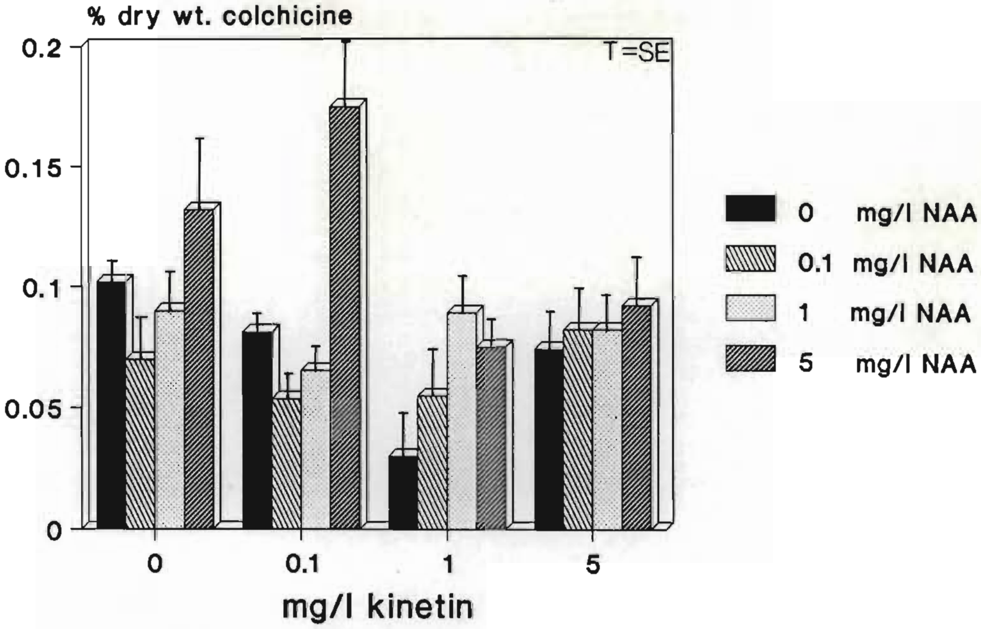

Figure 6.19 The effect of synthetic hormones on colchicine production in Gloriosa callus.

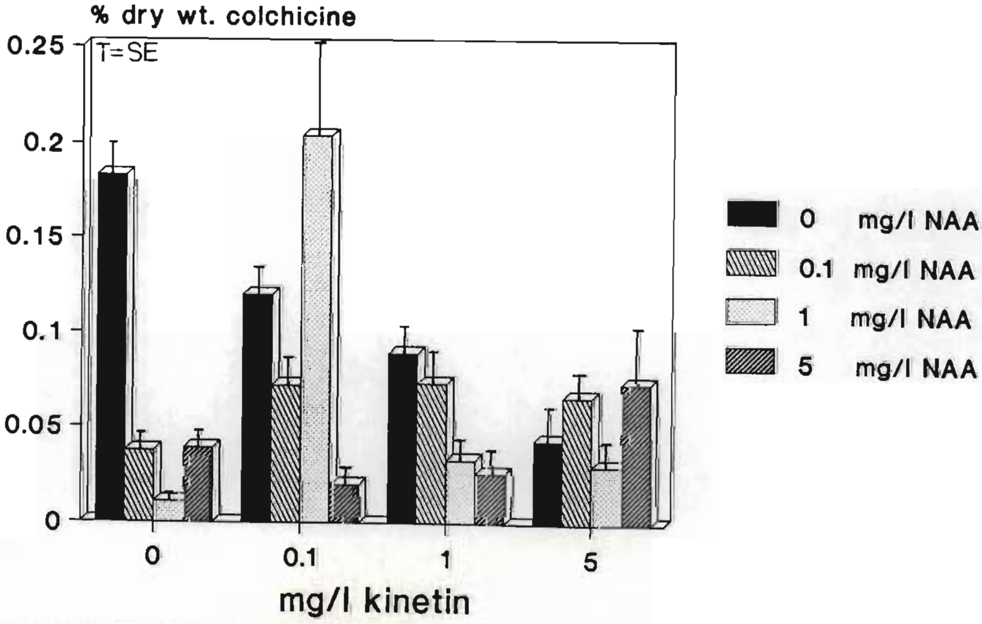

Figure 6.20 The effect of synthetic hormones on colchicine production in Sandersonia callus

There seems very little patterning in the response of colchicine production to hormones (kinetin, and napthaleneacetic acid). HAYASHI, YOSHIDA and SANO (1988) found that high levels of kinetin inhibited colchicine production in vitro, while indolebutyric acid stimulated cell growth in the presence of kinetin.

For Sandersonia cultures it appears that at a level of 0,1 mg ℓ^{-1} kinetin, colchicine production was at its highest, and at higher concentrations, colchicine levels decreased. At a level of 0,1 mg ℓ^{-1} kinetin and 1 mg ℓ^{-1} NAA a level of ± 0,2% colchicine per dry weight was recorded. This level was exceptionally high and was not related to the relative amount of cell growth. A similar high level was recorded for Gloriosa cultures at 0,1 mg ℓ^{-1} kinetin and 5 mg ℓ^{-1} NAA. It was speculated that the role hormones play in secondary metabolism is not on the intermediates of the biosynthetic pathway but rather that they act to shift cytological conditions so as to favour / inhibit secondary metabolite production (ZENK, EL-SHAGI, ARENS, STOCKIGT, WEILER and DEUS, 1977).

Figure 6.21 records the colchicine levels found in Gloriosa tissue grown on a source of natural hormones (zeatin and indoleacetic acid). The relative levels of colchicine recorded are much lower than those found for the synthetic hormones and there does not appear to be any inhibitory or stimulatory role for natural hormones in the production of colchicine in vitro. SCRAGG, MORRIS and ALLAN (1986) showed that a broad range of phytohormones did not have an effect on triggering or allowing accumulation of alkaloids, yet found that natural cytokinin had a greater stimulatory effect on quinidine production than did synthetic hormones, contrary to the results found for colchicine. (It is possible that on autoclaving, a high percentage of the natural hormones' activity was lost). In general indoleacetic acid has been put forward as the most effective source of auxin. HAYASHI, YOSHIDA and SANO (1988) however showed that indoleacetic acid was not an effective source of auxin for colchicine production stimulation. They concluded that indoleacetic acid is unstable, probably due to degradation by IAA oxidase.

In both Sandersonia and Gloriosa cultures (Figures 6.19 and 6.20) high levels of colchicine was recorded for cultures without any hormone supplementation. Low levels of hormones were able to increase the production of secondary metabolites (COLLIN, 1987). A feature that was remarkably clear especially in Gloriosa cultures was the fact that secondary metabolite production is inversely related to the amount of tissue growth. (This trend was more clearly defined than was the ability of roots / callus to produce colchicine). PINOL, PALAZON, ALTABELLA, CUSIDO and SERRANO (1985) also showed that nicotine synthesis was stimulated in hormone supplemented media that limited tissue growth. BECKER (1987) recorded that there appears to be no "unequivocal correlation" for the role of hormones on secondary metabolite production in culture.

The response of two different callus ages in the same hormone treatments and their respective ability to produce colchicine is shown in Figure 6.22. It is evident that the old callus produced more colchicine per dry weight than did the new callus. This observation may once again be a feature of the relative growth of the callus. Old callus grew much slower than did the new callus, showing an inverse relationship between secondary metabolite production and cell growth. A further explanation for the variation in colchicine levels, is the origin of the callus. Old callus was exclusively derived from the radicle portion of the embryo, while a percentage of the new callus was derived from callus produced by leaf explants. SASSE, HECKENBERG and BERLIN (1982a) found that the amount of metabolite produced did not vary with explants derived from different sources. KINNERSLEY and DOUGALL (1980) however, showed that the ability of the culture to produce nicotine varied depending on the position of the explant on the stem. According to KURZ and CONSTABEL (1985) the origin of the explant may be important, but

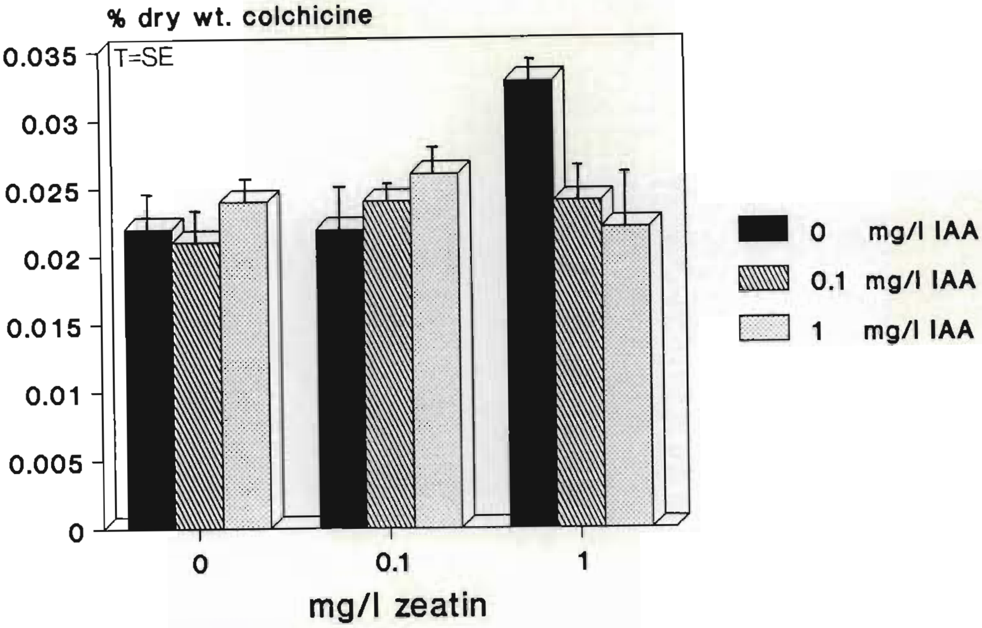

Figure 6.21 The effect of natural hormones on colchicine production in *Glorlosa* callus.

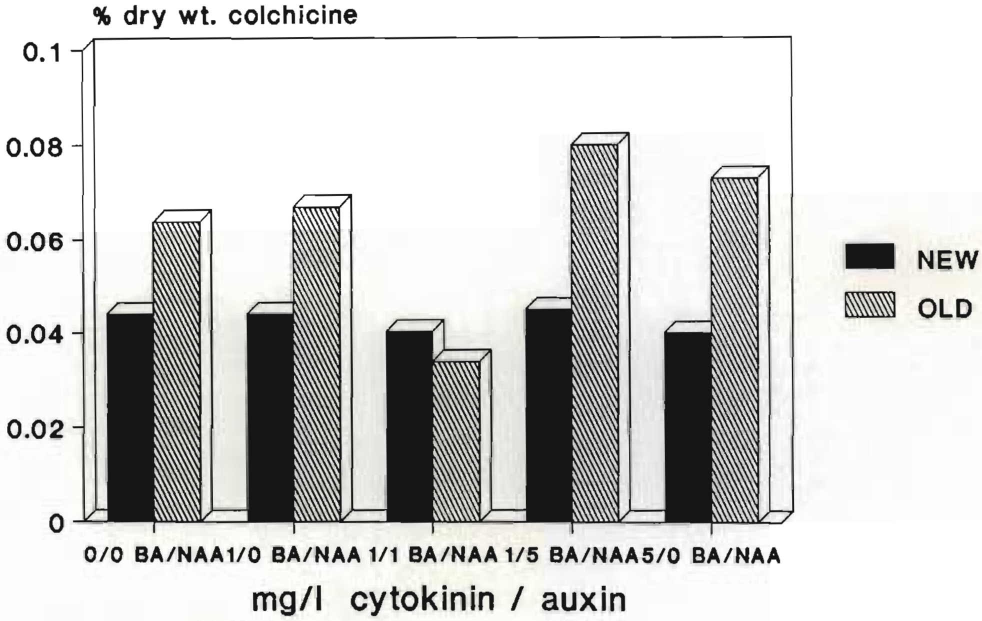

Figure 6.22 The ability of *Gloriosa* callus of different ages to produce colchicine *in vitro*.

when cells were grown for extended periods of time, the origin of the explant became irrelevant with respect to metabolite production in culture. An interesting feature of Gloriosa cultures is that the callus after prolonged culture, does not appear to lose its ability to produce colchicine. This is often reported for other species (HAGIMORI, MATSUMOTO and KISAKA, 1980). KURZ and CONSTABEL (1985) and COLLIN (1987) have both reported examples where there is a definite reduction in the ability of the culture to produce secondary metabolites, and posed the question whether the ability of the cultures to synthesize and accumulate metabolites had been lost over time. In the case of Gloriosa the ability of the cultures to produce colchicine was not lost over a ± three year period. BECKER (1987) reported that alkaloid degradation may be an important factor in the alkaloid content of a plant, but very little is known about the regulation mechanisms of alkaloid breakdown.

