THE REGENERATION OF *HYPOXIS ROOPERI* S. MOORE AND PRODUCTION OF HYPOXOSIDE *IN VITRO*

by

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Submitted in fulfilment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in the

Department of Botany, Faculty of Science,
University of Natal, Pietermaritzburg.

DECEMBER, 1984.

PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, from February 1981 to December 1983, under the supervision of Professor J. van Staden. These studies, except where the work of others is acknowledged, are the result of my own investigation.

Yvonne Margaret Page

December, 1984

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to the following people:

Professor J. van Staden for his guidance and encouragement during the past 4 years; Dr C.B. Rogers for his assistance on the hypoxoside quantification technique; Professor S.E. Drewes for supplying a pure sample of hypoxoside; Dr E. Schoonraad, Mr V.G. Naiken and members of the Electron Microscope Unit for their advice and assistance with light and electron microscopy; Mr V. Bandu, Mr J. Kundalram and Mr D. Tunnington for their assistance on photography; Professor R.N. Pienaar and Dr N.A.C. Brown who gave advice during my Research Committee meetings; Mrs D. Judkins for typing this manuscript; and the post-graduate students for encouragement and help on numerous occasions.

Most important of all I wish to specially thank:

My parents for their love and financial help during the past 8 years; Mr R.M. Pike for more help than there is space to enumerate upon here; Miss K. Kelly for both moral and academic support; and my sister for the most professional drawing of many of my figures.

I also gratefully acknowledge the receipt of bursaries from Essential Sterolin Products (PTY.) Ltd. and the Council for Scientific and Industrial Research.

TO MY PARENTS

ABSTRACT

Against the background of the increasing pharmaceutical importance of members of the genus *Hypoxis* L., methods for propagating these plants and for producing hypoxoside (the believed active compound found within *Hypoxis* species) using *in vitro* techniques, were investigated. These investigations were accompanied by anatomical observations. *Hypoxis rooperi* T. Moore was selected as experimental material because of its availability and common usage among researchers studying the genus *Hypoxis*.

Two aseptic procedures were developed for propagating *H. rooperi*. These being the only procedures as yet to be established and documented, using a member of the family Hypoxidaceae. The first procedure involved the induction of callus and adventitious shoots from flower bud explants of *H. rooperi*. For this response to be initiated, the buds selected for culture had to be of a specific morphological and physiological age. The best medium determined for inducing a callusing and shooting response from these explants, was a MURASHIGE and SKOOG (1962) medium supplemented with low levels of 1-naphthalene acetic acid and high levels of 6-benzylaminopurine. The rate of this response was enhanced by the wounding of flower bud explants (i.e. by the excision of the perianth segments, stamens and style from the buds). Investigations indicated that callus and adventitious shoot formation was inhibited by the acropetal positioning of damaged flower buds on the culture medium. This inhibition was not manifest when buds were placed basipetally or horizontally on the culture medium. Flower bud harvest time was not found to have a marked effect upon the numbers of explants responding in culture. On average 37,5 per cent of the buds formed callus and adventitious shoots throughout the flowering season.

The subculturing of callus tissue established from *H. rooperi* flower buds, onto a MURASHIGE and SKOOG (1962) medium supplemented with the same hormone levels as were initially used to induce callus and shoot formation, resulted in the production of multiple adventitious shoots. Serial subculturing of this tissue indicated that the shoot producing capacity of the callus, was maintained for at least a year. Shoots

produced via this method, when inoculated onto a hormone-free culture medium, formed roots. Seventy-five per cent of the plantlets regenerated *in vitro* were successfully "hardened-off". Theoretically it was calculated that using the micropropagation procedure developed, almost 81000 *H. rooperi* plantlets could be established from 100 flower bud explants, within a year.

The second aseptic procedure developed, involved the culturing of explants excised from the primary thickening meristem region of *H. rooperi* corms. The best medium determined for inducing the formation of adventitious shoots from these explants, was a MURASHIGE and SKOOG (1962) nutrient solution supplemented with: equivalent low concentrations of 1-naphthalene acetic acid and 6-benzylaminopurine; 30 rather than 20 or 40 $g\ell^{-1}$ sucrose; and 1,0 $g\ell^{-1}$ casein hydrolysate. Random as opposed to a basal or side positioning of corm explants upon the culture medium, resulted in higher numbers of adventitious shoots being produced. The location of explant excision from within the donor plant was also found to influence shoot productivity. No significant difference was detected in the total number of shoots produced from corm explants harvested at various times of the year.

The rooting of shoots differentiated from corm explants posed few problems, as most shooted explants eventually formed roots without being subcultured. Those which did not form roots could be induced to do so, by the inoculation of the shooted explants onto a culture medium either devoid of hormones or containing low 1-naphthalene acetic acid levels. Following a rather simple procedure developed, ninety per cent of the plantlets were "hardened-off". From 100 corm explants it was therefore possible to regenerate 104 to 112 plantlets within a 3 to 4,5 month period.

Prior to the assessment of the usefulness of *in vitro* cultures for producing hypoxoside, qualitative and quantitative techniques for detecting hypoxoside, were developed. Using these techniques it was established that only the root-like types of cultured tissue, contained hypoxoside. The levels of hypoxoside detected within these tissues were much lower than those found within mature *in vivo* grown plants. Using the cultured tissue

containing the highest levels of hypoxoside, it was shown that the subculturing of this tissue resulted in a decrease in hypoxoside content. This effect could be overcome by lowering the levels of nitrogen in the medium or by culturing the tissue in the dark. These results showed that the cultured tissue was able to synthesize hypoxoside. To what extent this synthetic rate can be increased remains very much an academic problem and one which deserves more attention.

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Hypoxis L. a member of the monocotyledonous family Hypoxidaceae R. Br., has until recently only been used by eastern and southern African tribes in folk medicine and as a food. Since 1970 however, reference has been made to the fact that various species of this genus are of economic value. Numerous patents have been published describing the preparation of *Hypoxis* corm extracts for curing prostate hypertrophy. Clinical investigations have shown that the plants belonging to this genus possess surprising medicinal properties. For these reasons, the demand for *Hypoxis* plants has increased tremendously over the past few years and a potential threat has been placed upon indigenous populations.

1.2 REVIEW OF *HYPOXIS* LITERATURE

Originally the family Hypoxidaceae was associated with the Amaryllidaceae. The first classification system produced which separated these families was that developed by BAKER (1878). Detailed chronological notes on the taxonomic alterations of this family are recorded by THOMPSON (1972), WOOD (1976) and HEIDEMAN (1979). It is evident from these that taxonomic opinions differ regarding the status of this group of plants. In the International code of Botanical Nomenclature (STAFLEY, 1972), Hypoxidaceae is regarded as a conserved family.

Except for Europe and North America, the family has a cosmopolitan distribution and genera belonging to it are: *Curculigo* Gaertn., *Empodium* Salisb., *Forbesia* Eckl., *Hypoxis* L. (type genus), *Ianthe* Salisb., *Molineria* Colla, *Pauridia* Harv., *Rhodohypoxis* Nel, and *Spiloxene* Salisb. (DAHLGREN and CLIFFORD, 1982).

In South Africa, the family Hypoxidaceae is represented by 5 genera: *Hypoxis*, *Empodium*, *Pauridia*, *Rhodohypoxis* and *Spiloxene* (THOMPSON, 1972), the latter 4 of which are endemic (DYER, 1976). Although taxonomic studies have been completed on the genus *Hypoxis* in Natal (WOOD, 1976) and along the Witwatersrand, Transvaal (HEIDEMAN, 1979), it is still extremely difficult to identify species correctly. Studies of the naturally occurring populations have indicated that in most cases it is almost impossible to decide where the morphological variation of one species stops and another begins. It

has thus been suggested that further revision of the genus should not be attempted nor new species described, until the reason(s) for polymorphism within *Hypoxis* have been analysed (WILSENACH and WARREN, 1967).

The name *Hypoxis* is derived from the Greek words: "hypo" - beneath and "oxys" - sharp, a description of the inferior ovary which possesses a sharply narrowing base. In South Africa, common names for this genus are Kaffertulp, Gifbol, Sterretjie and Inkbol (SMITH, 1966). A few of the more enchanting vernacular names which have developed among the African tribes include: ilabatheka (Zulu), inkomfe-enkulu (Zulu), lotsane (Sotho) and tshuka (Tswana),(WATT and BREYER-BRANDWIJK, 1962).

In general *Hypoxis* species are classified as perennial herbs with fleshy mucilagenous corms, which are bright yellow or white internally. Corm sizes range from 0,5 to 11,0 cm in diameter and vary in shape from oblong to globose. Knowledge of the patterns of growth of corms within the genus is very limited. However, some attention has been devoted to this aspect by WOOD (1976) and HEIDEMAN (1979). A mature corm usually has several parallel rows of root holes in its lower section and a ring of functional contractile roots just below its widest part. Last season's fibre relics crown the upper portion of the corm and surround the newly formed leaves. The ages of the corms are difficult to determine and according to HEIDEMAN (1979), large corms could be several decades old.

The leaves of *Hypoxis* plants arise in the apical region of the corm. They may vary from few to many and are arranged either rosulately or tristichously. There is considerable range in the erectness and rigidity of the leaves, however features such as leaf vestiture, venation and shape are useful criteria for species identification (WOOD, 1976). Classification within the genus *Hypoxis* depends mainly upon vegetative characters such as these, because of the high level of uniformity in floral structure. All species possess one or more axillary peduncles which are usually stellately hairy and terminate in an inflorescence. The inflorescence types within the genus range from corymbose to simple. All taxa possess either white or yellow "star-shaped" flowers. These appear profusely towards the end of September and continue flowering through to February. The fruits of *Hypoxis*, which are not at all attractive are capsular and dehisce circumscissille below the apex. From these fruits numerous small black seeds are shed during summer. Mature seeds are obconic in shape and possess a projecting

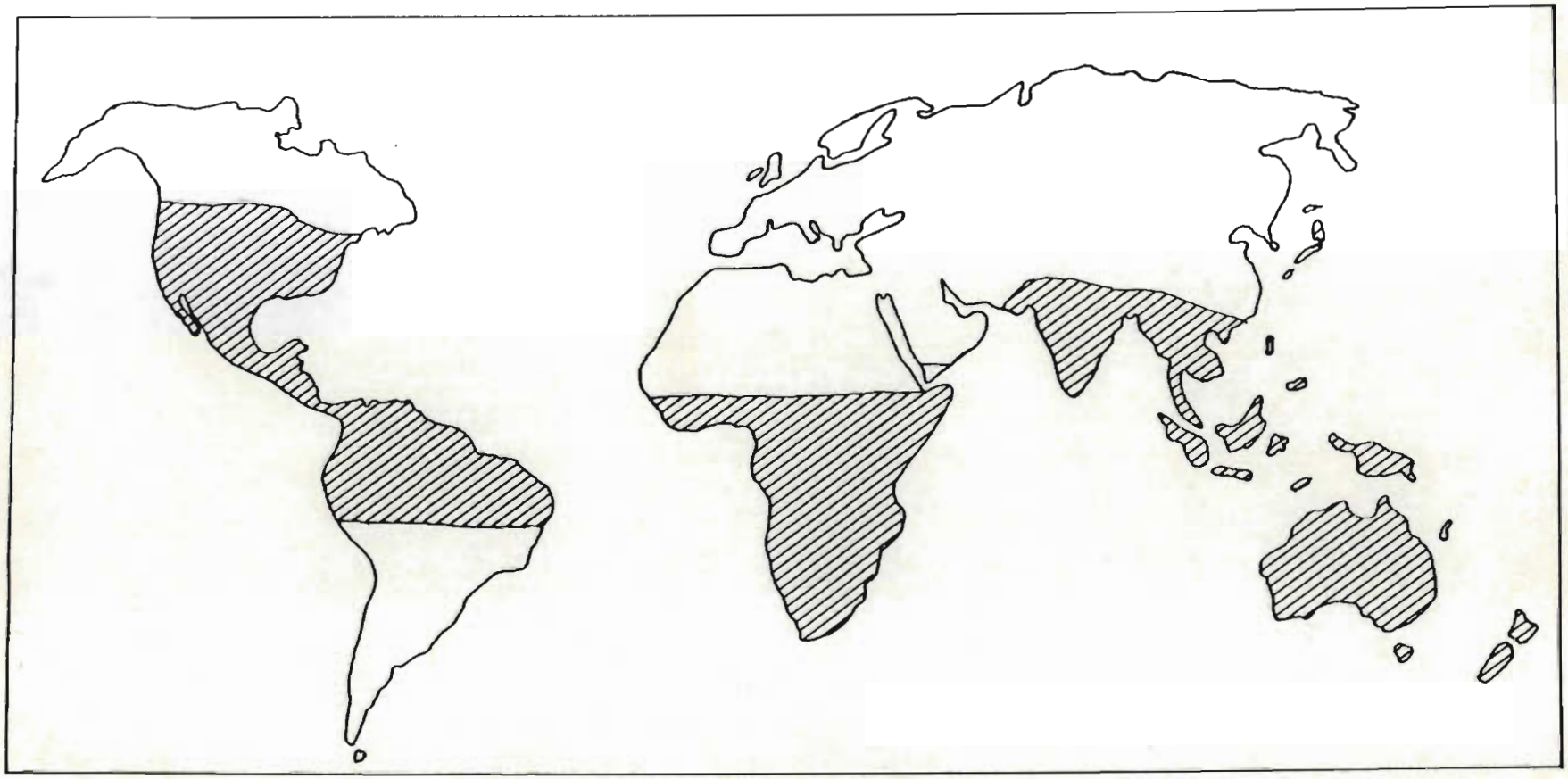


Figure 1.1: General known world distribution of *Hypoxis* L. (WOOD, 1976).

funicle and micropyle in close proximity to one another. The seed coat surface ranges from smooth to papillate and is reported to be of no taxonomic value (WOOD, 1976).

Geographically speaking, *Hypoxis* species are found to occur throughout most of the warm temperate and tropical zones of the world (Figure 1.1). However, their presence is not known in Europe, North and Central Asia, North Africa, Canada and extra-tropical South America (WOOD, 1976). In Africa, south of the Sahara, *Hypoxis* has been cited in Ethiopia, West Africa, Central Africa, Uganda, Malawi, Angola and South West Africa. Generally the number of species although small, increases southwards in Zimbabwe, Mozambique, Swaziland, Botswana and Lesotho (HEIDEMAN, 1979).

The largest number of *Hypoxis* species is recorded in South Africa. This is approximately 50 (DYER, 1976), with the greatest concentration in the Eastern Cape. Towards the winter rainfall areas of the Cape, the *Hypoxis* populations decrease markedly and only one species, *H. floccosa* Bak., is reported at the western limit of the genus (HEIDEMAN, 1979). Common small South African *Hypoxis* species are: *H. augustifolia* Lam., *H. argentea* Bak., *H. filiformis* Bak. and *H. membranacea* Bak., while the common larger species include *H. acuminata* Bak., *H. latifolia* Hook., *H. obtusa* Burch., *H. rigidula* Bak. and *H. rooperi* T. Moore.

The majority of *Hypoxis* species present in southern Africa are perennial grassland plants, although some may occupy a wider range of habitats. *H. rooperi* for example, has been found to occur in grasslands, sandy areas, dune forest margins and damp areas along streams. They are most common however in grassveld and disturbed areas (WOOD, 1976). Exceptions are however evident particularly among the more fragile species of the genus (*H. membranacea* and *H. parvula* Bak.) which prefer moister ravine habitats and forest margins.

Although numerous *Hypoxis* species have been utilized as food and medicine by eastern and southern African tribes for many centuries (Table 1.1), the medicinal potential of these plants has only recently been realised by Europeans. Besides these uses, the leaves of *H. rigidula*,

Table 1.1: Species of *Hypoxis* used by African tribes for food and medicine.

SPECIES	TRIBE	PLANT ORGAN USED	USE
H. argentea Bak. (WATT and BREYER-BRANDWIJK, 1962; WRIGHT, 1963; SMITH, 1966)	Congo Sotho Xhosa	Rootstock	-Remedy for chafes on horses -Food in times of famine -Ointment for anointing cracks on teats of cows -African medicine
H. gerrardii Bak. (RILEY, 1963)	Locally used	Corm	- To treat stomach trouble
H. haemerocallidea Fisch. and Mey. (WATT and BREYER-BRANDWIJK, 1962)	Lesotho	-	-Charm against thunder and storms
H. latifolia Hook. (WATT and BREYER-BRANDWIJK, 1962; BRYANT, 1966)	Congo Zulu	Bulb	-Poison for small vermin in food -Emetic for disagreeable dreams said to accompany heart weakness
		Plant	-Medicine for barrenness -To produce and remedy delirium or insanity -Purgative and ascarifuge -African medicine
H. multiceps Buching. (WATT and BREYER-BRANDWIJK, 1962)	Lesotho	–	-Charm against lightening

Table 1.1 continued

H. nyasica Bak. (WATT and BREYER-BRANDWIJK, 1962)	Nyanja	Plant	-Cough remedy
	Congo	Plant	-African medicine
H. obliqua Jacq. (WATT and BREYER-BRANDWIJK, 1962; WRIGHT, 1963; SMITH, 1966)	Xhosa	Corm/ Rootstock	-Lotion for septic wounds
	Congo	Plant	-African medicine
H. rigidula Bak. (WATT and BREYER-BRANDWIJK, 1962; SMITH, 1966)	Sotho	Rootstock	-Remedy for gallsickness in cattle
	Congo	Plant	-African medicine
H. rooperi S.Moore (WATT and BREYER-BRANDWIJK, 1962; SMITH, 1966)	Congo Kwena Lesotho Tswana	Plant	-Tonic for weakly children -Purgative -African medicine
		Rootstock	-Remedy for burns -Cure for headaches
H. villosa L.f. (WATT and BREYER-BRANDWIJK, 1962)	Lobedu Southern Sotho	Bulb/tuber	-Charm against thunder -Repel witches -To produce diarrhoea in domestic stock
H. villosa L.f. *var. scabra* (WATT and BREYER-BRANDWIJK, 1962)	Lesotho	Rootstock	-Charm against thunder
Hypoxis L. species (WATT and BREYER-BRANDWIJK, 1962)	Karanga Manyika Zulu	Rootstock	-Ingredient of an infusion taken as an "internal parasiticide" and purgative -Treatment for delirium -Remedy for wounds -Superstitious remedy for babies who inadvertently drink the milk of women who have conceived again -Remedy for vomiting, loss of appetite, abdominal pain and fever

H. rooperi and *H. villosa* L.f. var. *scabra* Bak. are utilized for making rope. In Brazil, *Tuber brasiliensis* a species believed to belong to the genus *Hypoxis*, is used as a forage for cattle (GUIMARAES , 1927). As yet no member of *Hypoxis* has been used as an accepted drug by pharmacists anywhere in the world. In the Transvaal, European farmers use a hot aqueous decoction of fresh *Hypoxis* corms as a remedy for enlarged prostate glands. Sun-dried corms of *Hypoxis* are even sold as a herbal remedy under the name PROSTAMIN in South Africa. This medicine is prepared as a tea, of which the patient has to drink three cups per day (PEGEL, 1976a).