The growth of Gloriosa cultures over an extended period of time (100 days) showed that while there was an increase in the amount of plant tissue, there was a decrease in the amount of colchicine produced by the culture (Figure 6.23). This pattern again showed the inverse relationship between growth and colchicine production. Although successive subculturing may result in the loss of the cells' potential to produce colchicine, within a subculture period the absolute levels of colchicine show great fluctuation. A noticeable peak in colchicine concentration was found at 30 days. Often the low levels of metabolites recorded in cultured tissue may be compounded by the fact that the quantification procedure was performed when metabolite production was at a low level. HAYASHI, YOSHIDA and SANO (1988) on the other hand showed that colchicine formation in Colchicum cultures was parallel with the culture's growth but decreased when cell growth ceased after seven weeks.

Perhaps the most interesting result was obtained with the environmental experiment (Figure 6.24). In this experiment the production of colchicine in vitro was not related to the growth of the culture. Cultures grown in the light showed very low levels of colchicine, with the other cultures having a colchicine content of more than double that of the light treatment. The highest levels were obtained when there was a fluctuation in the light regime (either when the culture was grown in the dark and then transferred to the light or when the tissue was grown "outside"). TABATA (1977) reported that most metabolites in cultures are stimulated by light which is unfortunate as it would be more economically advantageous if the cell cultures could grow in the dark!

HAHLBROCK (1972), BRUNET and IBRAHIM (1973) and MATSUMOTO, NISHIDA, NOGUSHI and TAMAKI (1973) all reported that metabolite synthesis was triggered by light. ALFERMANN and REINHARD (1971) replaced the light requirements of their cultures with auxin. TABATA, MIZUKAMI, HIRAOKA and KONOSHIMA (1974) showed that shikonin synthesis was inhibited by cool white light because of an inactivation of a co-enzyme. PAGE and VAN STADEN (1987) found that hypoxoside production was high in continuous darkness. HAYASHI, YOSHIDA and SANO (1988), when culturing Colchicum callus did so in the dark. The ability of Gloriosa to produce colchicine in vitro in the dark is advantageous, as the expense of providing light for culture growth is negated.

Very little research has been produced regarding the temperature optima for cell growth and metabolite production (MARTIN, 1980a). Figure 6.25 shows that more colchicine was produced at low temperatures. This may be explained by the fact that low temperatures may cause chilling stress resulting in inhibition of growth and intermediates are used for secondary pathways rather than growth (COLLIN, 1987). Membrane damage may have also resulted in a breakdown of compartmenta-

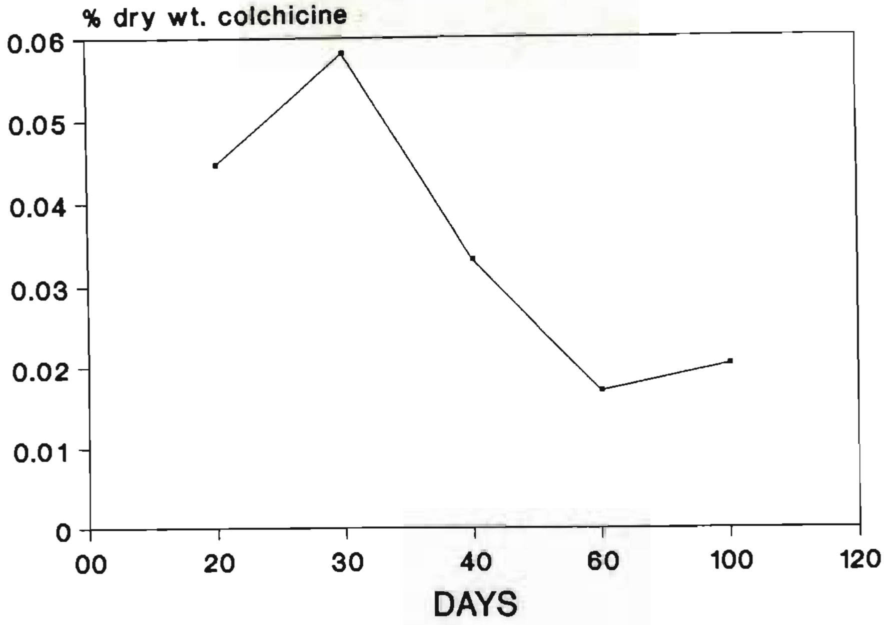

Figure 6.23 The production of colchicine in vitro over a period of 100 days using Glorlosa callus.

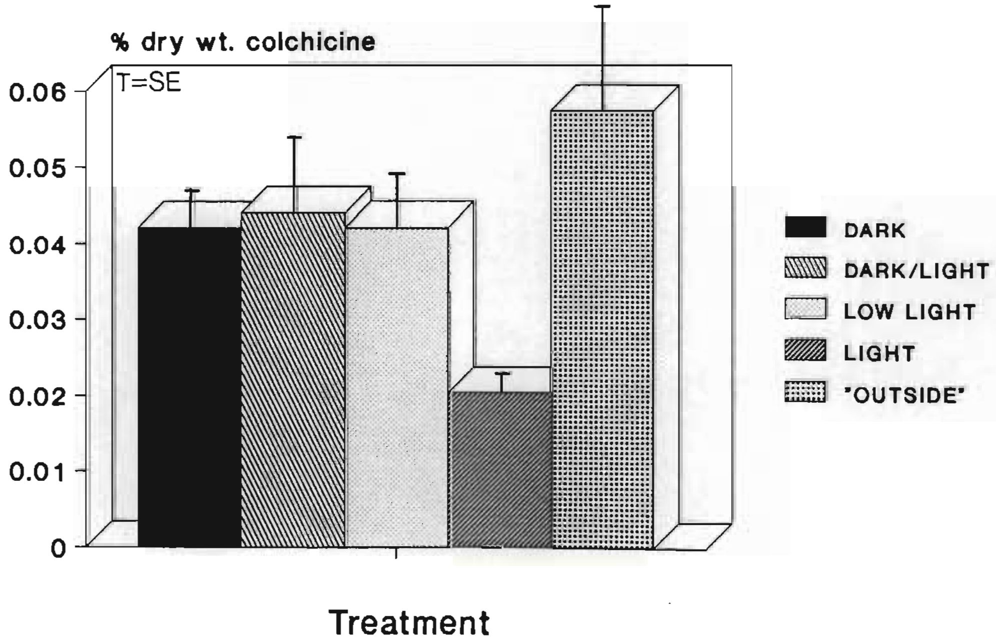

Figure 6.24 The effect of differing light regimes on the ability of Gloriosa callus to produce colchicine.

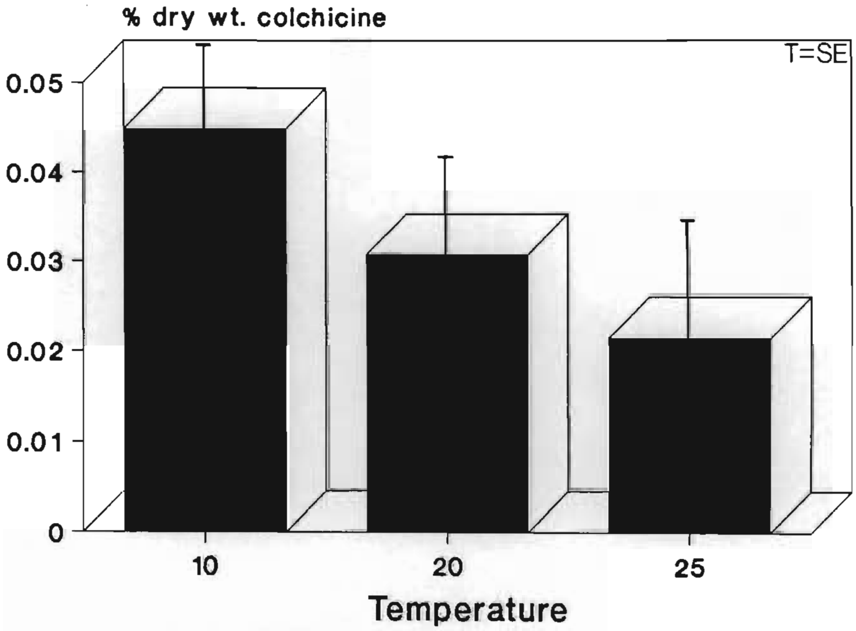

Figure 6.25 The effect of temperature on colchicine production in Gloriosa callus in vitro.

tion and a release of the metabolite. The majority of cultures grow best at 20 - 28°C. LOCKWOOD (1984) showed that when Papaver cultures were transferred from 36° to 5°C there was an increase in the amount of thebaine released into the medium, but at 5°C further metabolism was inhibited. At 10°C the amount of cell growth in Gloriosa cultures was low, while at 25°C the amount of colchicine produced was small. Thus for commercial application a temperature optimum that promotes both cell growth and colchicine production will have to be established.

Sucrose was added to the Gloriosa cultures at 2, 4 and 8 percent with the following results (Table 6.10).

Table 6.10 Sucrose and colchicine production in Gloriosa tissue *in vitro*

% sucrose	% dry weight colchicine
2	0,093
4	0,06
8	0,14

Although the absolute values for colchicine are elevated, the addition of sucrose at high levels (up to 8%) appears to stimulate colchicine production. HAYASHI, YOSHIDA and SANO (1988) showed that sucrose was the only effective source of carbon for colchicine formation, but high levels of sucrose (above 5%) were inhibitory, with an optimum at 3%. CONSTABEL (1968), DAVIES (1972) and COLLIN (1987) all reported that increasing the amount of carbohydrate resulted in an increase in the amount of secondary metabolites. Low levels of sucrose may result in low levels of metabolites because of a limitation in the amount of enzymes produced for metabolism. At low levels the sucrose is utilized for cell growth and secondary metabolite pathways are inoperative. SUZUKI, MATSUMOTO and

MIKAMI (1984) showed that high levels (5 - 7%) of sucrose increase anthraquinone production, this trend being also cbserved for Gloriosa and colchicine production in culture.

Fluctuation in nitrogen levels have often been shown to influence the production of secondary metabolites in culture. WESTCOTT and HENSHAW (1976), AMORIM, DOUGALL and SHARP (1977), MEHTA and SHAILAJA (1978) and DOUGALL (1980) all showed that by increasing nitrogen levels there was a resultant decrease in the amount of metabolites produced. Figure 6.26 represents the variation in colchicine content of Gloriosa cultures when grown on different concentrations of nitrogen. At low levels of nitrogen the production of colchicine is at its highest. HAHLBROCK, EBEL, OAKS, AUDEN and LIERSCH (1974) suggested that low levels of nitrogen may cause a switch of cellular metabolism to reactions forming nitrogen-free products. However as an alkaloid, colchicine contains nitrogen, and at low levels of nitrogen the colchicine concentration is at its highest. Perhaps variation in the relative levels of NH_4^+ and NO_3^- would have yielded more significant results as numerous reports have been presented on metabolite production at various ratios of the two forms of nitrogen. Suffice to say, that low levels of inorganic nitrogen enhances colchicine production. The ability of cultures to produce higher levels of metabolites at low nitrogen levels in darkness was also recorded by PAGE and VAN STADEN (1987), but is contrary to most of the reported literature.

KURZ and CONSTABLEL (1979a) have reported that ammonia and nitrate are poor sources of nitrogen and better levels of metabolites would probably be obtained using peptone, yeast extracts, glycine and casein hydrolysate. Kurz and Constabel have summarized the action of nitrogen as follows: "Any levels of nitrogen sufficient to reinitiate growth in cells which have already entered the stationary phase, would be detrimental to product synthesis."