Although there have been reports of toxic compounds in *Hypoxis* plants (WATT and BREYER-BRANDWIJK, 1962), these do not seem to have been supported by any definite species identification, or actual compound isolation. On the contrary, experimental investigations with rats have revealed that corm extracts of *H. rooperi* are neither toxic, carcinogenic nor teratogenic (PEGEL, 1976b). Since 1970, it has been found that *Hypoxis* plants are of considerable medicinal value. Numerous patents have been published describing the preparation of *Hypoxis* corm extracts for curing: prostate hypertrophy and its attendant phenomena; diseases of the gastro-intestinal tract and metabolic disturbances; diseases of the urogenital tract, blood and blood forming organs; the cardiovascular system; the skeletal system and muscles; and dermatological diseases (WARREN, 1972; PEGEL, 1973a, 1973b; REISCH and MÖLLMANN, 1974, PEGEL, 1975, 1979, 1980). From a commercial and pharmaceutical aspect it is evident that the mucilagenous corms of *Hypoxis* plants are very valuable.

With the rising interest in the medicinal importance of *Hypoxis*, there has been a concurrent increase in the number of reports on the chemical constituents of this plant. Some of the earliest work in this field was done by BEWS and VANDERPLANK (1930). They analysed the seasonal changes in the overall carbohydrate content of *H. rooperi* leaves and corms. More detailed analyses have since been done and the presence of glucose, fructose and sucrose unequivocally identified. The following sugars were shown not to be present: arabinose, galactose, lactose, mannitol, mannose, raffinose, rhamnose, ribose, sorbitol, sorbose and xylose (MARTIN and PEGEL, 1976).

Investigations on the alkaloids and saponins in *Hypoxis* and associated genera have been undertaken mainly for taxonomic reasons. Although Hypoxidaceae is closely allied to the Amaryllidaceae (alkaloid accumulators) there exist no substantial reports of either groups of chemicals in this family (WALL, KRIDER, KREWSON, EDDY, WILLAMAN, CORRELL and GENTRY, 1954; UNITED STATES DEPARTMENT OF AGRICULTURE TECHNICAL BULLETIN, 1961; WATT and BREYER-BRANDWIJK, 1962; HEGENAUER, 1963; KAPOOR, KAPOOR, SRIVASTAVA, SINGH and SHARMA, 1971; GIBBS, 1974; MARTIN and PEGEL, 1976).

The occurrence of steroids and their glucosides have been detected in *Hypoxis* species (UNITED STATES DEPARTMENT OF AGRICULTURE TECHNICAL BULLETIN, 1961; WARREN, 1972; PEGEL, 1973a, 1973b; REISCH and MÖLLMANN, 1974; PEGEL, 1975), and it is claimed in patent specifications that these chemicals are the pharmaceutically active agents in *Hypoxis* corm extracts.

Reports on the presence of phenolics and organic acids in *Hypoxis* corms are available. These are however, often vague and not based on sound chemical investigations (RAMSTAD, 1953; BATE-SMITH, 1956; HARBORNE, 1964; SKRYZYPCZAKOWA, 1970; KAPOOR, KAPOOR, SRIVASTAVA, SINGH and SHARMA, 1971). As a result of this a more detailed biochemical analysis was undertaken by MARTIN and PEGEL (1976). In *H. rooperi* the following cinnamic acids were not detected by comparative thin layer chromatography; caffeic acid, cinnamic acid, ferulic acid and sinapic acid. MARTIN and PEGEL (1976) also found that chelidonic acid, ellagic acid and gallic acid as well as numerous other organic acids were absent in *H. rooperi*. Many of the more common flavonoid compounds such as catechin, epicatechin, fisetin, kaemferol and quercetin were also shown to be absent.

The presence of fatty acids have been determined in dried and acid hydrolised *H. rooperi* corms. Mass spectrophotometry investigations have indicated that palmitic acid (~ 73 per cent), sepdecatrienoic acid (~ 10 per cent), septadecanoic acid (~ 6,0 per cent), stearic acid (~ 6,0 per cent), octadecenoic acid (~ 5,0 per cent), and octadecadienoic acid (~ 2,0 per cent) are present in this organ (MARTIN and PEGEL, 1976).

The most recent chemical publication on *Hypoxis* is a report on the isolation of an unusual phenolic glucoside from the corms of *H. obtusa* (BETTOLO, PATAMIA, NICOLETTI, GALEFFI and MESSANA, 1982). This new glucoside (1-(3',4'-dihydroxyphenyl)-5-(3",4"-dihydroxyphenyl)-1-penten-4-yne), hypoxoside, has been the subject of intense research amongst various groups of scientists for the past three years. It is now believed, contrary to previous ideas, that hypoxoside is the major active pharmaceutical constituent of *Hypoxis* corm extracts. Presently, clinical investigations to determine the curative properties of this chemical are being undertaken.

Despite the medicinal value of *Hypoxis* species, plants for commercial utilization are still collected from the wild. This is an exploitational operation because, although *Hypoxis* plants set copious healthy looking seed, very few apparently germinate each year. This is obvious by the lack of seedlings in the wild. Reports have been made that *Hypoxis* seeds are dormant (WOOD, 1976; HEIDEMAN, 1979) however, no clear description of the type of dormancy mechanism involved has been documented. Several conventional methods of overcoming the dormancy of these seeds have been attempted without success (WILSENACH, 1967). It would thus appear that *Hypoxis* seed germination is under hormonal or embryo control, and not coat imposed as suggested by WOOD (1976). Recent investigations of *H. floccosa* and *H. rooperi* have shown that a year after-ripening of the seed is necessary before germination can be achieved. Furthermore, the application of exogenous gibberellic acid had no effect on overcoming this after-ripening requirement (HEIDEMAN, 1979).

Although sexual propagation is regarded as being a most efficient and economical method of propagating plants, once seed dormancy problems (if any) have been overcome, the major disadvantage of this method is that it results in genetic variability. In *Hypoxis* this problem is compounded by the high somatic chromosome numbers, meiotic abnormalities and variable pollen grain chromosome numbers which have been observed to occur within species of this genus (WILSENACH, 1967; WILSENACH and PAPENFUS, 1967; WILSENACH and WARREN, 1967). Furthermore, any breeding programme utilizing sexual propagation is normally time and

space consuming, and seasonally controlled (HARTMANN and KESTER, 1983).

Tissue culture propagation presents one solution to these problems. It usually assures that the desired characteristics of the selected plant(s) is retained throughout its clone. The rate of multiplication of plants has also been shown to be enhanced substantially using this method. *In vitro* techniques also allow year round perpetuation of plants. In commercial nurseries, it can be used to minimize the growing space usually required for the maintenance of stock plants. Furthermore, when properly executed tissue culture may be utilized for the reproduction and maintenance of disease-free plants (MURASHIGE, 1974; QUAK, 1977).

In view of the above considerations, experimentation on the *in vitro* propagation of *Hypoxis* was initiated. It was hoped that in this way the destruction of natural populations could be prevented, and a contribution could be made towards establishing an economically viable, pharmaceutical industry around some of South Africa's indigenous *Hypoxis* species.

1.3 REVIEW OF TISSUE CULTURE LITERATURE WITH PARTICULAR REFERENCE TO PLANT PROPAGATION OF MONOCOTYLEDONOUS PLANTS

Tissue culture, a tool which has generated much interest during the last 20 years, is a principle which was initiated in the 1840's by SCHLEIDEN and SCHWANN. These two scientists postulated that the cell was the basic structural unit of all living creatures and that this unit was potentially capable of forming a complete new organism. The first botanists to realize the significance of this hypothesis was HABERLANDT, who in 1902 attempted to demonstrate totipotency. Unfortunately his efforts to induce the division of palisade, pith and glandular hair cells of *Erythronium* L. *Ornithogalum* L. and *Tradescantia* L., were unsuccessful.

During the period 1907 to 1920 attempts to grow mature plant cells *in vitro* were fraught with difficulties. Interest in this field waned but was once again stimulated by the successful establishment of excised

animal tissue in an artificial nutrient medium. In 1922 the first *in vitro* culture of plant root-tips was achieved (KOTTE, 1922; ROBBINS, 1922). However, it was soon realised that such cultures could not be used to demonstrate the theory of totipotency and could therefore not be classified as true plant tissue cultures. In 1934 the establishment of cultures from cambial tissue of trees were obtained (GAUTHERET, 1934) and by 1939 three scientists working independently (GAUTHERET, 1939; NOBÉCOURT, 1939; WHITE, 1939) managed to proliferate cultures of carrot and tobacco for a prolonged period of time. This was achieved by incorporating the newly discovered auxin, indole acetic acid in the culture medium (WENT and THIMANN, 1937). In most historical accounts on the development of plant tissue culture, this milestone is regarded as being the first establishment of a true plant tissue culture system; however it is not accepted as the first definitive experimental evidence of totipotency. This aspect was eventually demonstrated by VASIL and HILDEBRANDT in 1965. Their experimentation proved that single cells of hybrid tobacco (*Nicotiana glutinosa* L. and *N. tabacum* L.) grown in a defined medium and in isolation from other cells, formed completely organized plants capable of flowering.

Since the inception of tissue culture many plants belonging to different taxonomic groups have been established *in vitro*. Generally, progress made in this field has been achieved using dicotyledonous plants. In some respects this is not surprising since despite a number of attempts to establish monocotyledonous plants in culture, it was not until 1945 (i.e. 23 years after the first *in vitro* culture of dicotyledons) that this was successfully attained (LOO, 1945). In 1951 the first callus cultures of monocotyledonous plants were initiated. MOREL and WETMORE (1951) achieved this by using tuber tissue from *Amorphophallus rivieri* Dur. and *Sauromatum guttatum* Schott (Araceae). By 1959 out of approximately 100 species of flowering plants brought into culture only 11 were monocotyledons (Table 1.2) (GAUTHERET, 1959). It is for this reason that this group of plants was labelled "*difficult to culture*".

Table 1.2: Monocotyledonous plants which had been brought into culture by 1959 (adapted from GAUTHERET, 1959).

SPECIES	EXPLANT SOURCE	REFERENCE
Agave toumeyana Trel	Leaf	Personal communication NICKELL
Amorphophallus campanulatus Blume	Rhizome	Personal communication MOREL
A rivieri Dur.	Rhizome	MOREL, 1950a; MOREL and WETMORE, 1951
Bellevalia romana Rch	Embryo	Personal communication MOREL
Dioscorea composita Hemsl.	Tuber	Personal communication NICKELL
Gladiolus L. species	Bulb	MOREL, 1950b
Iris L. species	Embryo	Personal communication MOREL
Lilium formosanum Wallace	Embryo	Personal communication MOREL
Sauromatum guttatum Schott	Rhizome	MOREL, 1950a; MOREL and WETMORE, 1951
Zea mays L.	Endosperm	Personal communication LA RUE
Z. saccharata Sturter	Root, leaf	Personal communication NICKELL

Despite the fact that the initiation of monocotyledonous plants in culture was achieved later than that of dicotyledonous plants, the commercial and ornamental importance of the former, particularly the Orchidaceae, has resulted in the development of a considerable amount of interest in the *in vitro* culture of these plants. At present approximately 93 monocotyledonous genera (excluding the Orchidaceae) have been propagated aseptically (see APPENDIX 1A). The following review, although concerned with the *in vitro* propagation of monocotyledons, does not include the literature on orchid tissue culture as several excellent reviews are available concerning this aspect (MURASHIGE, 1974; ARDITTI, 1977; RAO, 1977). A list of orchid genera which have been propagated through tissue culture is also presented by HUGHES (1981).

The term "*tissue culture*" (alternatively and perhaps more correctly referred to as *in vitro* culture) is a blanket term which covers the aseptic cultivation of all plant parts, whether a single cell, a group of cells or an organ. This technique has found broad application in both economically and academically orientated activities. Still one of the major applications in research today, is the use of plant cell cultures to study the effect(s) of external factors on processes such as differentiation and growth. Another important aspect to which this technique is frequently applied is plant propagation. This subject is the major topic of this review and will therefore be covered in detail. For the sake of completeness other uses of tissue culture are briefly listed below. (MURASHIGE, 1974; DE FOSSARD, 1976; REINERT and BAJAJ, 1977; SPIEGEL-ROY and KOCHBA, 1977; GRESSHOFF, 1978; DE FOSSARD, 1981).

1. Plant improvement
   - recovery of virus free clones
   - haploid culture
   - embryo culture
   - *in vitro* pollination and fertilization
   - protoplast culture (changes in ploidy, para-sexual hybridisation)
   - screening for disease, toxin and stress resistance
   - mutant initiation and selection
   - preservation of valuable germ plasm
   - production of chimeras.

2. All-year-round production of clones.
3. Elucidation of natural interactions between bacteria and plants such as in bacterially induced tumours and symbiotic nitrogen fixation in legumes.
4. Expedition of plant material from one country to another.
5. Production of pharmaceuticals and other natural compounds.

Generally micropropagation is the term adopted for the process of multiplying plants vegetatively by means of *in vitro* culture. According to MURASHIGE (1977 , 1978) four sequential stages can be recognised in micropropagation systems: 1. Establishment, 2. Multiplication, 3. Pre-transplantation, and 4. Transplantation.

Stage 1. Establishment. The objective of this stage is to achieve prolonged survival *in vitro* of an infestation-free plant segment (MURASHIGE, 1977). The types and effectiveness of sterilants available to sterilize explants are listed in Table 1.3. The termination of Stage 1 is determined variously. With axillary and apical bud explants the criterion is moderate enlargement of the bud to enable transfer to Stage 2. In directly induced meristem explants, it is desirable that adventitious organs or embryos are generated before transfer to Stage 2. With indirectly induced meristem explants (i.e. in callus producing explants) vigorously growing callus, signals the fulfilment of Stage 1. Usually after 4 to 6 weeks the establishment stage is completed.

Stage 2. Multiplication. In this stage, propagule multiplication is accomplished. This can be achieved by enhancing axillary shoot formation or by initiating more adventitious shoots or embryos. Generally the latter method enables a substantially faster increase in propagules. Often however, this also introduces a high incidence of genetically aberrant plants (D'AMATO, 1977). Usually the bulk of culture activity and time is spent on this stage.

Stage 3. Pre-transplantation. The function of this stage is to prepare the plantlet for transplanting and establishment outside the artificial, aseptic environment of the culture flask. In Stage 3 the unrooted shoots are rooted, thus rendering the plants capable of autotrophic development and adaptable to changes in moisture stress and light intensity.

Table 1.3: A comparison of the effectiveness and properties of several sterilizing agents (YEOMAN, 1973).

STERILIZING AGENT	CONCENTRATION USED	EASE OF REMOVAL	TIME OF STERILI-ZATION (minutes)	EFFECT-IVENESS
Calcium hypochorite	9-10 per cent	+++	5-30	Very good
Sodium hypochlorite	2 per cent*	+++	5-30	Very good
Hydrogen peroxide	10-12 per cent	+++++	5-15	Good
Bromine water	1-2 per cent	+++	2-10	Very good
Silver nitrate	1 per cent	+	5-30	Good
Mercuric chloride	0,1-1 per cent	+	2-10	Satisfactory
Antibiotics	4-50 $mg\ell^{-1}$	++	30-60	Fairly good

* 20 per cent volume/volume of commercial solution

Furthermore, a degree of resistance is conferred upon the plants. Usually Stage 3 is accomplished in 2 to 4 weeks.

Stage 4. Transplantation. This stage involves the transfer of the plantlet from the aseptic culture environment to the free-living environment. Several environmental conditions are essential during the initial period after transplanting. These are aptly recorded by DE FOSSARD (1981) and SMITH (1981). Often some of the greatest losses in plant tissue culture occur at this stage of the propagation procedure.

In order to reduce the cost of commercial micropropagation operations, a fifth stage (Stage 0) has been advocated by various scientists. In this stage stock plants are maintained under hygienic conditions prior to *in vitro* propagation (HOLDGATE and AYNSLEY, 1977; DEBERGH and MAENE, 1981).

When developing an *in vitro* propagation programme, parameters which require serious evaluation include: the initial explant characteristics; nutrient formulations; culture environment and the finished product. Comprehensive coverage of these topics are provided by STREET (1973), MURASHIGE (1974, 1977), DOUGALL (1980) and SEABROOK (1980); hence only a brief outline of the salient points with particular reference to monocotyledonous plant examples, will be presented here.

The initial explant characteristics are of particular interest since no tissue culture has ever been done on the genus *Hypoxis*. In this study therefore, the best source from which to obtain explants needed to be determined. Virtually every organ or tissue can be used as a source of explants. For example, in monocotyledonous plants, plantlet regeneration has been obtained from anthers (BAJAJ and PIERIK, 1974), apical and axillary buds (FUJINO, FUJIMURA and HAMADA, 1972; MURASHIGE, 1974; HUSSEY, 1977b, 1978a; MATHEWS and RANGAN, 1979; MANTE and TEPPER, 1983), bracts (BAJAJ, SIDHU and GILL, 1983), bulbs (HUSSEY, 1975b; STIMART, ASCHER and ZAGORSKI, 1980; LESHEM, LILIEN-KIPNIS and STEIŃITZ, 1982), corms (HUSSEY, 1982b; PAGE and VAN STADEN, in press), filaments of stamens (MONTEZUMA-DE-CARVALHÒ, LUDOVINA and GUIMARAES, 1974), flowers (BAJAJ, SIDHU and GILL, 1983), inflorescence stalks (CHEN, STENBERG

and ROSS, 1977; CHEN, LO and ROSS, 1979; HOSOKI and ASAHIRA, 1980a; CHEN, CHEN, LO and ROSS, 1982; RANGAN and VASIL, 1983), immature embryos (EAPEN and RAO, 1982b; NAKAMURA and KELLER, 1982a; SKENE and BARLASS, 1983), leaves (HUSSEY, 1977a; NEL, 1981, 1982; BEYL and SHARMA, 1983), mature embryos and seeds (EAPEN and RAO, 1982a; SKENE and BARLASS, 1983), nodal stem segments (FORSYTH and VAN STADEN, 1982), ovary tissue (MAJUMDAR, 1970), protoplasts (BERRY, LU, PENTAL and COCKING, 1982), stem segments (HUSSEY, 1977a; YAKUWA, HARADA and TSUJI, 1982) and young flower buds (BAJAJ and PIERIK, 1974; PIERIK and STEEGMANS, 1975b).

Although a wide range of explants may be utilized for *in vitro* micropropagation, variations in regenerative characteristics do exist. Sometimes differences in the explants physiological age or extent of differentiation may affect their regenerative capacity. Generally the degree of success is inversely related to the explant age and differentiation. For example in *Freesia* Klatt, it has been shown that although corm, stem, leaf, pedicle and anthers are capable of forming callus, optimal callus induction was obtained from young flower buds (BAJAJ and PIERIK, 1974). In *Zea mays* L., GREEN and PHILLIPS (1975) discovered that whereas embryo explants from mature kernels produced only callus and roots, those from young caryopses (14 to 18 days after pollination) were able to develop both callus and plantlets.

The season in which explants are obtained may also influence the regenerative characteristics in tissue culture, particularly with plant varieties adapted to temperate climates (MURASHIGE, 1974). In *Lilium speciosum* Thunb., it has been observed that bulb scale explants obtained during spring and autumn regenerate freely, whereas those taken during summer or winter hardly ever produce bulblets (ROBB, 1957). Similarly in *Hippeastrum hybridum* Hort. successful *in vitro* results could be obtained only in autumn (MII, MORI and IWASE, 1974). It has been suggested that the capacity for explants to regenerate in culture may be related to periods of the donor plants vegetative growth (ROBB, 1957).

With cultivars that normally manifest seasonally recurring dormancy, the climatic requirements that underlie this condition must be satisfied before tissue cultures are attempted. This is particularly applicable to the regeneration of bulbous plants. For examples, in lilies and gladioli the chilling of explants prior to culture, enhanced their regenerative potential (NARAYANASWAMY, 1977).

Among other characteristics associated with the explant, which may influence the regenerative capacity, is the explant size. Generally the smaller the size the poorer the rates of survival and plantlet propagation in culture. In *Hyacinthus orientalis* L. it has been observed that larger bulb-scale segments markedly enhanced the number of adventitious bulblets produced (PIERIK and WOETS, 1971; PAEK, 1982). In *Lolium multiflorum* Lam. it was observed that the regeneration of plantlets was higher from larger stem-tip explants but their survival rate was low (DALE, 1975).

Even the orientation of the explant on the nutrient medium has been found to influence the regenerative capacity and efficiency of cultures. In *Hyacinthus orientalis* for example, it has been discovered that the regeneration from bulb-scale segments is inversely related to their natural orientation (PAEK, 1982). Similarly in *Lilium longiflorum* Thunb. explants positioned on the nutrient medium with their dorsal side down produced more bulblets (LESHAM, LILIEN-KIPNIS and STEIŃITZ, 1982).

During each *in vitro* stage the nutrient medium must be suitable in both chemical and physical qualities. Usually for Stage 1 and 2 a common formulation is satisfactory, however for Stage 3 a second is often required.

Actively growing plant tissue requires a continuous supply of inorganic elements and the composition of the mineral salt mixture must take this into account. Since the advent of tissue culture, numerous macro-nutrient and micro-nutrient formulations have been developed (THOMAS and DAVEY, 1975; DE FOSSARD, 1976; GAMBORG, MURASHIGE, THORPE and VASIL 1976; NARAYANASWAMY, 1977), because of their suitability for the promotion of organogenesis in a wider range of plants. Generally the view which has emerged is that there is not one ideal fixed nutrient solution with a single set of additive stimuli. In

Table 1.4: Revised MURASHIGE and SKOOG (1962) nutrient medium.

STOCK SOLUTION	CHEMICAL	MASS g 500 $m\ell^{-1}$ STOCK SOLUTION	ml STOCK SOLUTION USED ℓ^{-1} MEDIUM
1	NH_4NO_3	82,5	10
2	KNO_3	47,5	20
3	$CaCl_2$	17,2	10
4	$MgSO_4.7H_2O$	18,5	10
5	NaFeEDTA	2,0	10
6	KH_2PO_4	8,5	10
7	H_3BO_3	0,310	
	$MnSO_4.4H_2O$	1,115	10
	$ZnSO_4.7H_2O$	0,430	
	KI	0,042	
8	$NaMoO_4.2H_2O$	0,0125	
	$CuSO_4.5H_2O$	0,0013	10
	$CoCl_2.6H_2O$	0,0013	
9	Thiamine. HCl	0,005	
	Nicotinic acid	0,025	10
	Pyridoxin. HCl	0,025	
	Glycine	0,100	
Additional	Sucrose		30 $g\ell^{-1}$ medium
	Agar		10 $g\ell^{-1}$ medium
	myo-inositol		0,1 $g\ell^{-1}$ medium

pH adjusted to 5,8 with NaOH

fact, little critical work has been done on the necessity of the various macro-elements and micro-elements utilized in *in vitro* culture media (DOUGALL, 1980). Overall the MURASHIGE and SKOOG (1962) nutrient solution (Table 1.4) can support the growth of most plant cultures. For this reason, this salt mixture is commercially obtainable as premixes.

In addition to the inorganic additives, organic constituents need to be included in the culture media in order to support explant growth. These may include: vitamins, a carbon source, growth regulators, amino acids and amides, nitrogen bases and natural complexes (Table 1.5). Detailed accounts of the necessity of these compounds and their influence upon growth are recorded by DOUGALL (1980) and MURASHIGE (1974). Of all these constituents the growth regulators ,which shall be discussed in detail later, are regarded as being the most important component.

The physical qualities of the culture media, although often decided upon by the availability of facilities and the accustomed practice of the investigator, should be taken more seriously (MURASHIGE, 1977). Either a solid, semi-solid, stationary or agitated liquid medium may be used. Details on the qualities of each are recorded by STREET (1973). With some plants, the extent of explant survival and regenerative capacity may depend upon whether a liquid or an agar nutrient is employed. For example, *Cattleya* Lindl. cultures could only be started *in vitro* in a liquid medium (MURASHIGE, 1974). In contrast, shoot-tip cultures of *Asparagus orientalis* Bess. required initiation on an agar gel medium (MURASHIGE, SHABDE, HASEGAWA, TAKATORI and JONES, 1972). NAKAMURA and KELLER (1982b) observed that hexaploid *Triticosecale* Wittmack calli, initiated on agar had a higher morphogenetic capacity than those initiated in a liquid medium. Similarly in *Iris* L. cultures, the best bud formation was obtained on a nutrient medium containing 2,0 $g\ell^{-1}$ agar (FUJINO, FUJIMURA and HAMADA, 1972).

Propagation efficiency may also be affected by the solidity of the nutrient medium. As early as 1965 it was noted that explants of *Cymbidium* Sw. initiated development faster in an agitated liquid medium than on a solid medium (WIMBER, 1965). It has since been demonstrated that agitated liquid cultures can be used to double the number of plantlets produced from bulb-scale explants of various species

Table 1.5: Types of organic constituents added to the culture medium in order to support explant growth.

ORGANIC CONSTITUENT	COMPOUNDS
Vitamins	Glycine, inositol, nicotinic acid, pyridoxin, thiamine
Carbohydrates	Glucose, sucrose
Growth regulators	Auxin: chlorophenoxy acetic acid, 2,4-dichlorophenoxy acetic acid, indole acetic acid, naphthalene acetic acid Cytokinin: benzylamino purine, N^6 *iso* pentenyl amino purine, kinetin Gibberellin: gibberellic acid
Amino acids and amides	Arginine, asparagine and aspartic acid, glutamine and glutamic acid, tyrosine
Nitrogen bases	Adenine
Natural complexes	Brewers by products: malt and yeast extracts Endosperm fluids: coconut, corn Fruit pulp and juice: banana, orange and tomato Hydrolysed protein preparations: casein and lactalbumin hydrolysates, soy pepton

of *Lilium* L. (TAKAYAMA and MISAWA, 1982, 1983a, 1983b). MATHEWS and RANGAN (1979) have also shown that in *Ananas comosus* (L.) Merr. shake culture techniques could be used to increase the number of shoots produced per explant three-fold.

Among other important physical qualities of nutrient formulations, the pH of the medium may influence the regenerative capacity of explants. For example in Dutch *Iris* cultures, it has been observed that pH variations higher than 5,7 and lower than 5,0 inhibited adventitious bud formation (FUJINO, FUJIMURA and HAMADA, 1972). Usually the pH of the medium is adjusted to range between 5,0 and 6,0 for the following reasons (DE FOSSARD, 1976):

1. Most plant cells have pH readings between pH 5,0 and pH 8,0.
2. Different elements become less available or unavailable to plant cells outside a certain pH range.

Unfortunately pH drifts do occur during the course of culture (STREET, 1973, MURASHIGE, 1974), however little is known about the actual influence of this on culture development.

In tissue culture two major environmental factors which influence explant growth and development are light and temperature. Of the light requirements, intensity and quality are important (MURASHIGE, 1977). In experiments done on *Asparagus* it was observed that the percentage of callus formed from anther explants was highest under minimum light intensities and inversely proportional to length of light exposure (INAGAKI, HARADA and YAKUWA, 1980). Furthermore, experimentation has indicated that red-light irradiance mediates organogenesis in meristem-tip cultures of this genus (KADKADE and WETHERBEE, 1983), and enhances leaf emergence in *in vitro* growing bulbs of *Lilium longiflorum* (STIMART, ASCHER and WILKINS, 1982). Similarly, PIERIK and co-workers (PIERIK, 1976; PIERIK and STEEGMANS, 1976a, 1976b) have found that in *Freesia* and *Anthurium* Schott cultures, light duration, spectrum and intensity seem to be the most important factors modifying culture growth. It has hence been suggested that more careful manipulation of light regimes in *in vitro* cultures may enhance

the organogenetic influence of growth regulators (HEMPEL, 1979). Observations such as the above mentioned examples also indicate that key organogenetic processes in tissue culture, are probably regulated by phytochrome (MURASHIGE, 1974).

A general practice in tissue culture is to employ a constant temperature of 25 to 27 °C (MURASHIGE, 1974). This is a particularly undesirable practice as it fails to recognize diurnal and seasonal temperature fluctuations, under which plants normally grow. HILDEBRANDT'S (1971) studies with *Gladiolus hortulans* Bailey for example, disclosed that corms and plants obtained from tissue culture would not develop into plants upon transplanting unless, the cultures had been exposed for a period of 4 to 6 weeks to a temperature of 2 °C. Similarly in *Hyacinthus orientalis* (PAEK, 1982) and *Lilium longiflorum* (STIMART, ASCHER and WILKINS, 1982), a period of low and high temperatures respectively was necessary to enhance shooting from *in vitro* grown bulbs. Data has been presented which indicates that temperatures applied during *in vitro* development of easter lilies, affects bulblet dormancy (STIMART and ASCHER, 1981) and hence may be utilized to overcome post-harvest dormancy breaking treatments. In *Yucca gloriosa* L. the application of a slightly higher culture environment temperature enhanced the morphogenetic capacity of callus tissue derived from buds (DURMISHIDZE, GOGOBERIDZE and MAMALADZE, 1983). From these investigations, it is clear that perhaps more attention needs to be devoted to determining optimum culture environmental temperatures, as these may influence both *in vitro* and post culture efficiency.

The atmosphere component of the culture environment is one which is generally neglected. The premature deterioration of cultures is sometimes attributed to diverse gases released by the cultures (HUGHES, 1981). Experiments using charcoal filters to remove these gases have been tried but with limited success (MURASHIGE, 1977). The relative humidity of the culture environment is mainly of concern in extreme climates, where humidifiers need to be employed to prevent rapid drying of the culture medium.

According to MURASHIGE (1974; 1977) there are two main points regarding the plants produced through tissue culture which must be emphasised;

1. the proportion of genetically deviant plants produced or the consistency with which the desired plants are reproduced, and
2. the establishment of pathogen-free plants.

The former of these will be discussed later in relation to the type of multiplication technique adopted. As for the latter, it is sufficient merely to mention that plants obtained via *in vitro* culture which are claimed to be pathogen-free, should be accompanied with evidence from pathogen tests (MURASHIGE, 1974).

In vitro cultures are initiated by placing protoplasts, cells, tissues or organs of conventionally grown plants onto or into a sterile nutrient medium. In micropropagation, vegetative multiplication of such cultures requires the continual formation of shoot meristems. Two kinds of shoot meristems are involved namely; apical and axillary meristems, and adventitious meristems (HUSSEY, 1981a, 1981b). Every shoot meristem is a potential plant and the origin of these meristems are particularly important as they may affect the genotype of cultured plantlets (D'AMATO, 1977).

Apical meristem proliferation occurs merely by elongation of the shoot apex. Only a single plant per explant can be produced by this method and hence it connot be used to rapidly propagate desired plants. This procedure is used primarily to produce virus-free plants (QUAK, 1977; WALKEY, 1978). Amongst the monocotyledons at least 15 genera (Table 1.6) have been freed from viruses using this technique.

Axillary (lateral) meristem proliferation provides a more rapid multiplication system whereby the number of potential plants is increased exponentially by repeated subculturing (HUSSEY, 1981a, 1981b). In most plants every leaf has an axillary meristem that develops from a small group of cells derived from the main apical shoot meristem. Each axillary meristem has the potential to develop into a lateral shoot however, this process is regulated by a complex hormone system (SAUNDERS, 1978). Although this mechanism is not yet fully understood, it seems that

Table 1.6: Monocotyledonous species which have been propagated *in vitro* via meristem tips, and viruses eliminated (adapted from QUAK (1977) and WALKEY (1980)).*

SPECIES	VIRUSES ELIMINATED
Allium sativum L.	mosaic virus, onion yellow dwarf mosaic virus, unidentified virus
Ananas sativus Schult.	unidentified virus
Asparagus officinalis L.	unidentified virus
Caladium hortulanum Birdsey	dasheen mosaic virus
Colocasia esculenta (L.) Schott	dasheen mosaic virus
Cymbidium Sw. species	cymbidium mosaic virus
Freesia Klatt species	freesia mosaic virus, phaseolus virus
Gladiolus L. species	unidentified viruses
Hippeastrum Herb. species	mosaic virus
Hyacinthus orientalis L.	mosaic virus, lily symptomless virus
Iris L. species	iris mosaic virus, unidentified virus
Lilium L. species	unidentified viruses, cucumber mosaic virus, lily mosaic virus, hyacinth mosaic virus, lily symptomless virus
Musa L. species	cucumber mosaic virus, unidentified virus
Narcissus tazetta L.	arabis mosaic virus, narcissus degeneration virus
Nerine Herb. species	nerine latent virus, unidentified virus
Saccharum officinarium L.	mosaic virus
Xanthosoma brasiliensis (L.) Schott	unidentified virus

*See reviews by QUAK (1977) and WALKEY (1980) for references

cytokinin is one of the key hormones involved in this process (HUSSEY, 1981a, 1981b). Experimentation on *in vitro* grown axillary shoots has demonstrated that precocious axillary branching occurs when cytokinins are incorporated into the nutrient medium (HUSSEY, 1976b, 1977b, 1978a; ZEPEDA and SAGAWA, 1981; FORSYTH and VAN STADEN, 1982; HUSSEY, 1982b; SAWHNEY and NAYLOR, 1982). SAWHNEY and NAYLOR (1982) have even suggested that the quiescence of *Tradescantia paludosa* Anderson and Woods axillary buds *in vitro* and *in vivo*, may have its basis in the unavailability of cytokinins. Usually the presence of this hormone inhibits the formation of roots on the explant, thus in order to permit rhizogenesis, shoots need to be transferred to a basal medium containing low or no cytokinin concentrations (HUSSEY, 1976b, 1977a, 1978a; HEMPEL, 1979; ZIV, 1979; HUSSEY, 1982b). With some plants the addition of a rooting hormone to the medium is required to stimulate adequate root formation (FORSYTH and VAN STADEN, 1982; SAWHNEY and NAYLOR, 1982; MANTE and TEPPER, 1983).

Enhanced axillary branching is a technique of micropropagation which was first demonstrated using *Gerbera* Cass., *Fragaria* L. (Strawberry) and *Gladiolus* L. (HUSSEY, 1981a). It has since been extended to a wide range of plants including various monocotyledons (Table 1.7). The great advantage of this technique, although usually slower than adventive processes, is that it is the most reliable way of ensuring genetically uniform propagules (MURASHIGE, 1977 ; HARTMANN and KESTER, 1983).