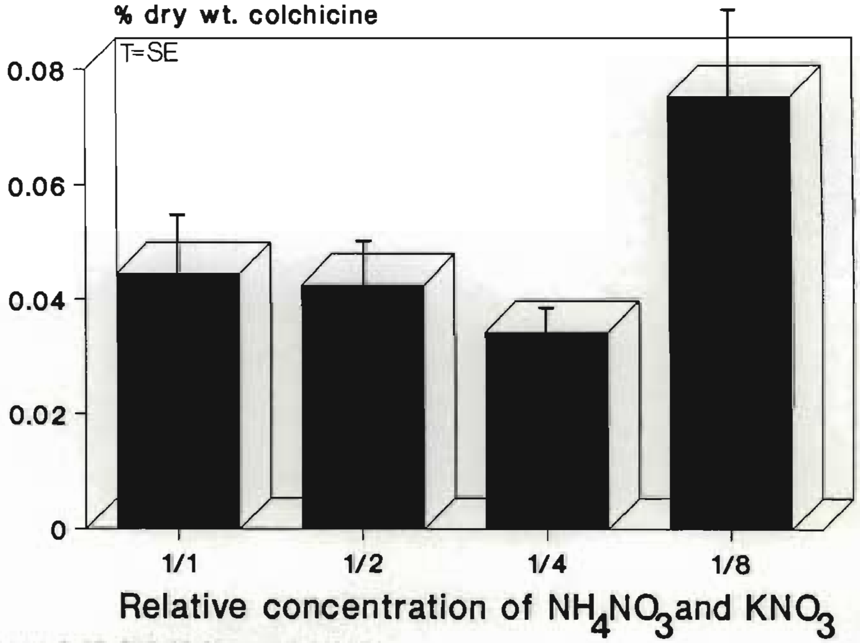

Figure 6.26 Colchicine production in Gloriosa callus grown on different relative levels of nitrogen.

Callus and roots grown in suspension / liquid culture were able to produce colchicine. Limited amounts of colchicine was released into the liquid medium (Figure 6.27 and Table 6.11). (See also Figure 3.36).

Table 6.11 Colchicine production in Gloriosa and Sandersonia tissue grown in liquid or suspension cultures (% colchicine dry weight or µg per 50 mls of medium)

Treatment		Plant tissue	Medium
Gloriosa roots on solid media		0,037	-
Gloriosa roots in liquid media		0,053	78 µg
Sandersonia roots in liquid media		0,045	-
Gloriosa suspension culture	0,1 kinetin 0,1 NAA (mg ℓ^{-1})	0,100	300 µg
	0,1 kinetin 1 NAA	0,064	241 µg
	1 kinetin 0,1 NAA	0,024	375 µg
	1 kinetin 1 NAA	0,051	367 µg
Sandersonia suspension culture		0,0693	180 µg

BARZ and ELLIS (1981) reported that surface grown cultures are of little value for commercial metabolite production, because of slow growth, the heterogenous nature of the callus, and the lack of correlation between levels of metabolites on solid media (high) to those in liquid media (low). From the results obtained it appears that levels of colchicine in suspension and liquid cultures are equal or slightly higher than those recorded for solid cultures. As reported in Chapter 3, growth of roots was easier to achieve in a liquid medium. Not only did enhanced root growth occur in a liquid medium but a certain amount of colchicine was released into the medium. TABATA (1977) recorded that for successful commercial application of plant cultures, one of the minimum requirements should be that the metabolite be preferably released into the medium without product degradation. This would facilitate recovery and purification.

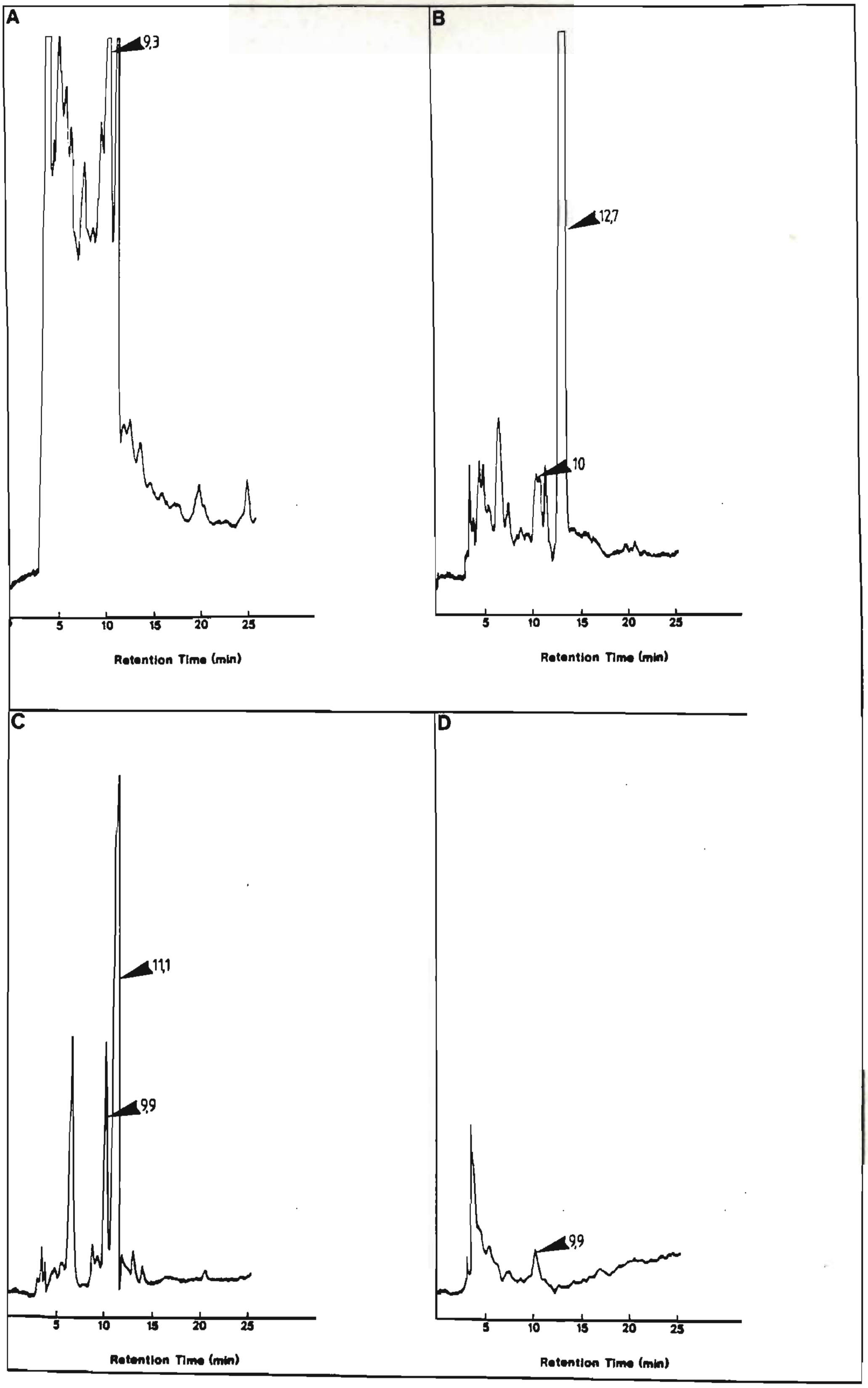

Figure 6.27 HPLC traces of a) Gloriosa roots from liquid culture
b) Sandersonia roots from liquid culture
c) liquid medium

The levels of colchicine extracted from suspension cultures of Gloriosa and Sandersonia are comparable with levels found for the hormone experiment (Figure 6.20). The highest levels of colchicine associated with solid cultures were found when differentiation of callus to roots occurred. Only minimal differentiation occurred in suspension. Although the levels of colchicine found in suspensions was relatively high these levels were derived from only 100 mℓ of culture. Whether these levels can be maintained when increasing the quantities for commercial application, remains to be seen. Despite the relatively high levels of colchicine produced in suspension the concentrations are still 10 to 20 times lower than those found within normal in vivo plants.

YOSHIDA, HAYASHI and SANO (1988a) reported that colchicine alkaloids are formed from phenylalanine, tyrosine and methionine. Ring A is said to be derived from tyrosine and ring C from phenylalanine while trans-cinnamic acid and p-coumaric acid have been identified as intermediates from phenylalanine (Figure 6.28).

Gloriosa plant cell cultures were fed with cold phenyalanine, tyrosine and cinnamic acid, as precursor feeding has been advocated to increase the production of secondary metabolites in culture. The results from precursor feeding are shown in Table 6.12.

A typical HPLC trace for Gloriosa tissue fed with one of the precursors and the trace of a solid medium extracted for colchicine fed with the precursors is shown in Figure 6.29.

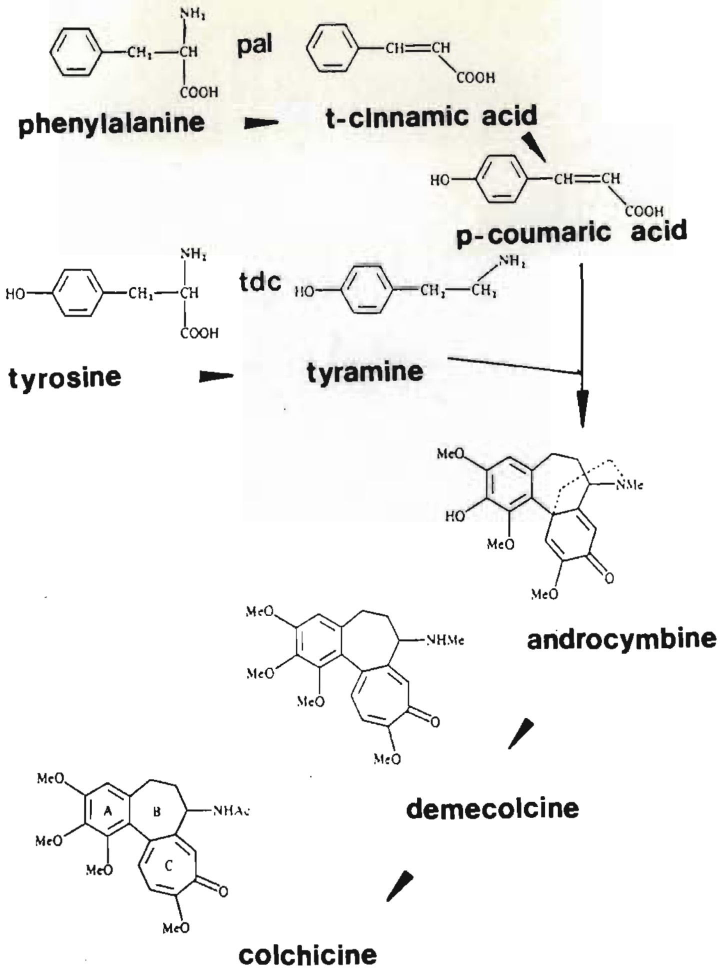

Figure 6.28 Biosynthetic pathway of colchicine alkaloids (adapted from YOSHIDA, HAYASHI and SANO, 1988a)

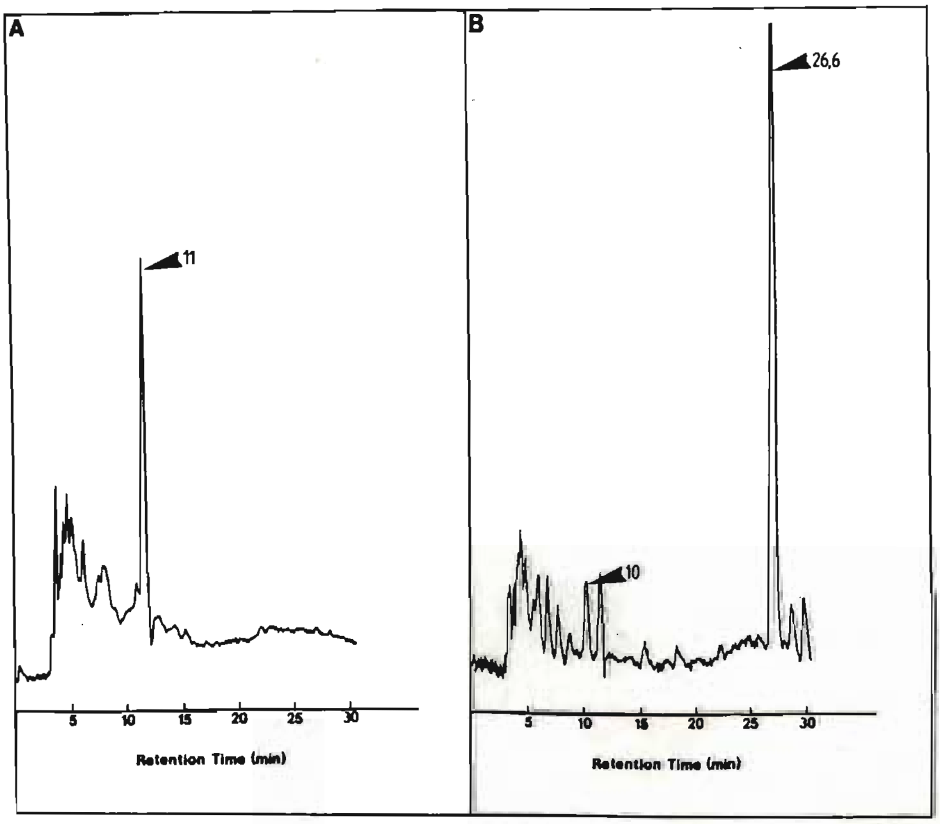

Figure 6.29 HPLC traces of a) callus fed with a precursor (phenylalanine)

b) media after growth with callus fed with a pre-

Table 6.12 Production of colchicine in precursor fed plant cultures of Gloriosa

Precursor	Concentration (molar)	% colchicine (dry weight)
	0	0,035
Phenylalanine ✓	10^{-4}	0,027
	10^{-6}	0,028
	10^{-8}	0,0316
	10^{-10}	0,0296
Tyrosine ✓	0	0,03
	10^{-4}	0,06
	10^{-6}	0,027
	10^{-8}	0,031
	10^{-10}	0,05
Cinnamic acid	0	0,03
	10^{-4}	0,026
	10^{-6}	0,021
	10^{-8}	0,022
	10^{-10}	0,018