While axillary shoots occur in most plants, they are not always formed in sufficient numbers for rapid propagation (HUSSEY, 1982b) and in some types of plants they are rare or absent (e.g. *Elaeis guineensis* Jacq.). Effective propagation of such plants therefore depends on the formation of meristems at sites other than leaf axils. These meristems are referred to as adventitious meristems, and can develop either directly from the explant or indirectly from callus that develops on the cut surface(s) of explants, or from cell cultures (HARTMANN and KESTER, 1983). Amongst monocotyledonous plants, propagation *in vitro* via adventitious processes is especially useful, as many increase very slowly under natural conditions (HUSSEY, 1981a).

Table 1.7: Monocotyledonous plants which have been propagated *in vitro* via axillary branching.

FAMILY	SPECIES	REFERENCE
Amaryllidaceae	*Hippeastrum hybridum* Hort	HUSSEY, 1976b
	Narcissus L. species	HUSSEY, 1976b
Bromeliaceae	*Ananas comosus* (L.) Merr.	ZEPEDA and SAGAWA, 1981
Commelinaceae	*Tradescantia paludosa* Anderson and Woods	SAWHNEY and NAYLOR, 1982
Dioscoreaceae	*Dioscorea alata* L.	MANTELL, HAGUE and WHITEHALL, 1978
	D. bulbifera L.	FORSYTH and VAN STADEN, 1982
	D. floribunda Mart and Gal.	LAKSHMI-SITA, BAMMI and RANDHAWA, 1976
	D. rotundata Poir.	MANTELL, HAGUE and WHITEHALL, 1978
Iridaceae	*Freesia* Klatt species	HUSSEY, 1976b
	Gladiolus grandiflorus Andr.	BAJAJ, SIDHU, GILL, 1983
	Gladiolus L. species	HUSSEY, 1976b, 1977a, 1977b
	Iris L. species	HUSSEY, 1976b
	Schizostylis coccinea Backh. and Harvey	HUSSEY, 1976b
	Sparaxis bicolor Ker-Gawl	HUSSEY, 1976b
Liliaceae	*Allium cepa* L.	HUSSEY, 1978b
	A. sativum L.	BHOJWANI, 1980
	Fritillaria meleagris L.	HUSSEY, 1976b
	Hyacinthus L. species	HUSSEY, 1976b
	Lilium pyrenaicum Gouan	HUSSEY, 1976b
	Lilium L. hybrid	HUSSEY, 1976b
Musaceae	*Musa accuminata* L.	SWAMY, RAO and CHACK 1983
Strelitziaceae	*Strelitziaceae reginae* Ait.	ZIV and HALEVY, 1983

With regard to the origin of direct adventitious shoots, histological studies have shown that in bulbous monocotyledonous plants, these shoots may arise from the division of epidermal cells (and at least one inner layer of cells) of twin scales and leaf bases (HUSSEY, 1982b). These cells become meristematic and pockets of small, densely staining cells termed meristemoids develop (HICKS, 1980). The response of explants to develop such meristems has been shown to be greatly influenced by the balance of hormones within the explant tissue, particularly the levels of cytokinin and auxin. In the Liliaceae, Iridaceae and Amaryllidaceae HUSSEY (1982b) reports that the induction of direct adventitious shoots from organ explants *in vitro*, is the principle method of propagation in these groups of plants. Furthermore he regards this mode of plant regeneration as being genetically stable and hence, similar to axillary meristem propagation.

In contrast to direct adventitious shoot development, indirect adventitious shoot formation first involves the initiation of callus from the original explant, whether cell, tissue or organ (HARTMANN and KESTER, 1983). This type of vegetative multiplication scheme, although allowing for the rapid propagation of plants, is fraught with certain obvious disadvantages. These are;

1. the decrease in frequency of plant regeneration and eventual loss of morphogenetic potential, and
2. karyological abnormalities which are often induced to occur.

Despite these disadvantages, callus cultures are particularly useful for analysing the influence of various factors on plant organogenesis and embryogenesis.

The development of callus from a fragment of tissue may be divided into three stages; induction of cellular division; continued proliferation and dedifferentiation, and structural and physiological redifferentiation (YEOMAN and AITCHISON, 1973; GRESSHOFF, 1978). Although these stages are distinctly different, the latter two often occur concurrently. Few plant tissues fail to respond to treatments designed to induce the formation of callus (EVANS, SHARP and FLICK, 1981), however they may vary in lag periods before active growth is induced (GRESSHOFF, 1978). For this reason the statement has been made that the isolation

and successful establishment of callus largely depends upon the culture conditions employed and not on the source of the explant material (YEOMAN, 1973). However, according to EVANS, SHARP and FLICK (1981) callus initiation is dependent on explant source. For example, different concentrations of 2,4-dichlorophenoxy acetic acid in the culture medium are necessary for obtaining callus from roots, scutella, cotyledonary nodes, coleoptiles or leaf sheath nodes of rice (WU and LI, 1971). These variations in culture medium requirements reflect physiological differences of the explant. Ploidy differences have also been reported to occur in calli obtained from different tissue explants of a single plant (YAMADA, NISHI, YASUDA and TAKAHASHI, 1967). Amongst the monocotyledons, plant regeneration from callus cultures have been derived from a number of genera and types of explants (see APPENDIX 1B).

Nearly all callus cultures are derived from two major types of cells in an explant; those of the vascular cambium which may already be in a state of division, and a variety of parenchyma cells which are quiescent and have to be induced to divide (YEOMAN and STREET, 1973). Two hypotheses have been proposed to try and explain the transformation of quiescent cells to the active form. BROWN and DYER (1972) suggest that quiescent cells retain their potential for a meristematic state, but are in some way repressed or inhibited. Therefore, to return to the dividing condition this repression needs to be removed. Alternatively and more popularly, the second theory states that the cell loses the power to divide as differentiation proceeds and acquires it again as a result of the induction and activation processes. Generally there is more evidence available in support of the first theory, however, evidence for the complete elimination of the second has not been presented (YEOMAN and AITCHISON, 1973).

In an explant not all cells respond in the same way to the aseptic environment. Only the cells at the outer surface of the inoculum or at the excised surface are induced to divide. The act of induction is followed by a succession of metabolic changes and syntheses which culminate in division. Adequate record of these are given by YEOMAN and AITCHISON (1973).

Besides physiological changes in an explant during the induction process, structural changes of the quiescent cells also occur. Detailed accounts of these structural changes are presented by YEOMAN and STREET (1973) and WILSON, ISRAEL and STEWARD (1974). Generally, one of the earliest detectable changes during this dedifferentiation process is an increase in the number of helical and spiral polyribosomes. Storage products characteristic of quiescent cells, tend to disappear and the arrangement and structure of the organelles change. The number of dictyosomes increase, suggesting that the cells become metabolically more active. The nucleus, which in quiescent cells is usually flattened and positioned against the cell wall, assumes a more rounded profile and central position in the cell. BAGSHAW (1969) from her study with vacuolated artichoke tuber cells in culture, observed this change very clearly. Dense aggregations of chromatin are more frequently observed in the nucleus, and whatever the position is of this organelle, it is always surrounded by perinuclear cytoplasm and often connected by cytoplasmic strands to the cell wall. Overall, the cell size decreases and the vacuoles present in the cell are small (YEOMAN and STREET, 1973). The division which occurs and results in the proliferation of the callus, takes place by means of the well documented process of mitosis.

Usually under optimal growth conditions callus has a white-yellowish appearance and a cauliflower-like topography. Calli derived from different species may however vary in texture, friability and colouration (GAUTHERET, 1959; YEOMAN and AITCHISON, 1973; NARAYANASWAMY, 1977). Although callus cultures may appear outwardly to be uniform masses of cells, in reality their structure is very complex (STREET, 1973).

Generally their growth follows a typical logarithmic pattern. There is a slow initial cell division induction phase, a rapid cell division phase followed by a gradual cessation of this activity and the differentiation of cells into parenchyma and vascular-type cells (HARTMANN and KESTER, 1983). In callus, cell division does not occur throughout the culture mass, but is primarily located to meristematic centres below the upper surface of cells. The cells of these centres are distinctively smaller and have less vacuolation, whereas the majority of callus cells are large and irregular in shape (GRESSHOFF AND DOY, 1973; BORNMAN, 1974; THOMAS and DAVEY, 1975). Because of this varied growth, the utilization

of callus cultures for investigations on the growth and metabolism of such tissue is fraught with numerous problems. These are;

1. the division of callus into equal pieces, usually by mass under aseptic conditions,
2. the selection of tissue with similar growth potential, and
3. the difficulty of restricting tissue damage during subculturing (YEOMAN and AITCHISON, 1973).

Following or occurring concurrently with the stage of cell division (or dedifferentiation), the stage of redifferentiation (which includes cytodifferentiation and organogenesis) occurs in callus cultures. During this process, new developmental patterns are superimposed upon the actively dividing tissue, resulting in the establishment of either roots, shoots or embryos. In spite of the usefulness of plant cell and callus cultures for studying these processes, only a few papers have been published dealing with the anatomical changes which occur during redifferentiation. Amongst the monocotyledons, investigations of this nature according to NAKANO and MAEDA (1979), are particularly rare.

Of the variety of factors which have been found to influence the induction, division and redifferentiation of callus, the hormonal components of the nutrient medium are regarded as being the most important. In dicotyledonous cultures, the synergistic action of cytokinin and auxin in callus induction is especially pronounced. Generally a high ratio between auxin and cytokinin levels must be kept to permit callus production. The precise levels which are optimal however, may vary depending on the tissue, the species, the basal medium and the technique of the investigator (GRESSHOFF, 1978). In monocotyledons, the necessity for this ratio between auxin and cytokinin to exist for callus induction and growth to occur, is according to GRESSHOFF (1978) less noticeable. There are however, numerous monocotyledonous examples in the literature which state that for successful callus induction, the auxin to cytokinin ratio must be kept high (ZIV, HALEVY, and SHILO, 1970; GRESSHOFF and DOY, 1973; CHENG and SMITH, 1975; MEYER, 1976b; CHEN, STENBERG and ROSS, 1977; CONGER and CARABIA, 1978; CHEN, LO and ROSS, 1979; LO, CHEN and ROSS, 1980; LARKIN, 1982). A number of these authors also advocate that the correct

phytohormone levels in conjunction with diffuse light or dark culture conditions, are optimal for callus production. In spite of this, it has been shown that for callus induction to occur in monocotyledonous cultures, auxin levels are of utmost importance. For example, in *Avena sativa* L., callus induction is dependent on auxin concentration, whereas kinetin is of little importance (CARTER, YAMADA and TAKAHASI 1967). Similarly FISHER (1977) found that to induce callus production from rhizome or tuber explants of *Cyperus rotundas* L., a naphthalene acetic acid supplemented medium was necessary. In *Saccharum officinarium* L. no callus could be induced to form from leaf, inflorescence and shoot apices, if the medium only contained kinetin (HEINZ and MEE, 1969). DUDITS, NEMFT and HAYDU (1975) noted that the addition of cytokinin to the basal medium inhibited callus growth from *Triticum aestivum* L. explants. Similarly in sugarcane cultures, the addition of 0,005 $g\ell^{-1}$ kinetin, regardless of the auxin concentration, inhibited callus growth and differentiation (BARBA and NICKELL, 1969). Contrary to this, SIMONSEN and HILDEBRANDT (1971) noted that callus induction from cormel stemtips of *Gladiolus*, was best on a basal medium containing kinetin.

Often what has been found to occur in monocotyledonous cultures, is that where explants are placed on a basal medium supplemented with low auxin and low cytokinin concentrations, adventitious organs develop. However, if the auxin concentration is increased, callus is induced to form (HUSSEY, 1975b, 1976c, 1977a; TISSERAT, 1979). In contrast to this general trend, examples can be cited in which an equivalent amount of auxin and cytokinin (BUI-DANG-HA, NORREEL and MASSET, 1975; HEUSER and APPS, 1976; SIMMONDS and CUMMING, 1976; ZEE, FUNG and YUE, 1977; YAKUWA, HARADA and TSUJI, 1982), or a higher cytokinin to auxin ratio is required (PIERIK, STEEGMANS and VAN DER MEYS, 1974; GAMBORG, SHYLUK, BRAR and CONSTABEL, 1977; GROENEWALD, WESSELS and KOELEMAN, 1977) for callus induction. For effective callus production amongst most of the cereal crops, media such as B5 (GAMBORG, MILLER and OJIMA, 1968) or other low ammonia media, supplemented with high auxin levels (particularly 2,4-dichlorophenoxy acetic acid), have been found to be most useful (NISHI, YAMADA and TAKAHASHI, 1968; RANGAN, 1974; GREEN and PHILLIPS, 1975; HENKE, MANSUR and CONSTANTIN, 1978; BAJAJ and DHANJU, 1981; RINES and McCOY, 1981; VASIL and VASIL, 1981a; EAPÉN and RAO, 1982a; HEYSER and NABORS, 1982). Often complete dedifferentiation of

monocotyledonous callus is difficult to achieve because of an intrinsic tendency for continual rhizogenesis (GRESSHOFF and DOY, 1973; GRESSHOFF, 1978). In some species such as *Zea Mays*, this rhizogenic potential is suppressible by high levels of 2,4-dichlorophenoxy acetic acid (SHERIDAN, 1975). This has resulted in the statement being made that 2,4-dichlorophenoxy acetic acid appears to be a strong dedifferential factor blocking organogenesis, and is therefore particularly useful for callus induction (HAVRÁNEK and NOVÁK, 1972; MURASHIGE, 1974). However, contrary to this, DURMISHIDZE, GOGOBERIDZE and MAMALADZE (1983) showed that in *Yucca gloriosa* cultures, an increase in the concentration of 2,4-dichlorophenoxy acetic acid facilitated morphogenesis.

In dicotyledonous callus, phytohormone requirements can be reduced or satisfied by the addition of other phytohormones. In monocotyledons, alternative auxins such as 2,4,5-trichlorophenoxy acetic acid and naphthalene acetic acid have been employed, at similar concentrations to achieve callus induction from various explants (GORTER, 1965; TAKATORI, MURASHIGE and STILLMAN, 1968; STEWARD and MAPES, 1971; INAGAKI, HARADA and YAKUWA, 1980; CHEN, CHEN, LO and ROSS, 1982; HEYSER and NABORS, 1982; NAKAMURA and KELLER, 1982a, 1982b; NABORS, HEYSER, DYKES and DEMOTT, 1983). Recently it has also been shown that higher yields of callus can be induced at a faster rate from monocotyledonous tissues using 4-amino 3,5,6-trichloropicolinic acid (picloram), instead of 2,4-dichlorophenoxy acetic acid (BEYL and SHARMA, 1983). Occasionally in dicotyledonous cultures, callus induction and growth rates have been promoted by the interaction of auxin and cytokinin with other phytohormones (NARAYANASWAMY, 1977; GRESSHOFF, 1978). However in monocotyledonous cultures, this type of interaction has apparently not yet been reported.

Although phytohormones are usually considered to be inductive agents, they are also required for continued growth of excised callus. It is however not always possible to distinguish between the requirements for these two states of growth. Often, the medium on which the callus is induced to form is sufficient for the maintenance of good growth (BUI-DANG-HA, NORREEL and MASSET, 1975; CHEN, LO and ROSS, 1979; LO, CHEN and ROSS, 1980; EAPÉN and RAO, 1982b). For dicotyledonous cultures, it has been noticed that optimal growth of excised callus is

governed mainly by auxin concentrations. In monocotyledons however, GRESSHOFF (1978) states that the culturing of callus is difficult and that there is an apparent lack of growth response to external cytokinins. These diverse insensitivities he regards as reflecting some special feature of monocotyledonous cells. In most papers on monocotyledonous callus culturing, the requirements for maintaining good callus growth are not clearly recorded. From the relatively few examples which are however available, the maintenance of callus has been best achieved by transferring the initial callus to a basal medium supplemented with lower auxin levels than those used for inducing callus growth (CHEN, STENBERG and ROSS, 1977; CONGER and CARABIA, 1978; LOWE and CONGER, 1979; BAJAJ and DHANJU, 1981; BAJAJ, SIDHU and GILL, 1983).

Following the period of callus induction, and usually occurring concurrently with the period of callus proliferation, is a phase of redifferentiation. It is generally accepted that this phase consists initially of cytodifferentiation processes followed by organogenetic processes. Based on the available information on tracheid formation, it has been concluded that cytodifferentiation is a phase in callus cultures which occurs immediately after cell division (KOHLENBACH, 1977). Phytohormones are known to induce the formation of differentiated tissues (cambia, vascular and meristematic nodules) associated with this phase (HALPERIN, 1973; KOHLENBACH, 1977). A tentative model (Figure 1.2) on the role of phytohormones on cytodifferentiation processes in callus cultures, was presented by GRESSHOFF (1978). In addition, a detailed account of the experimental evidence which is available and which assisted in the establishment of this model, is recorded by the same author. From this account it is obvious that studies of this nature on monocotyledonous cultures are lacking.

Since the elegant demonstration by SKOOG and MILLER (1957) that the relative ratio of cytokinin (kinetin) to auxin (indole acetic acid) determines the nature of organogenesis in tobacco pith tissue, interest in the development of complete organs from callus and the control of these processes by phytohormones has escalated. Despite the volume of literature available on this topic, no definite formulae can be presented which will ensure organogenesis in callus cultures. However, certain general recommendations

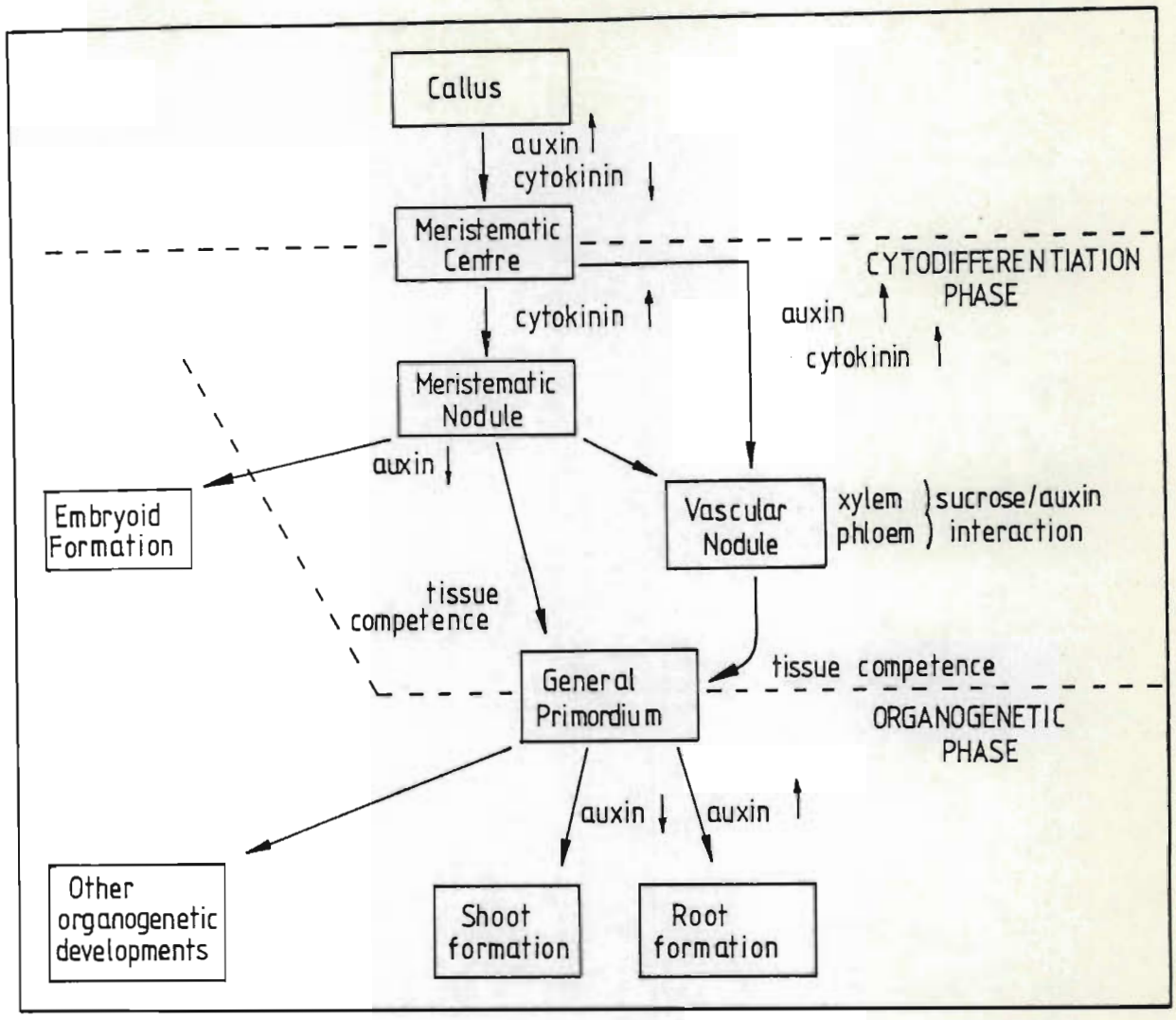


Figure 1.2: A diagrammatic representation of organogenesis in callus tissue. ↑ and ↓ denote high and low levels respectively of particular exogenous hormones (GRESSHOFF, 1978).

can be given. The two generalised hormonal treatments for root induction are;

1. that roots develop where callus is maintained on a medium with equimolar concentrations of auxin and cytokinin, and
2. that the withdrawal of cytokinin and/or auxin may induce rhizogenesis (GRESSHOFF, 1978). The latter treatment is regarded as representing the release of the root primordia from the suppressed situation.

Amongst monocotyledonous callus cultures, the supplementation of the nutrient medium with auxin alone (often a different auxin to the one which was used to induce callus formation) stimulates rhizogenesis (FRIDBORG, 1971; KAUL and SABHARWAL, 1972; BUI-DANG-HA, NORREEL and MASSET, 1975; RANGAN, 1976; CHIN and SCOTT, 1977; STIMART, ASCHER, and ZAGORSKI, 1980; EAPÉN and RAO, 1982a, 1982b; LARKIN, 1982; YAKUWA, HARADA and TSUJI, 1982). In many instances, merely the reduction of auxin utilized to induce and maintain callus (GROENEWALD, WESSELS and KOELEMAN, 1977; LOWE and CONGER, 1979; MATHEWS and RANGAN, 1979; BAJAJ and DHANJU, 1981; BAJAJ, SIDHU and GILL, 1983 , DURMISHIDZE, GOGOBERIDZE and MARMALADZE, 1983), or the transfer of callus onto a hormone-free basal medium (GORTER, 1965; DUDITS, NEMFT and HAYDU, 1975; DEBERGH and STANDAERT-DE-METSENAERE, 1976; FISHER, 1977; CHEN, CHEN, LO and ROSS, 1982; BEYL and SHARMA, 1983) causes rhizogenesis. According to GRESSHOFF (1978) this observation again points to the apparent cytokinin autonomy seen in monocotyledons (SHERIDAN, 1968).

In *Zea mays* callus cultures, GRESSHOFF and DOY (1973) found that the lowering of the initial auxin concentration, and increase of the cytokinin supplement in the basal medium (thereby decreasing the ratio between auxin and cytokinin) resulted in root development. Similarly in callus cultures of *Haworthia* Duval (KAUL and SABHARWAL, 1972) and *Triticum aestivum* (DUDITS, NEMFT and HAYDU, 1975), experimentation has shown that the addition of low but equal levels of auxin and cytokinin, caused root initiation. In fact, KAUL and SABHARWAL (1972), found that high concentrations of kinetin inhibited rhizogenesis. In contrast to these general trends, SANGWAN and GORENFLOT (1975) have indicated

that a high auxin to cytokinin ratio induces root formation in *Phragmites communis* L. callus cultures. Although a multitude of papers exist which mention root formation on certain media, the actual requirements for root induction are not recorded. Often it is merely stated that root formation occurred after successful shoot formation (CHEN, LO and ROSS, 1979; KASPERBAUER, BUCKNER and BUSH, 1979). As a result of this, there is a deficiency of factual information available upon which to establish more substantial generalizations about root formation in monocotyledonous callus cultures.

The utilization of phytohormones other than auxin and cytokinin to induce rhizogenesis in monocotyledonous callus has hardly been investigated. NAKAMURA and KELLER (1982b) are surely some of the pioneers in this field. They found that the transfer of hexaploid *Triticosecale* callus onto a gibberellic acid or triiodobenzoic acid supplemented medium, stimulated root development.

Since the discovery of cytokinin during the 1950's, the concept that balanced hormonal interactions control morphogenesis has been confirmed many times utilizing callus cultures (VASIL and HILDEBRANDT, 1967; BINDING, 1971; GRESSHOFF and DOY, 1972; KARTHA, GAMBORG, SHYLUK and CONSTABEL, 1976; SCOWCROFT and ADAMSON, 1976; VASQUEZ AND SHORT, 1978). Shoot formation in most dicotyledonous callus cultures is promoted by the transfer of callus to a medium in which the cytokinin to auxin ratio ranges between 10 and 100. This is quite the inverse of the ratio required to induce callus tissue in this group of plants. Thus the major technique leading to shoot development in dicotyledons has been the auxin/cytokinin reversal transfer (GRESSHOFF, 1978).

In monocotyledons it has been stated that cytokinin is not required to initiate shoot development (GRESSHOFF, 1978). Often auxin reduction (RANGAN, 1974; HUSSEY, 1975b, 1976b, 1976c; CHEN, LO and ROSS, 1979) or auxin removal (RANGAN, 1974; CUMMINGS, GREEN and STUTHMAN, 1976; HEUSER and APPS, 1976; HUSSEY, 1976a; NAKAMURA and KELLER, 1982a) is sufficient to induce organogenesis. In many liliaceous callus cultures where auxin and cytokinin were both utilized to induce callus formation, the reduction of auxin and/or increase of cytokinin resulted in shoot regeneration (HAVRÁNEK and NOVÁK, 1972; WESSELS, GROENEWALD

and KOELEMAN, 1976; ABO EL-NIL, 1977; EVANS, SHARP and FLICK, 1981). In some monocotyledonous cultures, shoot production could be induced by the tranfer of callus onto a nutrient medium containing completely different phytohormones to those used to induce callus formation. For example RANGAN (1976) noted that the transfer of finger millet, bullrush millet, and koda millet callus from a basal medium containing a high concentration of 2,4-dichlorophenoxy acetic acid onto a medium containing a low concentration of naphthalene acetic acid or indole acetic acid, resulted in the regeneration of shoots. Similarly, MASTELLER and HOLDEN (1970) observed that in *Sorghum bicolor* (L.) Moench cultures, shoots were induced to form when the callus was transferred from a basal medium containing 2,4-dichlorophenoxy acetic acid onto one supplemented with naphthalene acetic acid.

The only records of the utilization of gibberellic acid for shoot regeneration from monocotyledonous callus cultures, are those reported by NAKAMURA and KELLER (1982a) and THOMAS, KING and POTRYKUS (1977). Both groups of investigators noted that the addition of gibberellic acid to the basal medium enhanced shoot regeneration from callus cultures of graminaceous species.

In addition to the use of phytohormones to induce shooting of callus, alterations of the light conditions have also been observed to cause organogenetic processes to occur. For example in lily, shoot regeneration has been enhanced by the transfer of callus to a dark culture environment (HAVRÁNEK and NOVÁK, 1972; KATO, 1978; STIMART and ASCHER, 1978a). In contrast to this, *Lolium rigidum* Gaudin (SKENE and BARLASS, 1983), *Iris* (MEYER, FUCHIGAMI and ROBERTS, 1975) and *Anthurium andraeanum* Lind. (PIERIK, STEEGMANS and VAN DER MEYS, 1974) callus cultures, produced shoots when transferred from dark into light culture conditions.

Occasionally two media transfers are required to achieve efficient shoot differentiation. In *Agave* L., *Arabidopsis* Schur. and *Medicago sativa* L. this has been found to be necessary before shoots will develop (GROENEWALD, WESSELS and KOELEMAN, 1977; GRESSHOFF, 1978).

Another process whereby plants can be regenerated from callus or cell cultures is via somatic embryogenesis. The external prerequisites for this process are uniform in the examples thus far studied. It is necessary

that the cultures are in the presence of an auxin (particularly suitable is 2,4-dichlorophenoxy acetic acid) and a reduced nitrogen source (KOHLENBACH, 1977; MURASHIGE, 1977). The number of subcultures may require restriction, in as much as embryogenesis is often progressively repressed with each subculture. Transfer of the callus to an auxin-free or low auxin medium usually resulted in the emergence of embryos and plants. More detailed accounts of this aspect are presented by KOHLENBACH (1977), GRESSHOFF (1978) and STREET (1979). Amongst monocotyledonous plants which have been regenerated *in vitro*, 16 species (mainly cereal crops) have exhibited embryonic abilities (see Appendix I.B).

From the above discussion it is obvious that through the adjustment of auxin and cytokinin in the culture medium, a large number of callus cultures have been induced to undergo organogenesis. However, some tissues never initiate organs *in vitro* (regardless of the hormones to which they are exposed) and others lose their capacity to do so after a few subcultures (HALPERIN, 1969, 1973; HUGHES 1981). Thus the belief that exogenous phytohormones provide all the stimuli required for the many developmental phenomena seen in tissue culture, is a misconception which must be guarded against. NARAYANASWAMY (1977) in reviewing the process of plantlet regeneration in tissue culture, makes two statements that can well be introduced here:

1. "The source of the explant cultured is important in determining the regeneration potential", and
2. "The physiological age of the explant is another factor which exercises an influence on organ formation."

In fact, as stated by GRESSHOFF (1978), many internal biological factors as yet undefined must influence the interactions of tissues with a given medium. Concepts such as the one expressed by HALPERIN (1969) have emerged for this reason. He introduced the idea that callus inherited the ability to express totipotency from the primary culture and that these initial cultures contained mixed populations of competent and non-competent cells. He suggested that for callus to retain its regenerative capacity, active proliferation of competent cells was necessary. Today, the concept being advanced is similar. It is thought: that the morphogenetic competence of callus is determined from the time of culture initiation;

Table 1.8: Chromosome number of plants or shoots regenerated from callus or suspension cultures originating from explants of somatic, anther and pollen cultures (adapted from D'AMATO, 1977).

SPECIES	CHROMOSOME NUMBER	TYPE OF CULTURE	CHROMOSOME NUMBER OF REGENERATED SHOOTS OR PLANT
Asparagus officinalis L.	2n=20	Callus, suspension	20, 40
Festuca arundinacea Schreb	2n=49	Callus	25
Hordeum vulgare L.	2n=14	Callus	7,14,28
Lilium longiflorum Thunb.	2n=14	Callus	12, 24, 48, 24+1 isochrom
Oryza sativa L.	2n=24	Callus	12, 24, 36, 48, 60
Saccharum L. hybrid	2n=108-128 2n=106	Callus	94-120, 17-118 106
Secale cereale L.	2n=14	Callus	14, higher ploidy
Setaria italica (L.) Beauv	2n=18	Callus	9, 18
Triticum aestivum L.	2n=42	Callus	21, 42
T. aestivum L. hybrid	2n=56	Callus	28

that the growth regulators and other environmental conditions which result in callus morphogenesis merely play a permissive role in the expression of competent cells; and that if these cells were absent modification of the culture environment would not lead to organogenesis (STREET, 1979).

At the molecular level, the inability of some cultured tissue to form organs is ascribed either to epigenetic metabolic deficiencies (i.e. to the failure of the tissue to synthesize various limiting factors other than auxins or cytokinins) or to the retention of epigenetic characteristics of the differentiated function of the tissue of origin. It is also thought that genetic deficiencies associated with aneuploid chromosome numbers may also influence the regenerative capacity of callus tissue (HALPERIN, 1973). In many tissue culture systems, it has been shown that extensive subculturing of tissue results in a gradual change in ploidy and loss of regenerative vigour. Amongst the monocotyledons, this has been observed to occur in a number of callus cultures (Table 1.8).

In conclusion, it may be stated that a large number of factors are known to influence the behaviour of plant cultures however, many remain to be determined. In this review an attempt has been made to identify and summarize the pertinent literature regarding this aspect. In so doing, emphasis has been placed upon monocotyledonous cultures as *Hypoxis rooperi* (the plant of interest in this study), is a member of this large group of plants. Further much attention has been directed towards information available on callus cultures of these plants because, as it shall become apparent later, the cultures established in this study were mainly callus cultures.

1.4 BIOSYNTHESIS OF SECONDARY PLANT PRODUCTS *IN VITRO* WITH PARTICULAR REFERENCE TO PHENOLIC COMPOUNDS

Although the general trend over the past 50 years has been towards the artificial synthesis of important chemical and medicinal compounds, a number of these desired compounds still have to be directly extracted from plants; either because of costs or difficulties encountered in their synthesis. Plants, thus remain a fundamental source of these particular compounds. In recent years however, the quantity of plants available for extraction purposes has dwindled markedly. Factors regarded as being responsible for this include: natural restriction of geographical areas in which plants grow, political decisions such as closed frontiers of warfare,

human disturbances of the natural environment, ruthless exploitation, increasing labour costs and technical and/or economic difficulties in cultivating wild plants (TABATA, 1977; DOUGALL, 1979; KURZ and CONSTABEL, 1979). As a result of these problems it was suggested in the early 1950's, that plant tissue cultures be used as an alternative source of chemically useful compounds. The major advantages expected from cell culture systems over the conventional cultivation of whole plants were;

1. useful compounds could be produced under controlled environmental conditions,
2. cultured cells would be free of microbes and insects,
3. the cells of any plants could be easily multiplied to yield their specific metabolites, and
4. automated control of cell growth and regulation of metabolic processes would contribute to the reduction of labour cost and improvement of productivity (BUTCHER, 1977; TABATA, 1977).

Since the recognition of the potential of plant tissue culture in secondary plant metabolism, there has been continued exploitation of the biosynthetic capacity of various cultures and a considerable amount of data has accumulated during the last decade. The list of compounds already shown to be detected in culture is quite impressive (BUTCHER, 1977; NICKELL, 1980) however, there are relatively few examples in which specific compounds of the original plant are produced in comparatively high yields by cultured cells. Despite this, DOUGALL (1979) states that there are a sufficient number of examples to indicate that it is possible to obtain high yields of desired compounds via this technique.

One of the first class of useful products to be investigated in tissue culture were the alkaloids (KURZ and CONSTABEL, 1979; NICKELL, 1980). Amongst the monocotyledons, the Amaryllidaceae are known to contain high levels of this chemical (GIBBS, 1974). Two amaryllid species which have been studied *in vitro* to determine if the alkaloids produced in nature were produced in culture, are *Hippeastrum vittatum* Herb. and *Narcissus pseudonarcissus* L. (NICKELL, 1980). By far the greatest effort in the study of secondary metabolite production *in vitro* has been devoted to steroids, sterols, saponins and sapogenins (NICKELL, 1980). In the tissue

culture of monocotyledons, a number of genera have been analysed for these compounds (NICKELL, 1980). Of particular importance in this field is the genus *Dioscorea* L. from which diosgenin is extracted (KAUL and STABA, 1968; KAUL, STOHS and STABA, 1969; STABA, 1977). With regard to the phenolic content of plant cultures, NICKELL (1980) states that surprisingly little has been published on this topic. However, if one accepts the phenolic classification system of RIBÉREAU-GAYON (1972) and HARBORNE (1980), then a relatively large amount of literature is available on the analysis of these chemicals *in vitro*.