It is fairly evident that feeding of these basic precursors did not have an effect on increasing colchicine levels in vitro. The addition of tyrosine may have affected the level of colchicine but only slightly. YOSHIDA, HAYASHI and SANO (1988a) found that feeding of phenylalanine, tyrosine and methionine had no effect on the formation of colchicine. Feeding with p-coumaric acid, tyramine and demecolcine did increase alkaloid formation. The failure of precursors to increase alkaloid production may be due in part to the fact that the precursors were included in the solid medium. Application via a liquid medium may be more successful. ZENK, EL-SHAGI and SCHULTE (1975) reported that indirect precursors when added to the medium had no effect on metabolic stimulation. However, stimulation could be achieved using a more direct precursor. (This feature was also reported by YOSHIDA, HAYASHI and SANO (1988a) when they fed demecolcine to Colchicum cultures). KURZ and CONSTABEL (1979a) have reported that for successful use of precursors, the precursor should be applied when the cultures' physiological status favours the uptake and metabolism of the precursor.

A peak at 26 minutes (Figure 6.29b) on the HPLC trace was observed when media from the above experiments were chromatographed. Extraction of media that had not been used for culture did not show this peak. Fractions from peak "26" were collected and subjected to GCMS, however the structure and function of the compound remain unknown.

Results from radioactive feeding of callus tissue with ^{14}C phenylalanine, (Figure 6.30) confirm the results for cold feeding. Very little of the compound was found in the fraction co-chromatographing with colchicine on the HPLC. However this is not particularly surprising as phenylalanine is the precursor for numerous compounds in plants (incorporation into "other" compounds accounts for the high peaks observed from time 2 - 6 minutes).

The final extraction for colchicine from in vitro tissue was conducted on cultures that had regenerated into entire plantlets with leaves, stems and corms (see Chapter 3). Figure 6.31 and Table 6.13 represents a comparison of entire Gloriosa plants grown in vivo, as well as in vitro grown plants, callus and malformed roots grown in the same growth room using the same media and media supplements.

The results of Table 6.13 support the statement "Differentiation has been shown to increase product formation". The level of 0,51% colchicine in in vitro grown Gloriosa corms is the same as that found for in vivo grown Gloriosa plantlets collected during the mid-season. Retention or relocation of colchicine from the original mother explant cannot be considered a contributing factor as the corms

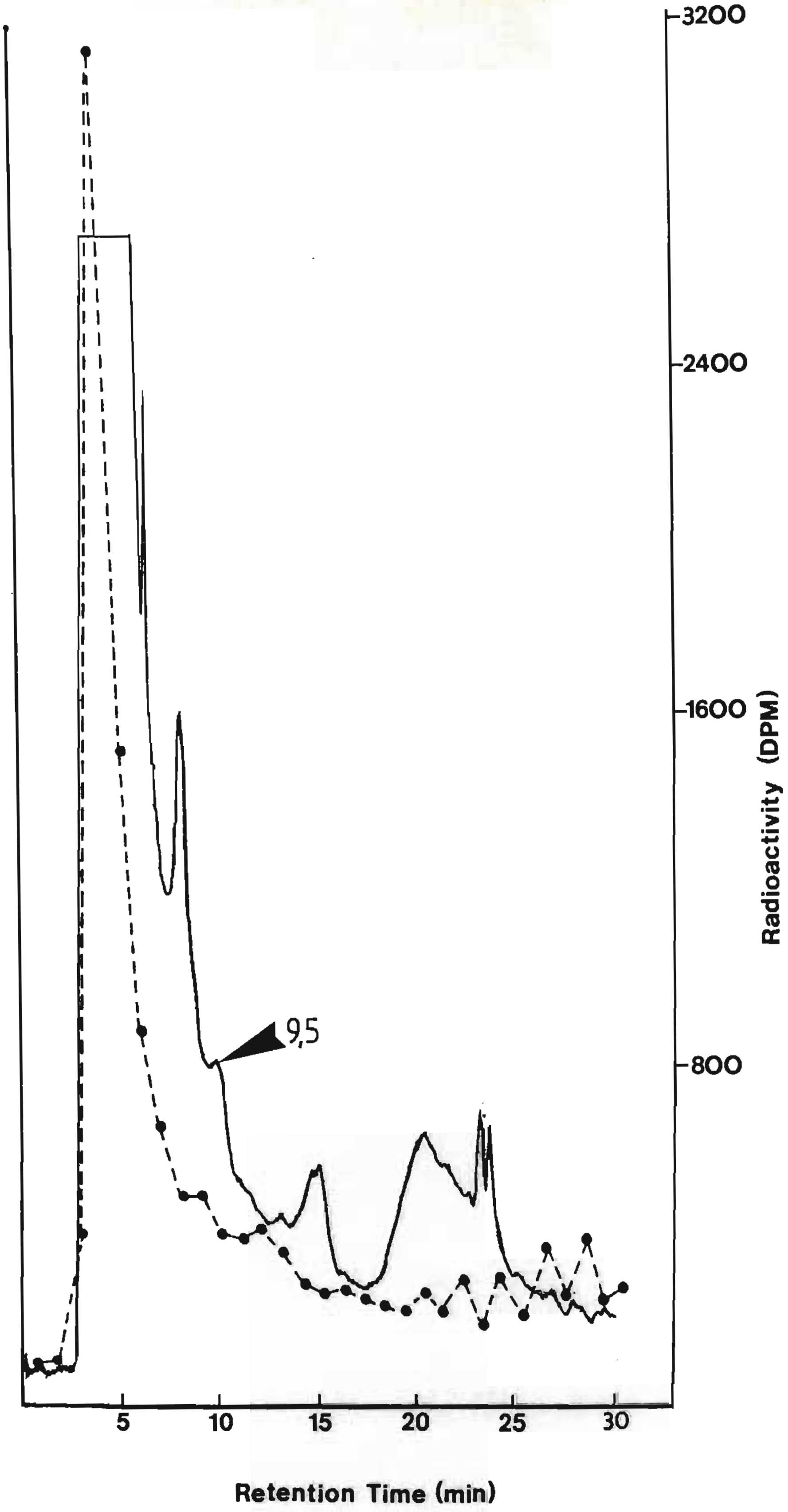

Figure 6.30 Relative radioactive levels (●- - -●) of Gloriosa callus fed with radioactive phenylalanine. (Solid line indicates the HPLC trace of the extract)

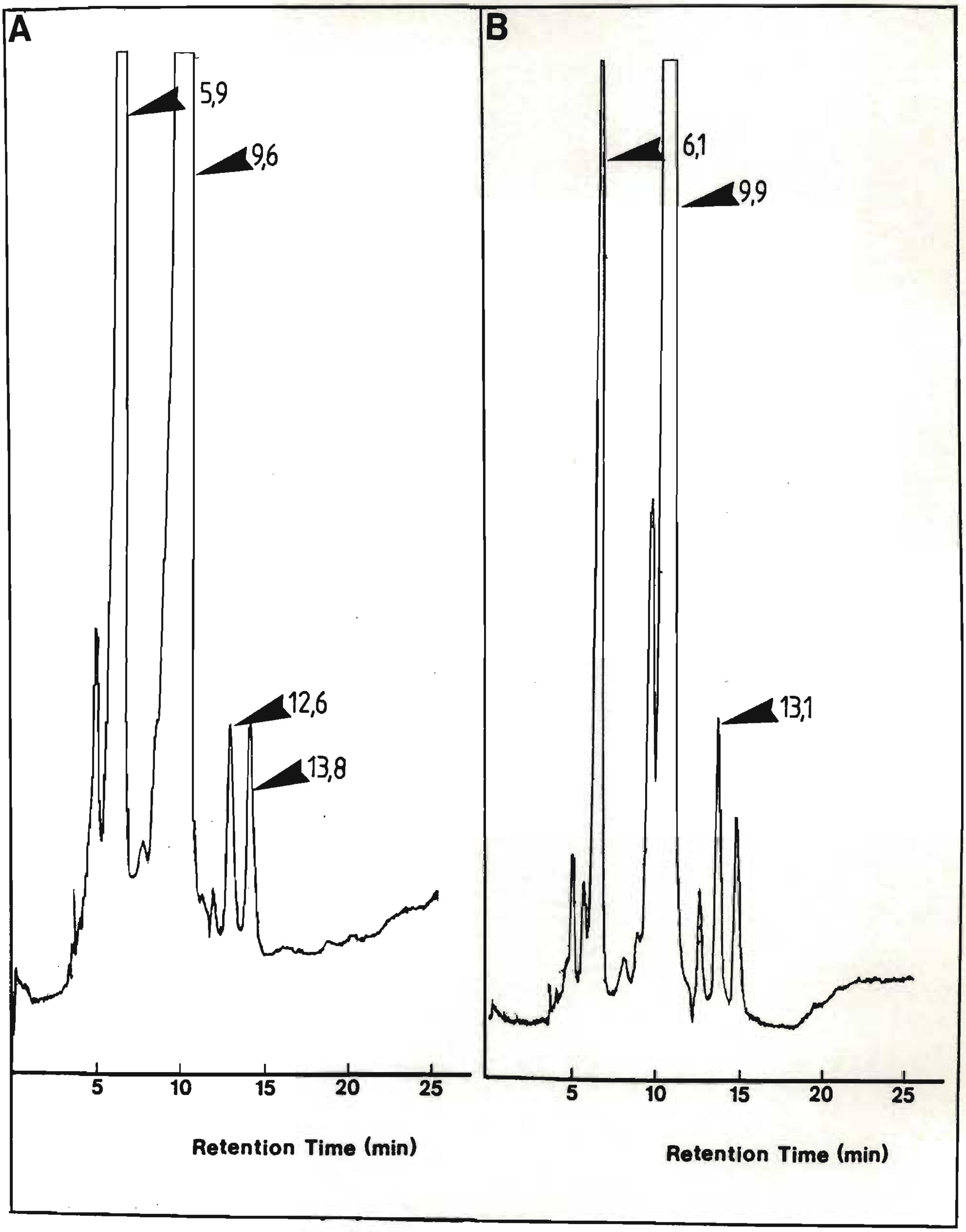

Figure 6.31 HPLC traces of Gloriosa a) corms grown in vitro
b) leaves grown in vitro

used for extraction were taken after two passages in culture. In addition explants used were very small and could not have accumulated these high levels.

Table 6.13 The effect of differentiation on tissue cultured Gloriosa plant's ability to produce colchicine in vitro. (± indicates standard error)

Explant	% colchicine (dry weight)
In vivo grown corms	0,9299 ± 0,151
In vivo grown leaves	0,7873 ± 0,35
In vitro grown corms	0,5153 ± 0,1
In vitro grown leaves	0,3621 ± 0,23
In vitro malformed roots	0,0451 ± 0,01
In vitro grown callus	0,0350 ± 0,01

To derive levels of colchicine in vitro equal to that found for in vivo plants, entire plantlet regeneration is necessary!

Levels of colchicine extracted from callus, malformed roots and entire plantlets respectively show an increase that can be directly related to the amount of differentiation in the culture. There is a great deal of debate as to the necessity of differentiated structures for secondary metabolite production in vitro. BECKER (1987) concluded that auxins lead to dedifferentiation via stimulation of cell division which ultimately results in diminished production of metabolites. BUTCHER (1977) recorded that spatial orientation, compartmentation of enzymes and a site for product accumulation are all factors that affect secondary metabolism in specialized tissue. HRAZDINA and WAGNER (1985) showed that compartmentation is vital for metabolite production. TABATA (1977) recorded that in a number of plants particular compounds are only synthesized or accumulated in particular tissues, and that differentiation in the cultures may induce the production of desirable compounds.

Differentiation has been shown to increase product formation (HIRAOKA and TABATA, 1974; VERZAR-PETRI, LADOCSY and OROSZLAN, 1982). Shoot forming callus was found to produce more digitoxin that was normally found in Digitalis plants (HAGIMORI, MATSUMOTO and KISAKA, 1980) while TABATA, YAMAMOTO, HIRAOKA, MARUMOTO and KONOSHIMA (1971) also showed that shooting callus produced elevated amounts of metabolite. The ability of rooting callus to produce higher concentrations of metabolites over levels found in dedifferentiated callus was shown by FREEMAN, WHENHAM, MACKENZIE and DAVEY (1974) as well as TABATA, YAMAMOTO, HIRAOKA and KONOSHIMA (1972). YEOMAN, MIEDZYBRODZKA, LINDSEY and McLAUCHLAN (1980) on the other hand showed that differentiation was detrimental to product synthesis. DOUGALL (1979) and KURZ and CONSTABEL (1985) have stated that it is the cultural environment that leads to structural specialization which also coincides with product formation. The fact that the gene required for accumulation is linked to morphological development is unlikely due to gene specificity!

A large number of reviewers of the literature on secondary metabolite formation, have stated that it should be possible to find cultural conditions that can enhance metabolite formation without stimulating morphogenesis. However, bearing in mind that all the Gloriosa explants were grown in the same cultural conditions, an increase in the amount of differentiation / organization lead to an increase in product formation.

Commercialization of colchicine production using an in vitro system where callus predominates may not at the present time be economically feasible due to the fact that the colchicine content is ± 20 times lower than that found for in vivo grown plants. At the present state of development the use of organ culture will provide a better system for commercial colchicine production. Should the levels of colchicine in Colchicum as recorded by BELLET and GAIGNAULT (1985) be comparable, then the use of Gloriosa and Sandersonia plantlets grown in vitro may provide an alternative source of colchicine using mass culture of plant corms as advocated in Chapter 3. The use of an in vitro system would overcome seasonality problems, especially when plants are grown in cool temperate climates. The reproduction coefficient of the plantlets is higher in culture than in the field. This proposal however, has major drawbacks in that labour costs and other factors such as limitation on the number of culture vessels, media costs and the availability of suitable growth chambers would provide a major cost component preventing commercialization of the in vitro organogenic technique.

On the assumption that there "must" be cultural conditions where metabolite formation may occur in high quantities, (and should the "search for speedier more wide ranging methods for plant cell and tissue culture" (FUJITA and TABATA, 1987) be successful, then the application of an in vitro technique using Gloriosa and Sandersonia tissue would be more profitable than using the traditional source of colchicine, Colchicum.

6.4 Conclusions

Colchicine produced commercially from Colchicum species has been widely used, in the areas of gout treatment, induction of polyploidy and cancer therapy. At present the use of colchicine in plant breeding is increasing. Gloriosa and Sandersonia plants contain the alkaloid colchicine at levels of ± 0,9%, and could be utilized as an alternative source for commercial colchicine production. Multiple plantlet regeneration as outlined in Chapter 3 provides an ideal system for selective breeding of high producing clones, and multiple plantlet / corm production, for rapid plant propagation.

Many useful secondary metabolites have been obtained from tissue culture in high quantities. This factor prompted HAYASHI, YOSHIDA and SANO (1988) to investigate the potential for colchicine production in tissue culture using Colchicum cells. In their "first report on the production of colchicine alkaloids by plant tissue culture" levels of colchicine extracted from callus cells was usually ten times lower than that found in intact corms.

In many cases it is now clear that many alkaloids are not inert end-products but are in a state of dynamic fluctuation in terms of both concentration and turnover (ROBINSON, 1974). Plants containing secondary metabolites have an optimum time for harvest that is seldom related to senescence, but may often be directly related to a stage of plant development. In the case of colchicine in Gloriosa, the levels are highest during the initial growth of the plant and these levels decline during growth, with a slight increase in the alkaloid content when corms become dormant at the end of the season. A question often asked is what selective advantage does the presence of an alkaloid confer on a plant? Often the answer to the question is that the production of alkaloids has no advantage. It is now widely believed that alkaloids function as poisons or repellants towards predators, parasites and competitors. In the case of colchicine-producing plants, there is an increase in the amount of colchicine during the early growth phase when the leaves have just emerged from the soil, and when they are most "palatable". It appears that a selective advantage can be realized as the "bitter principle" (WARDEN, 1881) confers a protective role against predators (the action of colchicine is more effective in warm blooded animals than cold blooded ones). However, it has been reported that porcupines can devour Gloriosa corms with impunity (WATT and BREYER-BRANDWIJK, 1962). Seeds of Gloriosa are red and prominantly displayed when the capsule opens, and it is assumed that the seeds are dispersed by birds. Does the colchicine affect the metabolism of the bird? The high levels of colchicine in the plant may also confer a degree of competitive advantage, however, it has yet to be ascertained why the alkaloid does not affect the plant itself. Plant tissues are generally less sensitive to colchicine than are animal cells. Normally plants require 1 000 times higher concentrations of the alkaloid to arrest mitosis (EIGSTI and DUSTIN, 1955; ZRYD, 1979).

Levels of colchicine in Gloriosa and Sandersonia are very similar, in the range of ± 0,9%. From evidence presented by BELLET and GAIGNAULT (1985) the levels in the two study species is much higher than the recorded level (0,62%) for Colchicum. The higher colchicine levels in these two species makes the plants a more viable source for commercial colchicine production. The manufacture of colchicine from plants would probably have to be restricted to tropical or sub-tropical regions, as successful cultivation of these plants, in cooler temperate climates, often has to be performed in hothouses. This results in an increase in the production cost and limits the space available for cultivation.

Production of metabolites in culture provides a viable alternative to the problems involved in the production of chemical, pharmaceutical and medicinal compounds (BARZ, REINHARD and ZENK, 1977; KURZ and CONSTABEL, 1979a; 1979b; STABA, 1980). In general yields of secondary metabolites in cell cultures is low, however recently many useful metabolites have been obtained in large amounts from tissue culture of higher plants (COLLIN, 1987).

Gloriosa and Sandersonia cultures were subjected to a number of nutritional and environmental variants. High levels of cytokinins and auxins are inhibitory to alkaloid synthesis, while low levels (0,1 mg ℓ^{-1} kinetin) of growth regulators may stimulate colchicine production. In general, cultural factors that result in retarded growth, were related to high levels of colchicine, showing an inverse relationship between metabolite production and growth. An increase in available sucrose levels results in an increase of colchicine, while a decrease in the relative amount of nitrogen can be related to an increase in colchicine levels. Contrary to most reports, darkness and variation (dark/light) in the light regime resulted in stimulated metabolite production.

The use of solid or liquid media did not affect the relative levels of colchicine produced, with comparable amounts of the alkaloid being produced in both types of media. A certain amount of colchicine is released into the liquid medium. The use of a liquid medium is more advantageous, as large fermenter tanks can be used for metabolite production.

Levels of colchicine found in in vitro grown roots and callus was lower than that found in in vivo plants. Levels of colchicine found in in vitro grown tissue was 10 - 20 times lower than in vivo tissue. Levels of colchicine found in plantlets grown in vitro was the same as that normally recorded for parent tissue. The higher levels of colchicine in malformed roots also adds to the evidence that differentiation affects colchicine production in vitro in Gloriosa tissue. It is thought that it should be possible to find cultural conditions where metabolite formation is enhanced without stimulating morphogenesis. At our present state of knowledge the use of differentiated structures for commercial colchicine production would be a far more economically feasible proposition than the use of undifferentiated callus.

From this investigation it has been shown that cultured Gloriosa and Sandersonia tissue can synthesize colchicine in vitro. The extent to which the cells' synthetic capacity can be enhanced has yet to be determined. The use of an in vitro system for commercial colchicine production at the present state of development is not recommended. BARZ and ELLIS (1981) have reported that cell cultures are only economically viable when those products selected for are costly or plant specific.

The view of BERLIN and BODE (1987) is more relevant, as they have stated that the feasibility of commercial product formation in tissue culture is too closely related to cost comparisons of field cultivation and that cost discussion is not yet meaningful. It is thought that speedier and more wide ranging methods for efficient production of metabolites in culture will be found in the near future (FUJITA and TABATA, 1987).

CHAPTER 7

THE EFFECTS OF COLCHICINE ON *in vitro* SYSTEMS

7.1 Introduction

Plant tissues are generally less sensitive to colchicine than are animal cells. Normally plants require 1 000 times higher concentrations of the alkaloid to arrest mitosis. Recently there have been an increasing number of reports concerning the production of polyploids via tissue culture (ESPINO and VAZQUEZ, 1981; CHAVADEJ and BECKER, 1984; CURRAH and OCKENDON, 1987). However, "alkaloids can be toxic to plants in high concentrations, even to the same plant that makes them. The powerful effects of colchicine are well known." (ROBINSON, 1974). Assuming that the levels of colchicine in *in vitro* cultures of *Colchicum*, *Gloriosa* and *Sandersonia* could be stimulated above that found in the mother plant, what would be the effect of these high levels of colchicine on the growth of the cells in culture?

The following research was directed towards the study of the effects of colchicine on "known" *in vitro* systems. Although the cells of *Colchicum*, *Gloriosa* and *Sandersonia* may be more "immune" to the effect of colchicine (or alternatively the cells may have developed induced resistance to colchicine (ZRYD, 1979)), the study of the effects of colchicine on *in vitro* systems will provide an insight into the inhibitory / stimulatory potential of colchicine on *in vitro* systems.

7.2 Materials and methods

7.2.1 Polyploidizing effect of colchicine on seeds prior to germination *in vitro*.

Seeds of *Lycopersicon esculentum* cv Moneymaker, *Nicotiana tabacum*, and *Parthenium argentatum* W10, were soaked in either distilled water or in a 10^{-2} molar solution of colchicine for 24 hours. The seeds were sterilized in 3,5% NaOCl and transferred to a full strength MURASHIGE and SKOOG (1962) medium supplemented with 30 g ℓ^{-1} sucrose, 8 g ℓ^{-1} agar and 0,2 mg ℓ^{-1} $GA_{4/7}$. The seeds were allowed to germinate and grow *in vitro* for four weeks, after which time chromosome counts were conducted on the roots.

7.2.2 Staining for chromosomes

Plant material (primarily root tips) was harvested in the early morning and placed in a saturated solution of p-dichlorobenzene for four hours at 10°C. Plant material was fixed in glacial acetic acid : ethanol (1 : 2) for one hour, followed by a soak in 45% acetic acid for 15 minutes. The material was then placed in a 2% solution of acetic orcein : HCl (9 : 1) and heated over a flame for five to ten seconds. The prepared material was positioned on a slide in a drop of 1% acetic orcein solution and "squashed". Chromosome squashes were viewed and counted under an Olympus BH_2 photomicroscope.

7.2.3 Colchicine and the soybean callus bioassay

Using the soybean callus bioassay (MILLER, 1965) (with the media supplemented according to the following grid (Figure 7.1)) three callus pieces were placed on each replicate. The cultures were grown in a growth room with the temperature set at 25° ± 2°C and a light intensity of 0,5 $\mu Em^{-2}s^{-1}$ for four weeks. After this period the cultures were harvested and weighed.

		μg ℓ^{-1} kinetin			
		0	1	10	50
% colchicine	1				
	0,1				
	0,01				

Figure 7.1 Media supplementation with colchicine and cytokinin for the Miller callus bioassay.

7.2.4 Colchicine and soybean / tobacco tissue culture

Soybean callus and tobacco pith explants (SKOOG and MILLER, 1957) were cultured on a full strength MURASHIGE and SKOOG (1962) medium supplemented with 30 g ℓ^{-1} sucrose, 8 g ℓ^{-1} agar, 0,5 mg ℓ^{-1} kinetin and 3 mg ℓ^{-1} IAA. Various concentrations of colchicine were added to this medium (0; 10^{-2} M; 10^{-3} M; 10^{-4} M; 10^{-6} M; 10^{-8} M; 10^{-10} M). The colchicine was either added to the medium and autoclaved or the colchicine was filter sterilized and added to a warm sterilized agar medium. Standard size pieces of sterile tobacco pith and soybean callus were placed on the media, and grown in a culture room set at 25° ± 2°C with a 16-hour light : 8 hour dark cycle. Cultures were grown for six weeks and then harvested, weighed and extracted for cytokinin. (A Dowex 50 cation exchange resin was used to purify samples (VAN STADEN, 1976) which had been extracted overnight in 80% ethanol at 5°C). The extracts were assayed for cytokinin activity using the soybean callus bioassay (MILLER, 1965).