The production of secondary metabolites by plant tissue culture systems is largely dependent on environmental and biological factors as is *in vitro* plant regeneration (STABA, 1977; TABATA, 1977; DOUGALL, 1979, KURZ and CONSTABEL 1979). The following discussion will consider factors relating to phenolic synthesis *in vitro*.

As indicated previously there are a number of nutrient media employed in tissue culture for the regeneration of plants. However, very little attention has been devoted to the effect of media components and their interactions on secondary metabolite production. In rose cell cultures, MEHTA and SHAILAJA (1978) observed progressive inhibition of synthesis and accumulation of phenolics when the levels of nitrogen compounds were increased in the media. Maximum accumulation of phenolics was registered with media supplemented with yeast extracts. Similarly in *Acer pseudoplatanus* L., the increase in nitrogen source in the nutrient media, delayed the beginning of tannin production (WESTCOTT and HENSHAW, 1976). Furthermore, the addition of urea to the basal medium caused a decline in tannin metabolism. WESTCOTT and HENSHAW (1976) thus suggested that an antagonistic regulation exists between tannin synthesis and nitrogen metabolism.

Usually carbohydrates are supplemented to the culture media as sucrose or glucose, in concentrations of 2 to 3 per cent. In *Juniperus communis* L. cultures, an increase from 1 to 6 per cent sucrose enhanced the production of tannin (CONSTABEL, 1968). Similarly in suspension cultures of *Coleus blumei* Benth., ZENK, EL-SHAGI and ULBRICH (1977) found that a sucrose concentration of 7 per cent increased yields of rosmarinic acid. The highest concentration found in the culture exceeding that of the parent plant by a factor of five. In rose cell suspension cultures, increasing concentrations of glucose (0,05 to 0,2 mol ℓ^{-1}) in the culture medium significantly promoted the synthesis of phenolics. This effect was reversed by the addition of

nitrate (0,224 mol ℓ^{-1}) to media containing high glucose levels (AMORIM, DOUGALL and SHARP, 1977). These results suggest that the effect of carbohydrates on the production of secondary metabolites, depends on concurrent nutritional factors (KURZ and CONSTABEL, 1979) and may be under an antagonistic regulation with nitrogen metabolism (TABATA, 1977).

As the production of secondary metabolites in plant tissue cultures is a function of cell multiplication and differentiation (TABATA, 1977), the quantity and quality of hormones added to the nutrient medium is of considerable importance. Hormone type and product formation have been well studied and it may be generalized that indole acetic acid is a more favourable auxin for permitting product synthesis and accumulation than naphthalene acetic acid or 2,4-dichlorophenoxy acetic acid. Furthermore, higher concentrations of auxin suppress and lower concentrations permit the production of metabolites (KURZ and CONSTABEL, 1979).

With regard to phenolic compound synthesis, in *Linum usitatissimum* L. callus cultures, high levels of auxin caused a marked decrease in the amount of p-coumaric acid and ferulic acid, with a concomitant increase in the amounts of p-hydroxybenzoic acid and vanillic acid. In contrast, high levels of kinetin led to an increase in p-coumaric acid and ferulic acid (LIAU and IBRAHIM, 1973). SKOOG and MONTALDI (1961), and FURUYA, KOJIMA and SYONO (1971), observed that in *Nicotiana tabacum* callus cultures, the amounts of coumarins produced was markedly influenced by the levels of auxin and kinetin in the culture medium. Both auxins and cytokinins have also been found to affect lignin formation. Kinetin, when added at the appropriate concentration, enhanced the amount of lignin in callus of *Daucus carota* L. (KOBLITZ, 1962) and *Nicotiana tabacum* (BERGMANN, 1964). On the other hand, auxin and sucrose levels in the medium of callus cultures of *Syringa vulgaris* L. and *Phaseolus vulgaris* L. greatly influenced the formation of xylem and its degree of lignification (WETMORE and RIER, 1963; JEFFS and NORTHCOTE, 1967). In the above studies it is difficult to decide whether the effects of the growth hormones are directly on lignification or on vascular differentiation. In *Acer pseudoplatanus* suspension cultures in which no tracheids were formed, CARCELLER, DAVEY, FOWLER and STREET (1971) have however shown,

that the lignin content is very much affected by the concentration of sucrose, 2,4-dichlorophenoxy acetic acid and kinetin. These results would suggest that the effect of the substances on lignin biosynthesis are not dependent on the differentiation of xylem-like elements.

With regard to the influence of plant growth regulators on anthocyanin production *in vitro*, CONSTABEL, SHYLUK and GAMBORG (1971) have shown that high concentrations of naphthalene acetic acid and 2,4-dichlorophenoxy acetic acid suppress the accumulation of these compounds in *Haplopappus gracillis* A. Grey cultures. Similarly STICKLAND and SUNDERLAND (1972a) observed that 2,4-dichlorophenoxy acetic acid added to the medium reduced the amount of anthocyanin produced by *Haplopappus gracillis* callus. In contrast ALFERMANN and REINHARD (1971) were able to replace the light requirement for anthocyanin biosynthesis in cultures of *Daucus carota* with auxin. On the other hand, the presence of gibberellic acid in the medium inhibited both light-induced and auxin-induced anthocyanin formation (SCHMITZ and SEITZ, 1972). From the above discussion, it is clear that plant growth regulators are important factors controlling phenolic product metabolism.

One of the most extensively studied influences on secondary product metabolism is light. It has been shown in a number of plant tissue cultures, that light generally stimulates the formation of secondary compounds (TABATA, 1977), particularly anthocyanins (REINERT, CLAUSS and VON ARDENNE, 1964; STICKLAND and SUNDERLAND, 1972b), flavonoids (FORREST, 1969a; HAHLBROCK and WELLMANN, 1970; HAHLBROCK, 1972; KREUZALER and HAHLBROCK, 1973) and polyphenols (DAVIES,1972a; BUTCHER, 1977).

Optimization of conditions for secondary product formation in cell cultures is not limited to modification of the nutrient medium and physical environment, but may also include the regulation of secondary compounds. Often precursor feeding can increase yields of metabolites, and may result in the production of compounds other than those found in the parent plant. In *Linum usitatissimum* callus cultures, it has been observed that higher levels of cinnamic acids could be obtained when phenylalanine (a likely precursor) was added to the basal medium (LIAU and IBRAHIM, 1973). It has also been shown with suspension cultures of *Solanum tuberosum* L. that ^{14}C labelled shikimic acid, quinic acid, p-coumaric acid and caffeic acid can serve as precursors

of chlorogenic acid (GAMBORG, 1967). ZENK, EL-SHAGI and ULBRICH (1977), found that the addition of L-phenylalanine increased the yield of rosmarinic acid in cell suspension cultures of *Coleus blumei*. Similarly, LOH, HARTSEL and ROBERTSON (1983), observed that in *Cannabis sativa* L. cultures, cannabidiol served as a useful precursor for the production of cannabielsoin phenolics. With regard to polyphenolic production, it has been demonstrated that shikimic acid, phenylalanine, p-coumaric acid and ferulic acid are good precursors for lignin biosynthesis (HASEGAWA, HIGUCHI and ISHIKAWA, 1960). Similarly ^{14}C labelled cinnamic acid, p-coumaric acid and caffeic acid were incorporated into lignin of suspension cultures of potato (GAMBORG, 1967).

As the above examples show, it is possible to increase desired chemical compounds by precursor feeding however, it is not a simple task. Often the administration of a direct precursor is not effective or may be toxic (TABATA, 1977). Moreover, from a practical viewpoint, the precursor may be too costly for this type of procedure to be commercially viable.

Other than the environmental factors which affect secondary product metabolism *in vitro*, various biological factors also play a significant role in controlling these processes. The biological factors include growth, morphological differentiation and variation in biosynthetic activity of cultures (TABATA, 1977).

Experimental data on the relationship between product formation and culture growth, has indicated that three major types of product-growth patterns may be identified (TABATA, 1977). In the first type of pattern, product formation proceeds almost parallel with cell growth. In the second, product formation is delayed until cell growth declines or ceases. According to DAVIES (1972a),polyphenol production belongs to this type of pattern. In the third type of pattern, the production curve is diphasic and lags behind the growth curve. In contrast to these product-growth patterns, YEOMAN, MIEDZYBRODZKA, LINDSEY and McLAUCHLAN (1980) state the following:

> "that an inverse relationship exists between secondary metabolite production and the growth rate of tissue and cell cultures."

In other words, either as a response to slow cell growth, or as a response elicited by environmental conditions inducing slow cell growth, there seems to be a switch from primary towards secondary metabolism.

Amongst phenolic producing cultures, there are a number of variations on the product-growth pattern theme. For example, in Paul's Scarlet Rose cultures, it was observed that cells produced phenolics during all phases of growth, but production was greatest during the stationary phase (AMORIM, DOUGALL and SHARP, 1977). In *Juniperus communis* cultures, CONSTABEL (1968), noted that tannin concentration reached a maximum at the onset of rapid cell growth, 30 days after inoculation, and then declined. Similarly in *Perilla ocymoides* L. (IBRAHIM and EDGAR, 1976) and *Daucus carota* (SUGANO, IWATA and NICHI, 1975) suspension cultures, phenolic compound biosynthesis was highest in the early exponential growth phase. In contrast to these examples, where it would appear that cell growth and phenolic production are complementary, there are a number of examples which suggest that these two processes are antagonistic. For example, FORREST (1969b) stated that anthocyanin concentration in *Camellia sinensis* L. cultures, was inversely related to growth rate. Thus a decline in culture growth which could be caused by various environmental conditions such as intense light or nutritional abnormalities, gave rise to an increase in polyphenolic synthesis. Similarly in Paul's Scarlet Rose cultures, the total phenolic content per culture declined to almost 50 per cent of the initial inoculum during the lag and early exponential growth phases. However, by the end of the exponential phase the phenolic content had begun to rise rapidly and continued to increase over the post-exponential period (NASH and DAVIES, 1972). In sycamore cultures, a similar situation has been observed to occur (WESTCOTT and HENSHAW 1976).

While the latter two studies both demonstrate that substantial accumulation of phenolic compounds is restricted to the late or post-exponential phase of cell growth, the number of cells continued to increase for some time after the onset of phenolic accumulation. Thus the relationship between secondary metabolism and cell cycle is uncertain. PHILLIPS and HENSHAW (1977) tried to clarify this aspect. Utilizing cultures of *Acer pseudoplatanus*, they managed to indicate that phenolic accumulation could be induced in stationary phase cells. In this instance, it would suggest that cell division is not essential for phenolic production to occur.

In higher plants, there are certain compounds which are synthesized or accumulated only in particular organs or tissues. Often the failure of unorganized cell or tissue cultures to produce desired compounds, is attributed to the lack of morphological differentiation (TABATA, 1977) and it has been suggested and shown that the induction of differentiation, will result in compound production (STABA, 1969; HIRAOKA and TABATA, 1974; TABATA, 1977; VERZÁR-PETRI, LADOCSY and OROSZLÁN, 1982). In *Allium cepa* L. cultures for example, the production of flavonoid compounds reached intensities approaching that of fresh onions only in cultures in which roots had differentiated (FREEMAN, WHENHAM, MACKENZIE and DAVEY, 1974). Light regimes inducing tissue differentiation and lignification also stimulated flavonoid production in *Citrus* L. tissue cultures (BRUNET and IBRAHIM, 1973).

DOUGALL (1979) provides some interesting ideas on this aspect. He states that the requirement for specific structures to be present in the medium, implies that the genetic information required to accumulate the desired compound, is part of the information required for the construction of the morphological structure. This, he notes seems unlikely on the basis of gene specificity and thus suggests that the close association of these processes is controlled by the culture conditions having a permissive effect on the expression of various genes. If this is so, he then hypothesizes that it should be possible to find conditions in which secondary products can be produced in the absence of specific morphological structures.

It is however not always essential and occasionally detrimental to compound synthesis, if tissue cultures become organized. YEOMAN, MIEDZYBRODZKA, LINDSEY and McLAUCHLAN (1980) for example state that, mature cultures which exhibit a marked amount of cellular differentiation tend to accumulate secondary products, normally in very small amounts and often different from those which can be isolated from the intact plant. An example of phenolic production without morphological differentiation is that observed by TABATA, MIZUKAMI, HIRAOKA and KONOSHIMA (1974). In *Lithospermum erythrorhiza* Sieb. and Zucc., they noted that shikonin derivatives were localised only in the cork layers of the roots, while in cell cultures lacking in cork cells, these derivatives could also be detected.

Another factor which can regulate secondary metabolism and has potential use in improving biosynthetic capabilities of culture strains, is cellular variation. According to DOUGALL (1979), one of the main reasons why plant cell cultures often yield low levels of secondary products is because the conditions in which cultures are maintained are conducive to the development and selection of low-yielding cell lines. Furthermore, in order to obtain high yields of specific compounds in culture, three conditions must exist within the cells;

1. the rate of degradation must be lower than the rate of synthesis of these compounds,
2. intermediates in synthesis must be present and
3. the reactions for synthesis must be possible.

BÖHM (1982) regards the latter condition (which may include the lack of specific enzymes) as the most important reason for the inability of plant cell cultures to produce secondary products. Thus in order to overcome these problems and improve secondary product metabolism, selection of genetically stable cell variants or mutants with the capacity to yield high levels of the desired compound(s), is necessary. Recently DEUS and ZENK (1982), established a four-step strategy which has proved to be a practical procedure for isolating cell culture strains yielding substantial amounts of natural products. The four steps include: the development of a suitable selection method; the selection of plants with high contents of natural products; the development of a production medium and the selection of variant strains. Utilizing this procedure, they maintain that the chance of using plant cell cultures for bioproduction of desired compounds should improve.

In conclusion, it may be stated that with regard to the production of phenolics *in vitro*, there have been significant advances over the last 30 years. From research already undertaken, a basic strategy for obtaining high yields of these compounds is emerging. As a result, the biotechnological application of these techniques is becoming a more practical and economically viable proposition.

CHAPTER 2

REGENERATION OF *HYPOXIS ROOPERI IN VITRO*

2.1 INTRODUCTION

Although the genus *Hypoxis* has for many centuries been recognised for its medicinal properties, it is only since research activities have disclosed that members of this genus possess unusual chemical constituents, that an interest in the pharmacological potential of these plants has developed. This has subsequently resulted in an increasing demand for *Hypoxis* plants. As yet, no method has been devised to ensure a continuous supply of these plants for commercial and research activities, and wild resources of this genus are rapidly being exploited. The following study was therefore initiated in an attempt to develop a technique for regenerating numerous *Hypoxis* plants.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

The experimental plant used in this investigation was *H. rooperi* (Figure 2.1). This species is one of the more important medicinal species within the genus *Hypoxis*. In Natal, its distribution is quite extensive (Figure 2.2), and members of this taxon may be easily identified by their many tristichously arranged, sickle-shaped leaves. A voucher specimen (PAGE 156) of a typical *H. rooperi* plant, is housed in the Natal University herbarium, Pietermaritzburg. For all experiments, plants of *H. rooperi* were collected from the natural grasslands in the environs of Pietermaritzburg. Material was always utilized within 24 hours of collection. As *H. rooperi* plants possess no axillary meristems (WOOD, 1976), the approach adopted in developing this *in vitro* propagation technique, was to establish meristems either directly or indirectly (via callus) from explants of this species. In order to determine the best source from which to obtain explants, the following material was selected and cultured *in vitro*; flower buds, inflorescence peduncle segments, leaf segments and corm segments. For all *in vitro* experimentation these explants were orientated in a basipetal position, unless otherwise stated.

Figure 2.1: *Hypoxis rooperi* S. Moore.

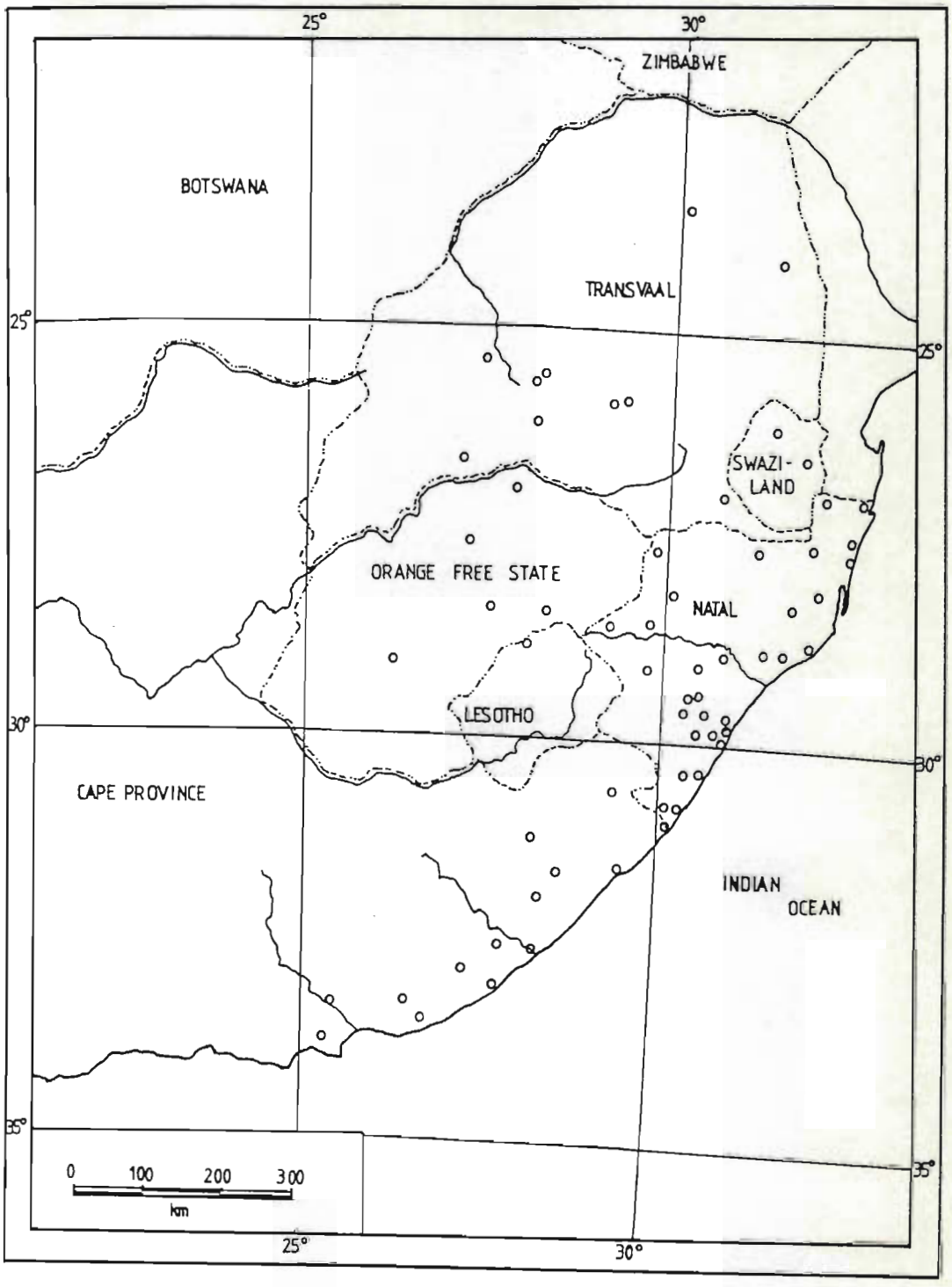


Figure 2.2: *H. rooperi* - known distribution in Southern Africa up to 1975 (WOOD, 1976).