7.2.5 The effect of colchicine on the *in vitro* flowering of *Kalanchoë*

Based on the *in vitro* flowering system of DICKENS and VAN STADEN (1988) the effects of colchicine on *in vitro* flowering was tested. Plants of *Kalanchoë* blossfeldiana Poellniz. were cultured in vitro on one-fifth of the salts of MURASHIGE and SKOOG's (1962) medium. The medium was supplemented with 30 g ℓ^{-1} sucrose and 9 g ℓ^{-1} agar. Stock plants were grown under non-inductive long days (18 hours) in an external light intensity of 100 $\mu Em^{-2}s^{-1}$ at 25°C. Nodal explants (containing two leaves) were cultured in 100 mℓ Erlenmeyer flasks containing 40 mℓ basal medium supplemented with various concentrations of colchicine (0; 10^{-4} M (filter sterilized); 10^{-4} M; 10^{-6} M; 10^{-8} M; 10^{-10} M autoclaved). The cultures were grown under inductive short day (eight hour) conditions (DICKENS and VAN STADEN, 1988). After the required period of time, plants were harvested and a variety of vegetative and reproductive characteristics were measured.

7.3 Results and discussion

7.3.1 Polyploidizing effect of colchicine in cultured tissue

The ability of colchicine to induce polyploidy in the various tissue culture systems is summarized in Table 7.1.

Table 7.1 The polyploidizing effect of colchicine on in vitro systems

Treatment	% diploids in the control	Treated % diploid	 % polyploid
Lycopersicon seeds soaked in colchicine	85	60	22 ± 7
Parthenium seeds soaked in colchicine	90	67	11 ± 6
Nicotiana seeds soaked in colchicine	93	50	27 ± 15
Tobacco explants grown on colchicine (10^{-2} M)	90	85	7 ± 2
Kalanchoë explants grown on colchicine (10^{-2} M)	94	90	5 ± 1

It was apparent that a higher percentage of polyploids could be induced using the seed soak method. The levels of polyploidy found in cultures grown in colchicine containing agar was exceptionally low. ZRYD (1979) found that cultures of Daucus died when cultured on a medium containing 2,5 x 10^{-3} M colchicine. Ploidy levels were raised when Valeriana cultures were treated with 0,05% or 0,2% colchicine, with a higher percentage being obtained after a second treatment. Continued culture in the absence of colchicine, resulted in a large number of the cultures returning to the original chromosome number (CHAVADEJ and BECKER, 1984). CHEN and GOEDEN-KALLENMEYN (1979) grew daylily callus on a medium containing colchicine and found that 50,9% of the plantlets derived from the callus were polyploids. A treatment level of 20 mg ℓ^{-1} was found to be most effective in producing tetraploidy. CURRAH and OCHENDON (1987) reported that injection treatment with colchicine may have an advantage over application via soaked cotton wool as the stabbing action facilitates entry of colchicine into the second layer of the apical meristem. PRYOR and FRAZIER (1968) recorded that colchicine treatment was most effective when using actively growing vegetative tissue.

The lower levels of polyploids found in the above experiment may have been a result of a number of factors; cultures were grown in light and the colchicine may have been photoconverted to lumicolchicine (SABNIS, 1981), and the levels of colchicine available to the plant may have been low because it was applied in the agar. The tissue used as explants for Kalanchoë and tobacco cultures was relatively well differentiated. For this reason it is likely that the differentiated tissue in contact with the source of colchicine was not active, and only small amounts of colchicine was transferred to the actively growing portions of the explant.

Direct application of colchicine to growing points, soaking seeds in solution, injection, and the growth of cultures in suspension will give higher levels of polyploidy than that found with cultures grown on agar. However certain precautions must be followed; application during active growth phases, application to undifferentiated or differentiating tissue and preventing photoconversion by light.

7.3.2 Colchicine and the soybean callus bioassay

A three dimensional representation of the effect colchicine and cytokinin has on soybean callus growth in culture is presented in Figure 7.2. Application of colchicine at 1% to the culture medium markedly inhibits cell growth, however, at 0,1%, and 0,01% colchicine does not appear to stimulate or inhibit cell growth. The presence or absence of cytokinin played a greater role in the stimulation of cell growth. CHAVADEJ and BECKER (1984) treated cell cultures of Valeriana with 0,05%, 0,2% and 0,5% colchicine. The treatment with 0,05% and 0,2% colchicine resulted in well growing cultures, while the highest dose caused the cells to die. Cells treated with 0,05% grew to 70% of the weight of the control. On transfer of colchicine treated Valeriana cultures onto a medium containing the same colchicine solution, the cells died (even after five colchicine free passages, the cells died when returned to the colchicine medium). This effect was explained as being a factor of accumulation of colchicine in the cytoplasm. ZRYD (1979) reasoned that colchicine resistance may be due to a loss of membrane permeability.

The fact that only 1% colchicine affected cell growth, is indicative of the fact that plant cells are generally insensitive to colchicine.

7.3.3 Colchicine and soybean / tobacco tissue cultures

Results from the culture of soybean callus and tobacco pith explants is shown in Figures 7.3, 7.4 and 7.5. It is again evident from these graphs that colchicine applied to the medium at high concentrations (10^{-2} M; 10^{-3} M and 10^{-4} M), de-

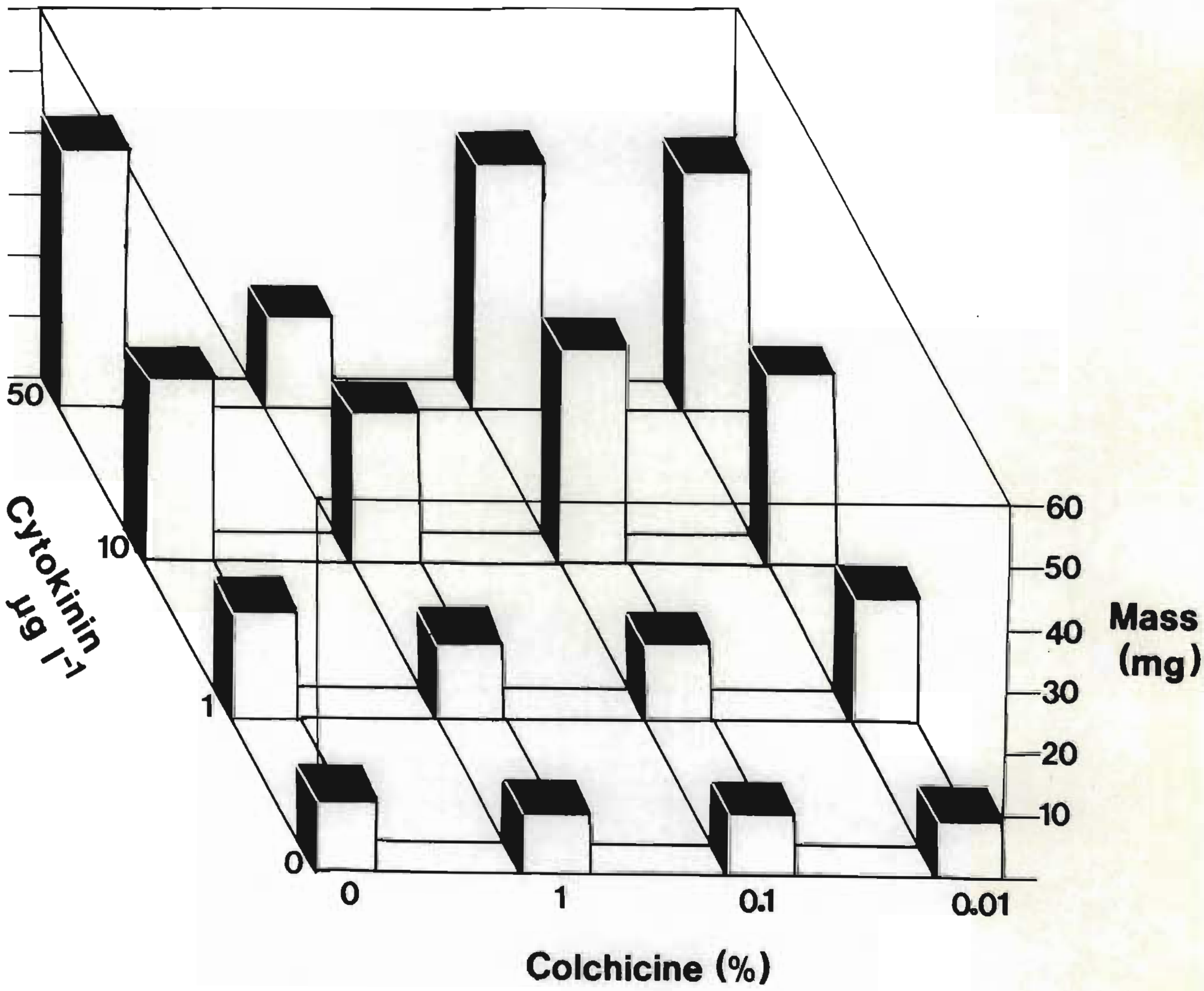

Figure 7.2 Three dimensional representation of the effect colchicine has on soybean callus growth in vitro

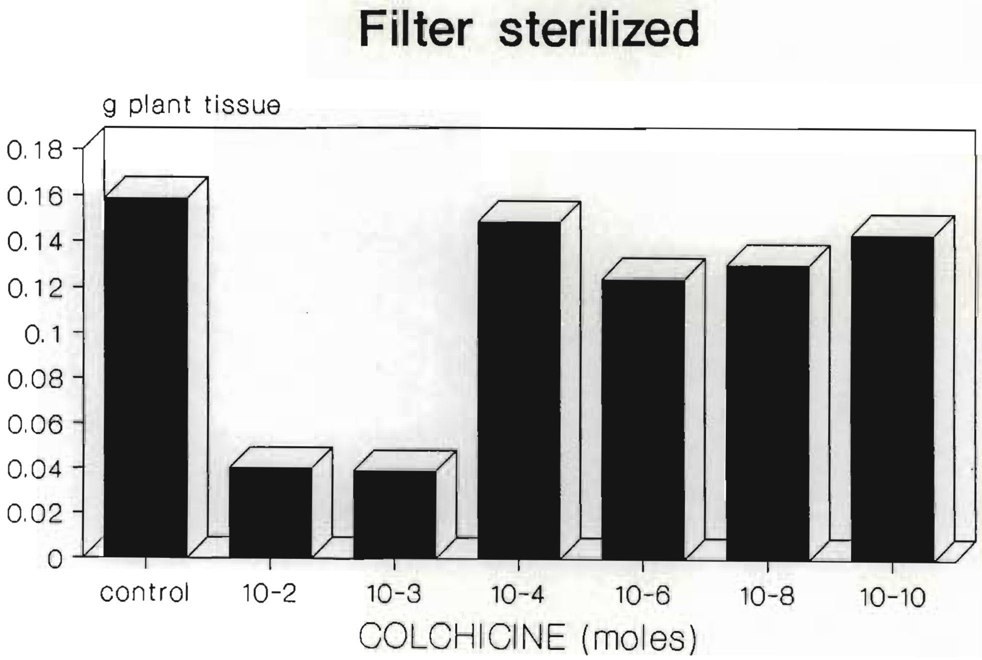

Figure 7.3a Soybean callus growth on colchicine supplemented media (filter sterilized).

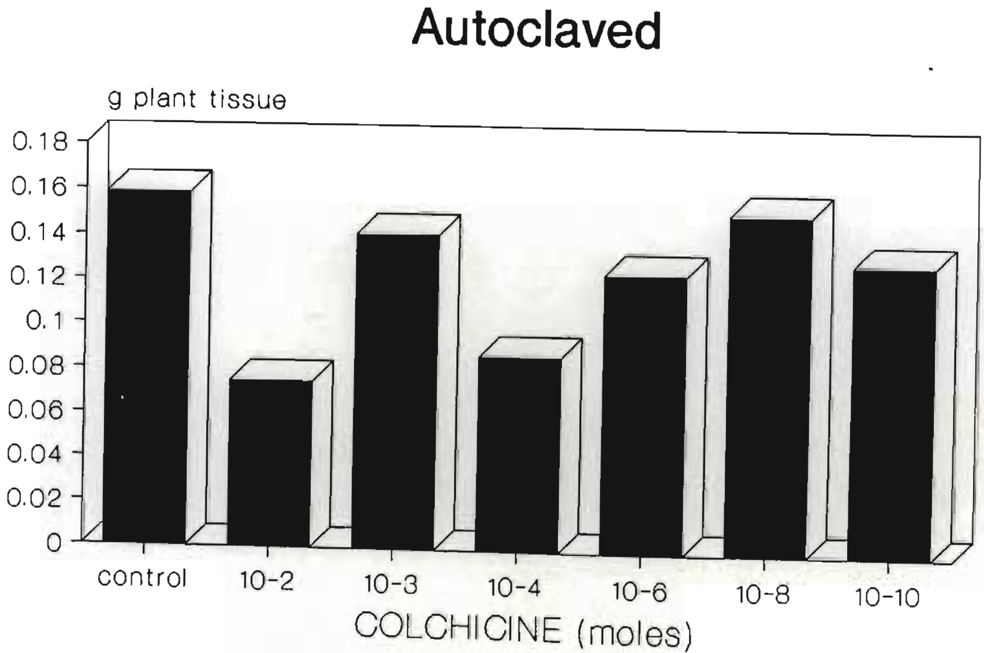

Figure 7.3b Soybean callus growth on colchicine supplemented media (autoclaved).

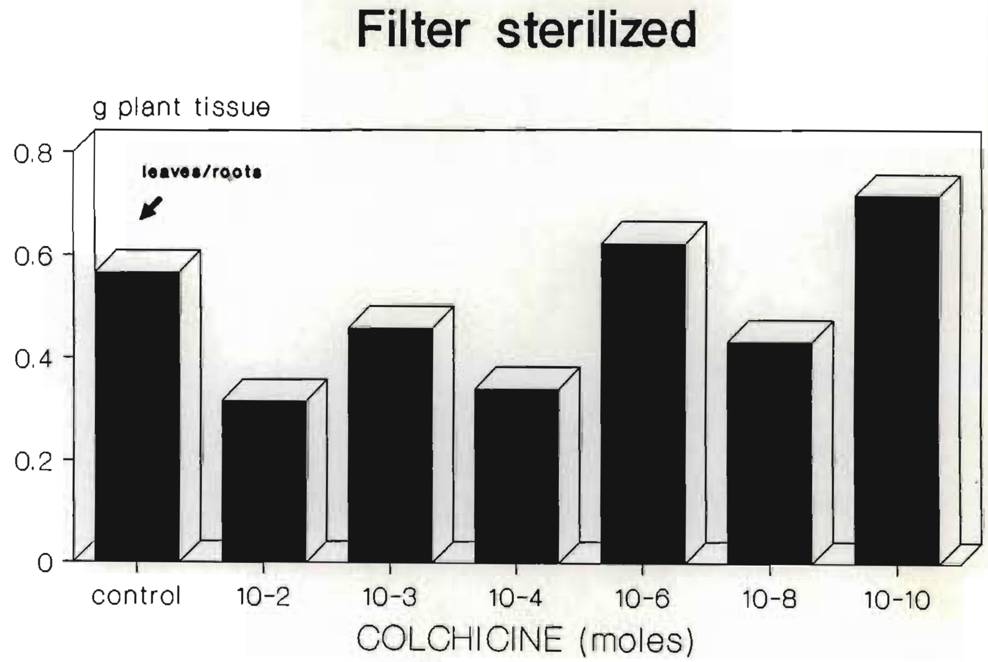

Figure 7.4a Tobacco pith explant growth on colchicine supplemented media (filter sterilized).

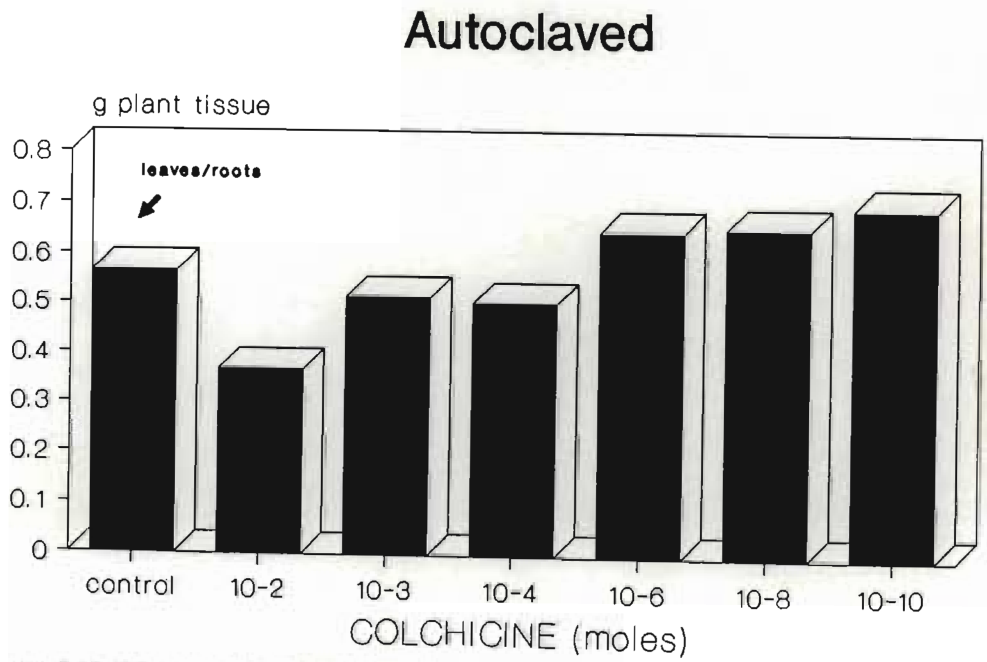

Figure 7.4b Tobacco pith explant growth on colchicine supplemented media (autoclaved).

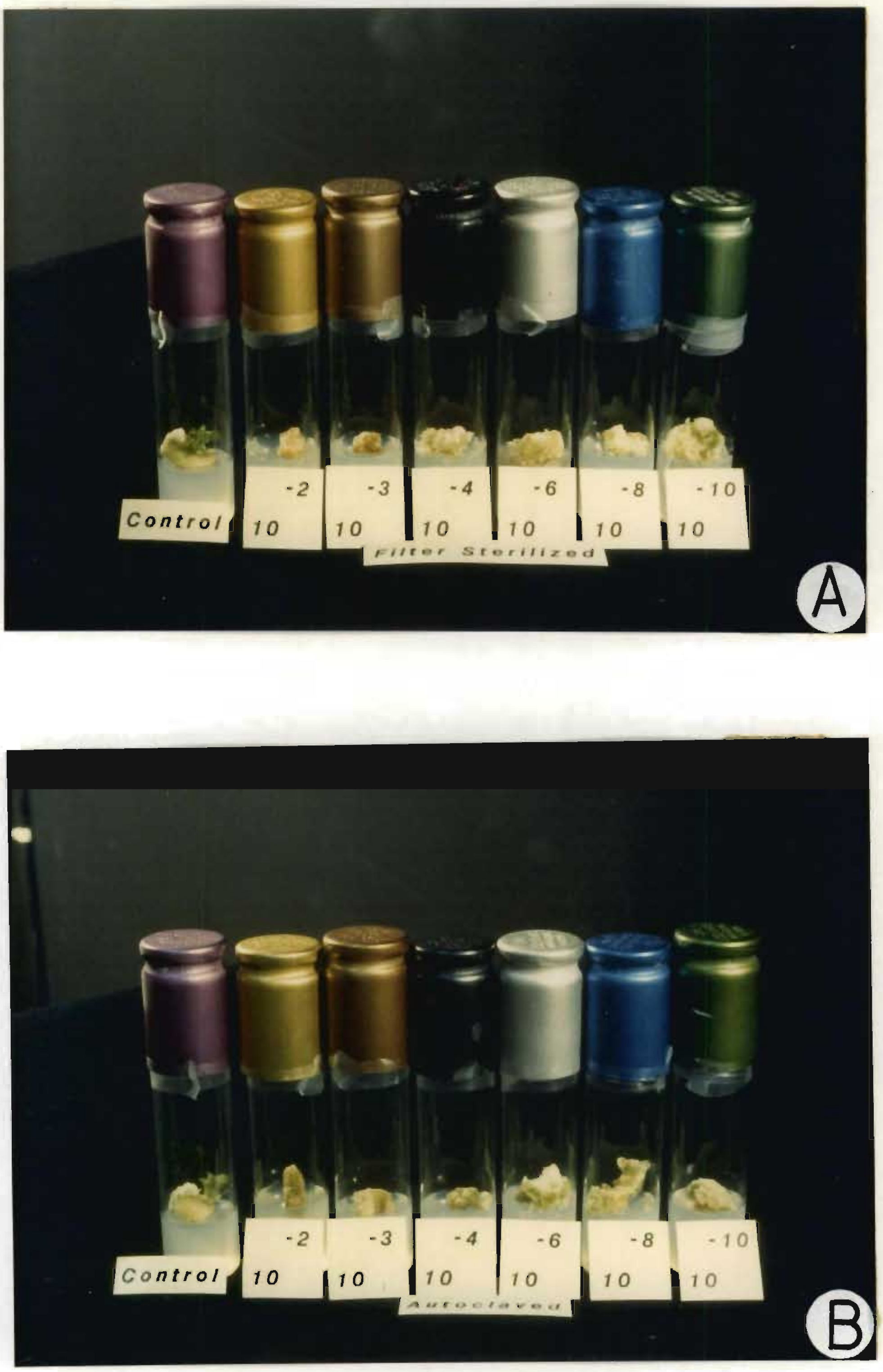

Figure 7.5 Colchicine and tobacco pith explant growth

a) filter sterilized

b) autoclaved

creased plant growth. At lower levels the amount of growth recorded was similar to that found for the control. Cultures growing on autoclaved colchicine, had a slightly higher growth rate than did the cultures supplemented with filter sterilized colchicine.

"It was thought at one time that colchicine increased the rate of cell division in plants" (HAMERSLAG, 1950). It is evident that at high concentrations of colchicine cell division is retarded, while at low concentrations the growth pattern is similar to the control. A noticeable feature was the production of leaves and roots in the control cultures, whereas morphogenetic development was retarded in the colchicine treatments. GOTTLIEB and GOLDSTEIN (1979) found that colchicine disrupted normal colony development in Eudorina. The colchicine effect on development is probably due to its action on the deposition of fibrils in cell walls. Colchicine has been shown to affect the dedifferentiation of adventitious root, and growth occurred by cell enlargement only (OPPENOORTH, 1978). UPADHYAYA and NOODEN (1977; 1978) and HOGETSU and SHIBAOKA (1978) showed that colchicine inhibited root elongation, but caused a swelling in the zone of elongation probably due to a change in the deposition of microfibrils.

It is also feasible that colchicine affects endogenous levels of growth regulators. ZATYKO (1973) found that pinto bean leaves treated with colchicine resulted in a high concentration of cytokinins. Logically this makes sense as cell division is inhibited, which should result in an accumulation of cytokinin. Results from the bioassay for cytokinins (from extracts made from the soybean and tobacco cultures) showed no significant difference in the relative activity of the cytokinins. Cytokinin activity was slightly higher in tobacco extracts, however, this can be explained by the presence of endogenous growth regulators in the original pith explant, and production of hormones by the organs regenerated in culture. (Pith explants contained sections of the cambium, a plant part speculated to be a site of cytokinin synthesis (VAN STADEN and DAVEY, 1979)). Soybean callus used for the bioassay is dependent on an exogenous supply of the hormone, and cytokinin activity found in these extracts was probably due to the cytokinin added to the medium.

7.3.5 Colchicine and in vitro flowering

Results from in vitro flowering Kalanchoë explants are presented in Figures 7.6 and 7.7. Once again it is evident that high concentrations of colchicine retard culture growth. Kalanchoë explants grown in cultures treated with colchicine, were able to produce morphological structures, however the number of flowers, size of the leaf and relative weights of the organs were markedly affected by the presence of colchicine. As evident in Figures 7.6 and 7.7, at low concentrations of colchicine (10^{-8} M and 10^{-10} M) the number of flowers produced was similar to that of the control. However, treated flowers were smaller and never fully opened in culture. There appears to be very little difference between the response of plants grown in autoclaved media and those grown on filter sterilized colchicine. However, the relative amount of morphological development was greater in autoclaved cultures. In the case of Kalanchoë tissue culture, colchicine did not totally inhibit morphological development, contrary to the reports of UPADHYAYA and NOODEN (1977; 1978) and OPPENOORTH (1978).

It was hoped that the colchicine applied to the medium might cause a change in the flower colour or other noticeable vegetative features. Apart from the size of the cultured plants, no changes of this nature were observed. Chromosome squashes of the roots and leaves showed that only a limited amount of polyploidy had occurred in culture (± 5% in cultures grown on 10^{-2} M colchicine).