2.2.2 Explant material

Flower buds. Investigations using flower explants of *H. rooperi* included the random use of buds of all ages. Initially, these explants were retained as whole units, however during later more detailed investigations the perianth segments, stamens and style were excised (Plate 2.1a and b). As *H. rooperi* plants flower most profusely in Spring, all investigations using flower buds were undertaken during September, October and November of the years 1981, 1982 and 1983.

Inflorescence peduncles. For the establishment of cultures from inflorescence peduncles, segments of material approximately 1,5 cm long, were removed from the entire length of peduncles of varying ages. These explants were only used for the sterilization procedure determination and hormone treatment experiments. Further use was discontinued as the response of this material *in vitro*, was relatively poor.

Leaves. The suitability of leaf material as an explant source, was determined using leaf segments (1,5 cm^2) taken from the midrib region of the lower half of young leaves. As the growth response of these explants *in vitro* was negligible, the use of leaf material in this study was not extensively pursued.

Corms. For plantlet formation from corm explants of *H. rooperi*, segments of corm material approximately 1,0 to 1,5 cm^2 , were taken randomly from regions 1, 2 and 3 (Figure 2.3), from the upper half of mature corms. Following the development of a suitable sterilization procedure for this material, it became apparent that only explants obtained from region 2, underwent organogenesis *in vitro*. For this reason, corm material from this region was used in all further experiments. The availability of corms is not seasonal and hence experimentation utilizing this material could be undertaken throughout the year.

2.2.3 Culture medium

The standard culture medium used throughout this study contained the macro-nutrients, micro-nutrients and vitamins as outlined by MURASHIGE and SKOOG (1962). Details of these constituents are presented in Table 1.4. This medium was chosen because it can be bought in a premix

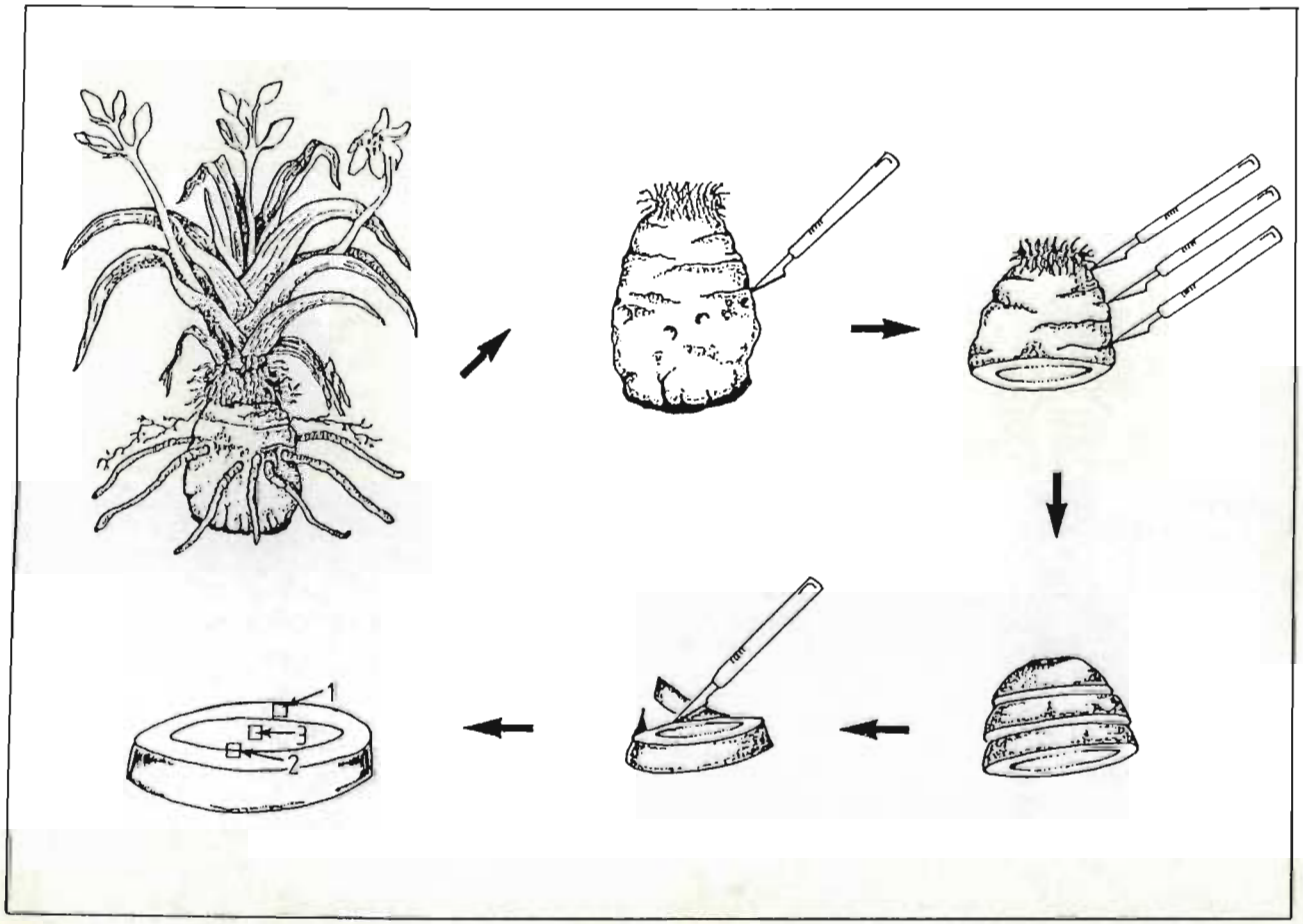


Figure 2.3: Diagrammatic representation of the origin of *H. rooperi* corm explants from within the host plant.

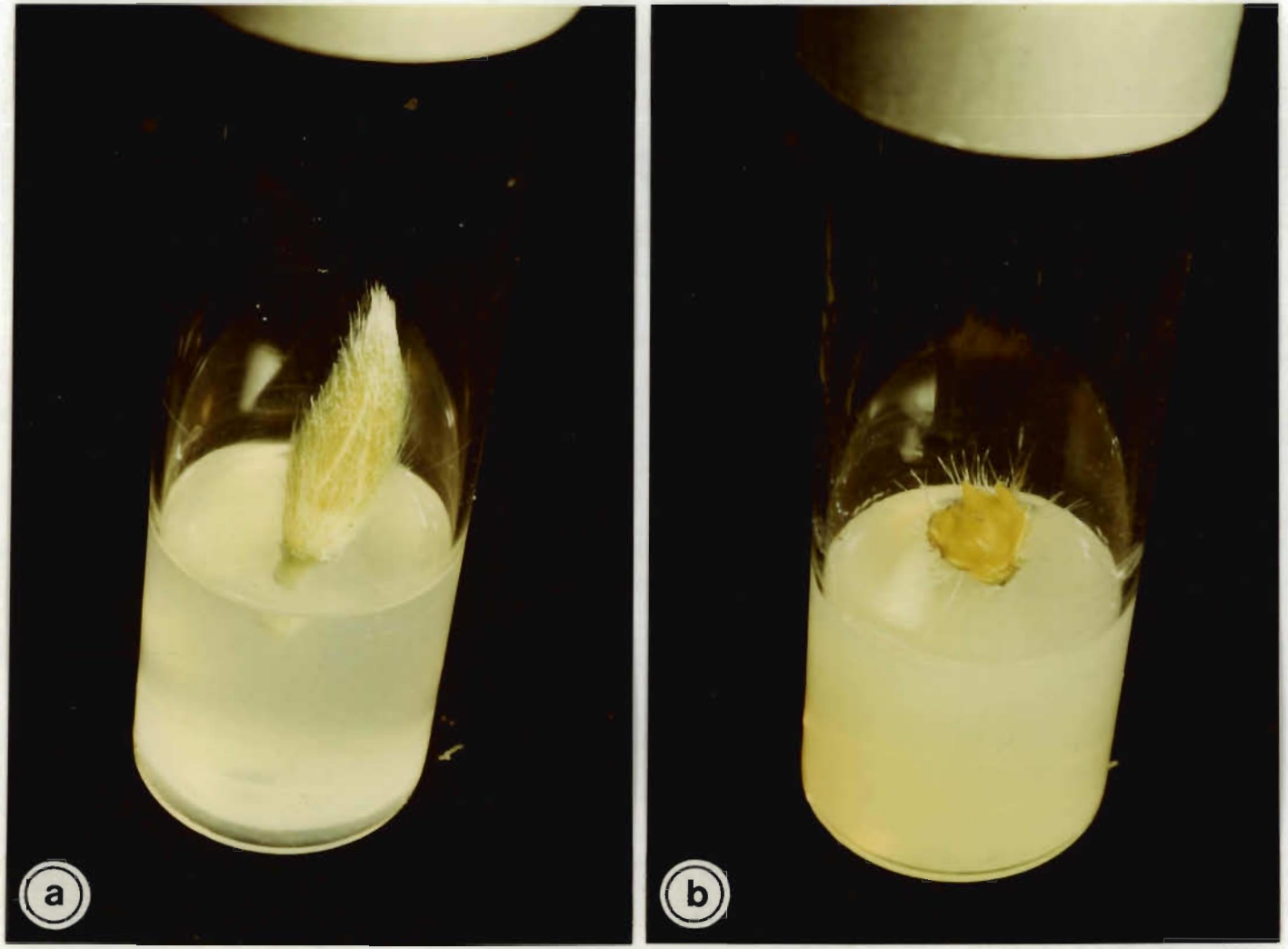


Plate 2.1: *H. rooperi* flower bud explants. Whole bud (a) and bud from which the perianth segments, stamens and style have been excised (b). x 1,8.

form. Therefore, if this technique proved to be suitable for commercial application, the problems involved in making up media would be kept to a minimum. For the present investigation however, all constituents of this medium (excluding sucrose), were made up as stock solutions. These solutions were obtained by dissolving the required amounts of analytical grade macro-nutrients, micro-nutrients and vitamins in pyrex-distilled water, and making the final volume up to 500 ml. All stocks were stored in glass containers at 5 °C. Those stocks which contained light-sensitive constituents such as ferric monosodium ethylene-diaminetetra-acetic acid (FeEDTA) and vitamin complexes, were stored in containers wrapped in aluminium foil to exclude light.

To obtain the complete culture medium, stock solutions were combined in the volumes as indicated in the last column of Table 1.4. This was supplemented with sucrose (30 g ℓ^{-1}), meso-inositol (0,1 g ℓ^{-1}) and thiamine-HCl (0,001 g ℓ^{-1}), and made to volume with pyrex-distilled water. The pH of the medium was adjusted using a sodium hydroxide (NaOH) solution, to 5,8. To each litre of culture medium, 9,0 g of Difco bacto agar was added to solidify it. This was dissolved in the medium, by steaming in an autoclave for 10 minutes, prior to dispensing into the culture vessels. All cultures were initiated in 25 mm x 80 mm glass tubes, each containing 15 ml of medium. Tubes were sealed with Cap-O-Test tops. The culture medium, instruments and glassware were sterilized in an autoclave at one bar pressure and a temperature of 121 °C, for 20 minutes.

2.2.4 Sterilization procedures

The first objective of this study was to develop a suitable sterilization procedure for each type of explant selected for use *in vitro*. To achieve this, the pre-sterilization procedures were standardised and the subsequent sterilization procedures varied. Two pre-sterilization methods were adopted one for flower bud, inflorescence peduncle and leaf material, and a second for corm material.

Pre-sterilization. For flower bud, inflorescence peduncle and leaf material, the pre-sterilization procedure adopted was as follows:

Initially, all material was washed in running tap water for 30 minutes, to remove dust and debris. The tissues were then trimmed to a workable size (Figure 2.4), and immersed for 2 minutes, in 95 per cent ethanol to which was added Tween 20 (2 drops per 100 ml ethanol).

For corm material, the procedure used prior to sterilization was as follows:

Firstly, the leaves and roots were severed from the corm. The upper half of the corm was then cut horizontally into three slices and the outer epidermis of each removed (Figure 2.4). The slices were then washed under running tap water for 20 minutes. The material was subsequently immersed in a 1,0 per cent solution of benzimidazol (Benomyl), for 5 minutes. The slices were then washed for a further 5 minutes, in 100 per cent ethanol.

Sterilization. Following the pre-sterilization procedures several sterilization techniques were tested under aseptic conditions. These were performed on a laminar flow bench. Prior to use, the surface of the bench was swabbed and the surrounding atmosphere sprayed with 95 per cent ethanol. For the sterilization procedures, the common sterilants sodium hypochlorite (NaOCl) and mercuric chloride ($HgCl_2$) were tested. The explants were immersed in sterilant solutions of these chemicals (1,75 per cent and 3,5 per cent NaOCl; 0,1 per cent and 0,5 per cent $HgCl_2$), for varying lengths of time. They were then rinsed three times in sterile distilled water, the final rinse lasting at least 30 minutes. Following this rinse, explants were removed individually and placed in a sterile petri dish for the final aseptic trimming. This involved the removal of all tissue visibly damaged by the sterilant and the cutting of explants to the sizes previously described. These were then transferred to tubes containing a sterile, hormone-free culture medium. The mouths of all culture vessels and the instruments used, were flamed prior to and following the transfer process. Occasionally during the operation, the bench surface and worker's hands and arms were swabbed with 95 per cent ethanol.

For all sterilization treatments, 20 or more replicates, of each type of explant, were used. After a month the number of explants surviving and sterile was recorded, and expressed as a percentage of the initial

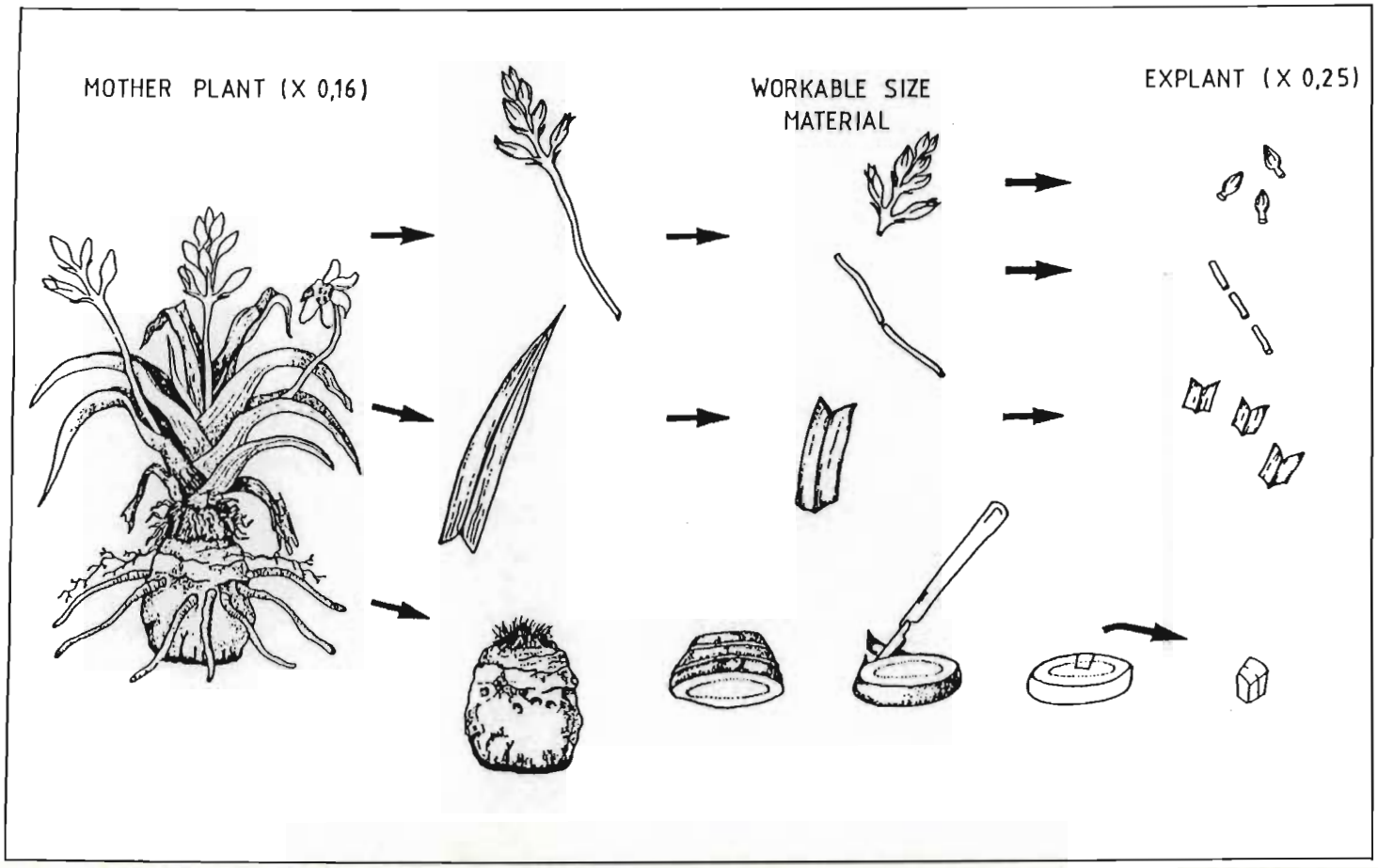


Figure 2.4: Diagrammatic outline showing the procedure followed to obtain workable size material.

number of explants used. From this experiment, a suitable sterilization regime for each type of explant was determined. These regimes were routinely employed for all further *in vitro* investigations.