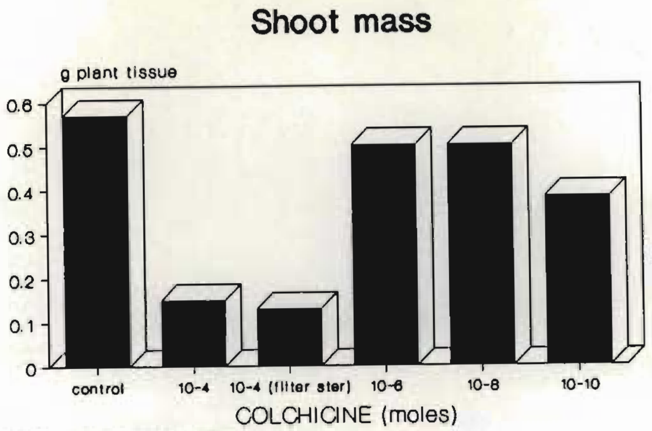

Figure 7.6a Shoot mass of Kalanchoe explants grown on colchicine supplemented media.

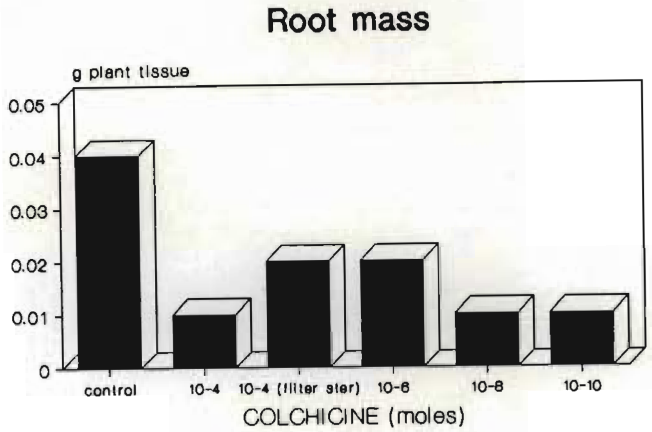

Figure 7.6b Root mass of Kalanchoe explants grown on colchicine supplemented media.

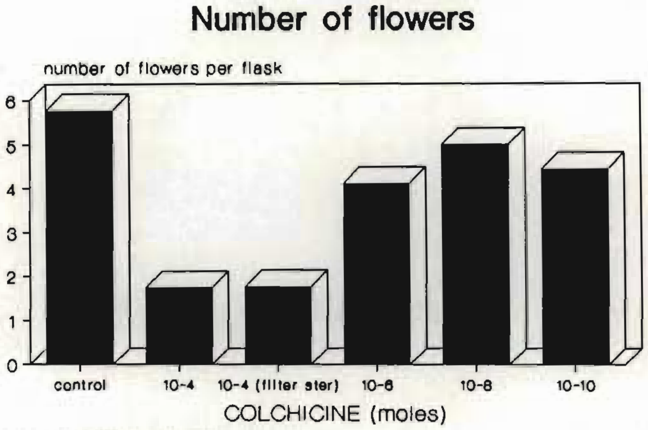

Figure 7.6c Number of flowers produced on Kalanchoe explants grown on colchicine supplemented media.

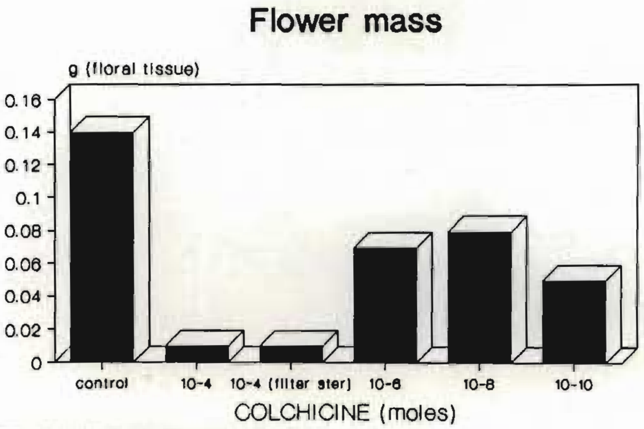

Figure 7.6d Mass of flowers produced on Kalanchoe explants grown on colchicine supplemented media.

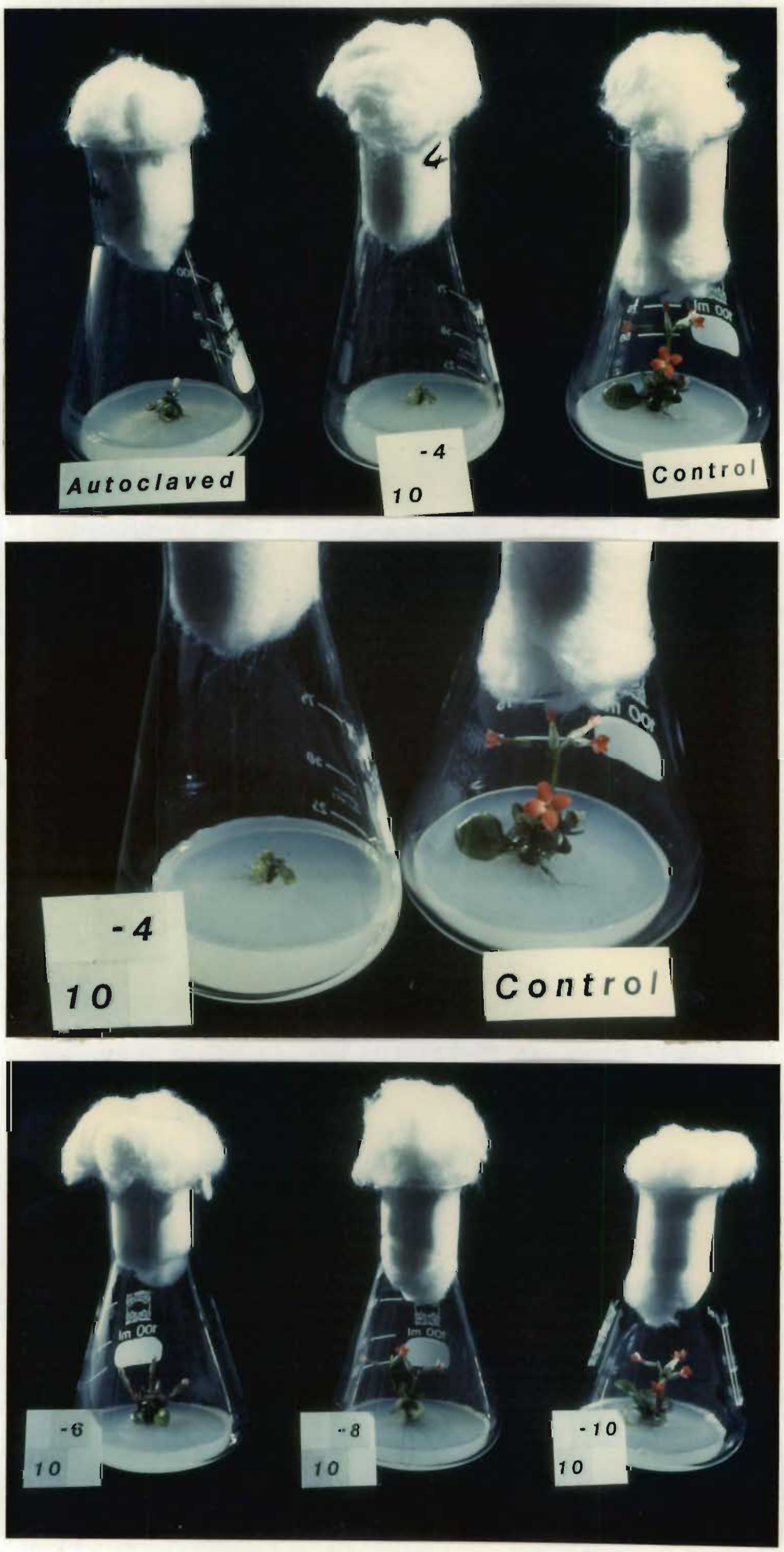

Figure 7.7 Kalanchoë tissue culture and colchicine (note the differerence in morphological development of Kalanchoë tissue on the autoclaved and filter sterilized media)

7.4 Conclusions

The general observation made from the above experiments is that colchicine is required at high concentrations to evoke a change in the growth pattern of the culture. Plant tissues are generally less sensitive to colchicine than are animal cells. The method of colchicine application to plant tissue greatly affects the ability of the alkaloid to elicit a response within the plant. Growth of differentiated tissue on media supplemented with colchicine was not markedly affected by the presence of the alkaloid, and the percentage of polyploids that resulted was low. Perhaps the best means of colchicine application to cultures to induce polyploidy are; direct injection into the plant, soaking of actively growing growth regions (or seeds) or growth of cultures as a suspension.

The effect of colchicine on tissue cultures is limited to a reduction in growth. In certain types of cultures, the colchicine may affect the morphological response of the culture, while in others morphological response is retarded but not totally inhibited.

As mentioned in the introduction the reasoning behind this section of research was to establish whether or not colchicine inhibited culture growth. If so, whether the production of high levels of colchicine in *Colchicum*, *Gloriosa* and *Sandersonia* cultures would have an effect on their *in vitro* response. Although the majority of the above research was conducted on solid media, it is evident that for inhibition of cell growth exceptionally high levels of colchicine are necessary. It is unlikely that levels of colchicine produced *in vitro* will ever be elevated sufficiently to be inhibitory to cell growth. (Levels of 10^{-2} M will have to be produced in the cultures to have any effect on cell growth). This together with the plant's "natural immunity" to colchicine, indicates that even with enhanced levels of colchicine production *in vitro*, the likelihood of the colchicine retarding the growth of the cells in commercial cultures is small.

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APPENDIX I

PROGRAMME FOR COLCHICINE DETERMINATION USING HPLC (VARIAN 500)

```
SINGLE CHANNEL METHOD:  COL
SECTION  1:  BASIC
PAGE  1
     ANALYSIS PARAMETERS
     CHANNEL:  1
               calculation:  A%
     AREA/HT:  A
     STOP TIME:  30.00
     NUMB EXPECTED PKTS:  100
     EQUILIBRIUM TIME:  0
     UNRETRAINED PK TIME:  0.00
     UNIDENT PK FACTOR:  0.000000
     SLICE WIDTH:  10

PAGE  2
     SAMPLE PARAMETERS
     RUN TYPE:  A
     SAMPLE ID:
     DIVISOR:  1.000000
     AMT STD:  1.000000
     MLTPLR:  1.000000

PAGE  3
     REPORT INSTRUCTIONS
     WHERE TO REPORT:  L
     COPIES:  1
     TITLE:  COL
     FORMAT:  N
     DECIMAL PLACE:  4
     RESULT UNITS:
     REPORT UNIDENT PKS:  Y
     REPORT INSTRUMENT CONDITIONS:  N
```

```
PAGE 4
    PLOT INSTRUCTIONS
    PLOT: Y
    ZERO OFFSET: 5
    ANNOTATION
            RETENTION TIME: Y
            PLOT CONTROL: N
            TIME TICKS: Y
            TIME EVENTS: Y
            PK START/END: N

PAGE 5
    CHART SPEED
            PAGES OR CM/MIN: C
            INIT VALUE: 0.3

PAGE 6
    PLOT ATTEN
            INIT PLOT ATTEN: 32

SECTION 2: TIME EVENTS
PAGE 1
    LINE NO.    TIME     EVENT    VALUE

       1        0.00      PR      50000
       2        0.00      SN          2
       3        0.00      T%        5.0
       4        0.00      WI          4

SECTION 5: LC INSTRUMENT CONTROL
PAGE 1

    PMAX: 350
    PMIN: 10
    COL TEMP: 30
    ANALOG OUT: %B
```

PAGE 2

LINE NO.	TIME	EVENT	VALUE
1	0.0	FL	1.0
2	0.0	RS	ABC
3	0.0	EV	0
4	0.0	%B	50
5	0.0	%C	50
6	30.0	%C	80
7	30.0	%B	20
8	40.0	%C	100
9	50,0	%C	100
10	55.0	%C	50
11	55.0	%B	50

SECTION 7: POST RUN

PAGE 1

FILE NAME : COL

SAVE INSTRUCTIONS

TYPE: TAR

WHERE TO SAVE: U

TRANSMIT/REPLOT INSTRUCTIONS

TRANSMIT RAW DATA: N

REPLOT WITH BASELINES: N

RAW DATA LOCATION: U

TRANSMIT REPORT: N

PAGE 2

METHOD LINKING INSTRUCTIONS

METHOD:

LINK CALC RESULTS: N

PROGRAM EXECUTION

PROGRAM:

PARAMETERS:

RESERVE PRINTER: Y