2.2.5 Culture conditions

For all experiments, cultures were maintained in a growth room on individually illuminated racks. The spectal irradiance measured was 1,15 Wm^{-2}. The photoperiod was 16 hours light followed by 8 hours darkness. The room temperature varied between 23 and 27 °C, and the relative humidity was 40 per cent.

2.2.6 Hormonal investigations

Following the development of a sterilization procedure for the different types of explants, the second objective of this study was to establish plantlets from the various types of explant material. This phenomena is commonly achieved in tissue culture by supplementing the culture medium with plant growth hormones. It was this aspect which formed the basis of the following series of investigations. The plant growth regulators selected for use in these analyses were the auxins 2,4-dichloro-phenoxy-acetic acid (2,4-D) and 1-naphthalene acetic acid (NAA), and the cytokinin 6-benzylaminopurine (BA). These were chosen, because of their chemical stability and frequent use in culture programmes (DODDS and ROBERTS, 1982). For each treatment at least 10 replicates were used. These replicates were examined and the growth response for flower bud, inflorescence peduncle and leaf explants recorded, after an incubation period of 6 weeks and corm explants after a period of 10 to 12 weeks.

Experiment 1. The initial experiment performed involved the culturing of flower bud, inflorescence peduncle and leaf explants, on a culture medium supplemented with varying concentrations and combinations of 2,4-D and/or BA (Table 2.1).

Table 2.1: Table to illustrate the various concentrations and combinations of 2,4-D and BA used in Experiment 1 .

BA CONCENTRATION ($g\ell^{-1}$)	2,4-D CONCENTRATION ($g\ell^{-1}$)				
	0	0,0005	0,001	0,002	0,004
0 0,002 0, 005 0, 01					

Experiment 2. Following the rather disappointing results obtained from the above experiment, the next experiment undertaken involved the culturing of flower bud, inflorescence peduncle and leaf explants, on a culture medium supplemented with NAA and/or BA. These two hormones were used in the concentrations and combinations as outlined in Table 2.2.

Table 2.2: Table to illustrate the various concentrations and combinations of NAA and BA used in Experiment 2 .

BA CONCENTRATION ($g\ell^{-1}$)	NAA CONCENTRATION ($g\ell^{-1}$)				
	0	0,0005	0,001	0,005	0,01
0 0,001 0,005 0,01					

Experiment 3. Finally an analysis of the response of corm explants on a culture medium supplemented with NAA and/or BA in varying concentrations and combinations (Table 2.3), was undertaken. The influence of 2,4-D upon corm material was omitted as results from Experiments 1 and 2 clearly indicated that NAA was a more inductive auxin than 2,4-D.

Table 2.3: Table to illustrate the various concentrations and combinations of NAA and BA used in Experiment 3 .

BA CONCENTRATION ($g\ell^{-1}$)	NAA CONCENTRATION ($g\ell^{-1}$)		
	0	0,001	0,005
0 0,0005 0,001 0,003 0,005			

From the above hormonal experiments, the following information was obtained;

1. that flower buds and corm explants were the two most responsive types of *H. rooperi* material analysed *in vitro*, and
2. that the most effective hormonal treatments for inducing shoot formation from flower buds and corm explants were 0,001 $g\ell^{-1}$ NAA and 0,005 $g\ell^{-1}$ BA; and 0,001 $g\ell^{-1}$ NAA and 0,001 $g\ell^{-1}$ BA, respectively.

Experiments henceforth conducted were designed to improve the shooting response obtained from flower bud and corm explants. All culture media used in these investigations, were supplemented with the most inductive shoot forming hormone concentrations for the respective types of explants. All experiments were repeated at least twice.

2.2.7 Flower bud investigations

From the preliminary experiments on flower buds, it was apparent that the response of this type of material *in vitro*, varied depending upon the morphological and physiological age of the buds. Thus an investigation was begun to analyse which age bud produced the best growth response *in vitro*. Buds were therefore divided into three categories (Plate 2.2);

1. young buds, less than 1,0 cm in length,
2. buds more than 1,0 cm in length, but which had not yet flowered, and
3. old buds, which had already flowered.

These buds, 10 per treatment, were then cultured *in vitro*. From this investigation, the most responsive category bud was selected. More comprehensive studies were then undertaken utilizing buds of this age. These included;

1. an analysis of the effect of wounding and the influence of bud orientation on bud response,
2. the re-analysis of the most effective sterilization procedure and hormone treatment for these buds, and
3. a more definite establishment of the number of buds which respond to inductive *in vitro* conditions.

The objectives of these analyses were to improve the *in vitro* growth response of the flower buds. For all these experiments at least 10 replicates per treatment were used.

2.2.8 Subculturing investigations

From the above experiments, a technique for producing callus *in vitro* from flower buds, was determined. Subculturing experiments could thus be embarked upon. To increase stocks of callus whole callusing flower buds were transferred to fresh culture medium, 12 weeks after inoculation.* Following a further two transfers of this material onto

*Preliminary subculturing experiments had indicated that the transfer of this material 6 weeks after inoculation, was detrimental for further growth.

Plate 2.2: *H. rooperi* inflorescence. (a) Young bud less than 1,0 cm in length. (b) Bud more than 1,0 cm in length, but which has not yet flowered. (c) Old bud which has already flowered. x 1,5.

fresh medium, each for a period of six weeks, a substantial stock of callus was established. During these two subculturings, the callusing flower buds were sufficiently large to be divided.

The culture media utilized during the entire 24 week subculturing period were supplemented with 0,001 $g\ell^{-1}$ NAA and 0,005 $g\ell^{-1}$ BA (the best "callus-shoot" inducing hormone treatment for flower buds). All callus transfers were made into 100 ml conical flasks, each containing 50 ml of solidified culture medium. These flasks were sealed with non-absorbant cotton wool bungs and covered with aluminium foil. Subculturing experiments were performed either on a laminar flow bench or in a sterile transfer cabinet. Similar precautions were taken as described for the initial culturing of material, to sterilize the culture medium and ensure aseptic conditions.

The main objective using the stock of well established callus, was to develop a continuous shoot producing culture. The first experiment performed to try and achieve this, involved the transfer of callus onto a culture medium containing various concentrations of NAA and/or kinetin (K) or BA (Table 2.4). The use of K was included in the analysis at this stage to determine whether this cytokinin was more effective in inducing shoot formation than BA. To determine the response of callus on these different media, cultures were maintained on them for 6 weeks. For each treatment 10 replicates were used. From this experiment, shoot producing, root producing and callus maintenance conditions were determined.

Following the above subculturing experiment, the next objective of this study was to determine the number of plantlets which could be produced *in vitro*, using the technique thus far developed. To calculate this, 30 week old callus (obtained from the above experiment), and 66 week old callus (obtained after the callus had been subcultured for a further 36 weeks), was treated in the following manner. Under aseptic conditions, callus blocks approximately 0,5 cm^2 and 0,7 g, were excised from the periphery of the different aged callus masses. These blocks were then transferred, in groups consisting of three pieces, onto a shoot producing culture medium, (i.e. a medium supplemented with 0,001 $g\ell^{-1}$ NAA and 0,001 $g\ell^{-1}$ BA). After various periods of time (2; 4 and 6 weeks), 10 flasks

Table 2.4: Table to illustrate the various concentrations and combinations of NAA, K and BA used to develop a continuous shoot producing culture.

NAA CONCENTRATION ($g\ell^{-1}$)	K CONCENTRATION ($g\ell^{-1}$)	BA CONCENTRATION ($g\ell^{-1}$)
0	0	0
0,001	0	0
0,001	0,005	0
0,001	0	0,001
0,001	0	0,005
0	0,001	0
0	0,003	0
0	0,005	0
0	0,010	0
0	0	0,001
0	0	0,003
0	0	0,005
0	0	0,010

of material were harvested and the callus therein massed. At 6 weeks, the number of shoots produced per flask was also counted. From this experiment, a theoretical number of shoots which could be produced *in vitro*, was calculated.

To achieve the rooting and "hardening-off" of shoots produced *in vitro*, several procedures were tested. The following technique was eventually found to be the most suitable:

1. Individual shoots or shoot clusters produced *in vitro*, were subcultured into Magenta GA-7 vessels containing 100 ml of hormone-free culture medium.

2. Once the shoots had rooted (Plate 2.3) (usually after a period of 4 to 6 weeks), the lids of the culture vessels were removed and replaced by plastic bags.
3. After 2 weeks, holes were cut in the corners of these bags.
4. Following a further 2 week period, the bags were removed and the plantlets recovered. The agar was then washed from their well developed roots.
5. The plants were then transferred to small plastic pots containing a sand and an organic fertilizer (Gromore) mixture (2 : 1).
6. The pots were maintained in a 60 per cent shade-house for approximately 1 month. They were then transferred to a 10 per cent shade-house for a further 1 month period. While in the shade-houses the plants were watered regularly.
7. After this procedure, the plants were sufficiently hardy to be transferred to natural growing conditions.

2.2.9 Corm investigations

As investigations thus far completed indicated that the corm of *H. rooperi* offered an adequate source of material for the micropropagation of this plant, more attention was devoted to this aspect. The design of further experiments was thus twofold in its objectives;

1. to enhance plantlet production from corm explants, and
2. to achieve this via a more direct and rapid pathway to that developed from flower buds.

Experiments embarked upon in an attempt to achieve these objectives, included an analysis of the influence of the following factors upon explant response;

1. corm explant orientation on the culture medium (Figure 2.5),
2. the presence of casein hydrolysate (1,0 $g\ell^{-1}$) in the culture medium,
3. varying sucrose concentrations (20, 30 and 40 $g\ell^{-1}$) in the culture medium, and
4. explant origin from within the donor plant (Figure 2.6).

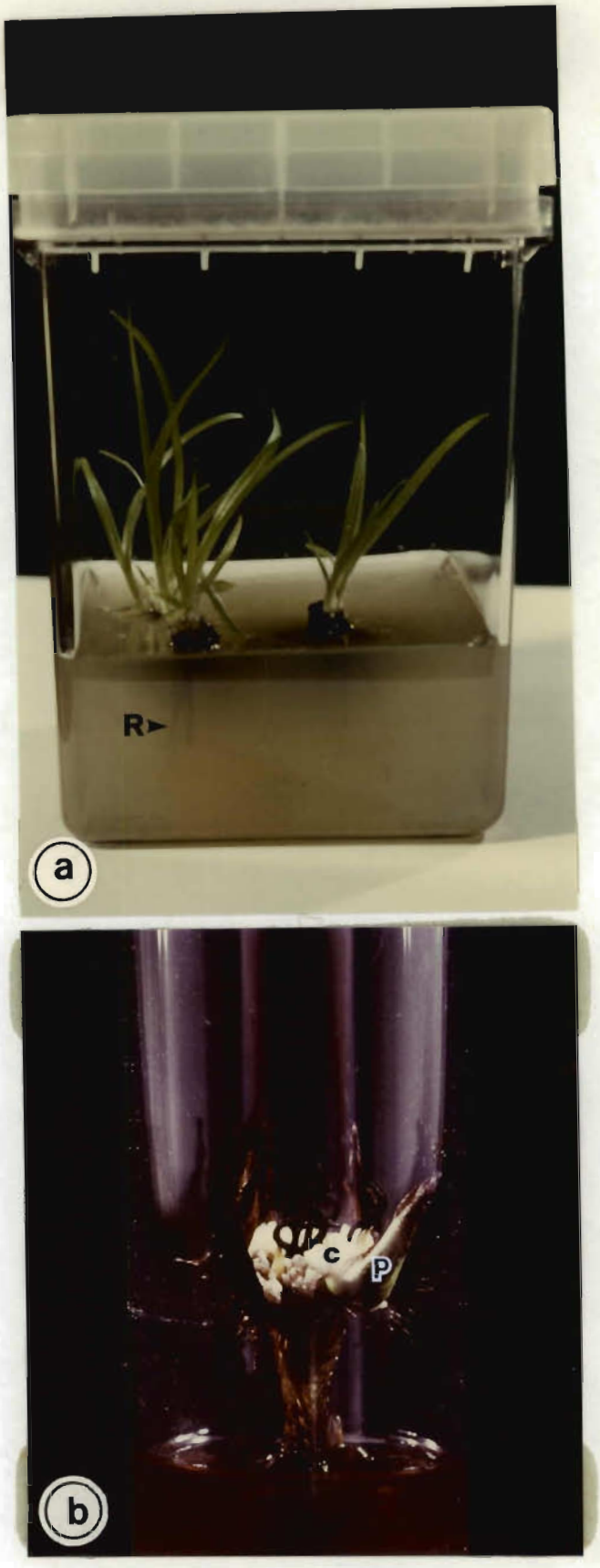


Plate 2.3: (a) Plantlets of *H. rooperi* after the first stages of the "hardening-off" process. R = roots. x 1,0.

(b) Cultured *H. rooperi* flower bud. P = reflexed perianth segments, c = callus. x 1,7.

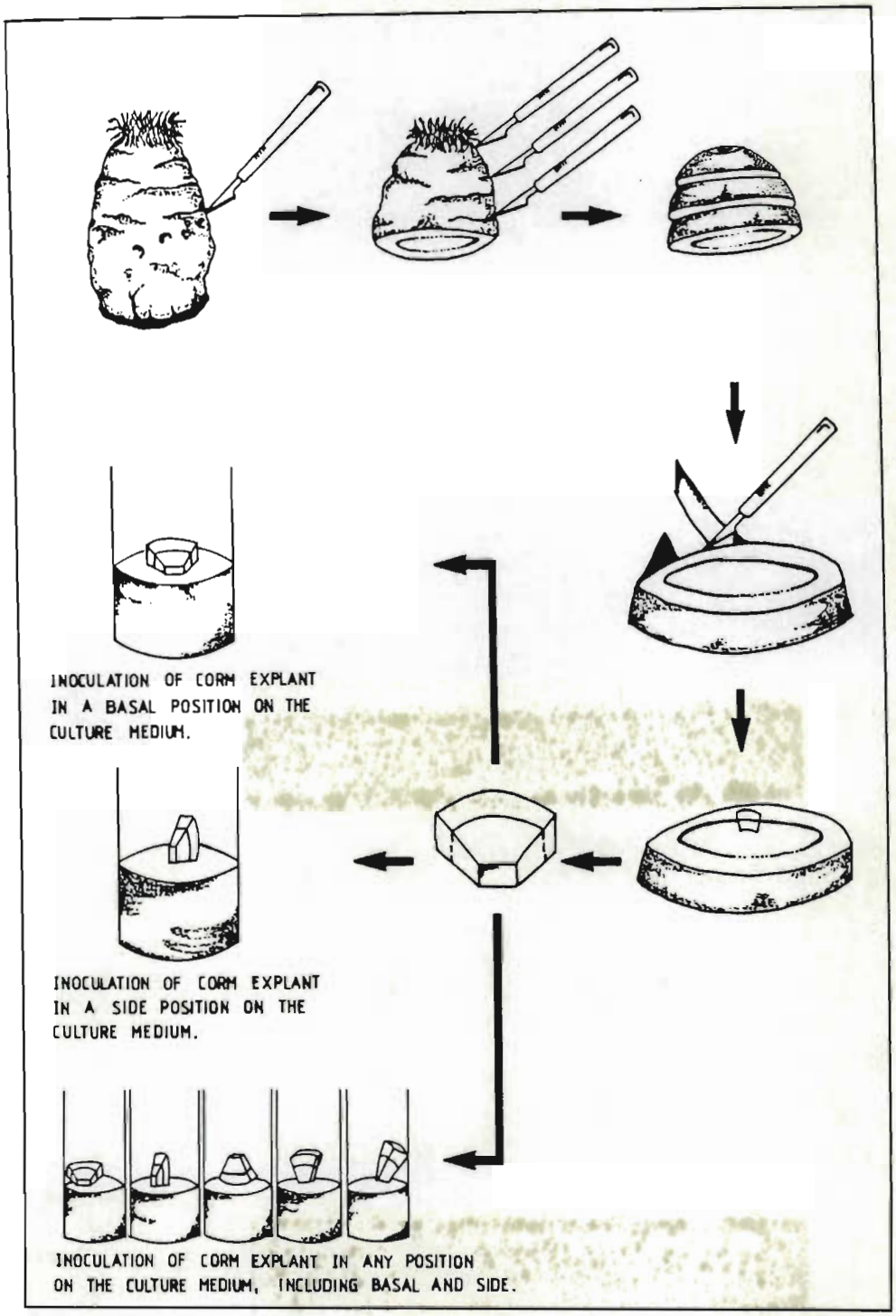


Figure 2.5: Diagrammatic representation of *H. rooperi* corm explant orientation upon the culture medium.

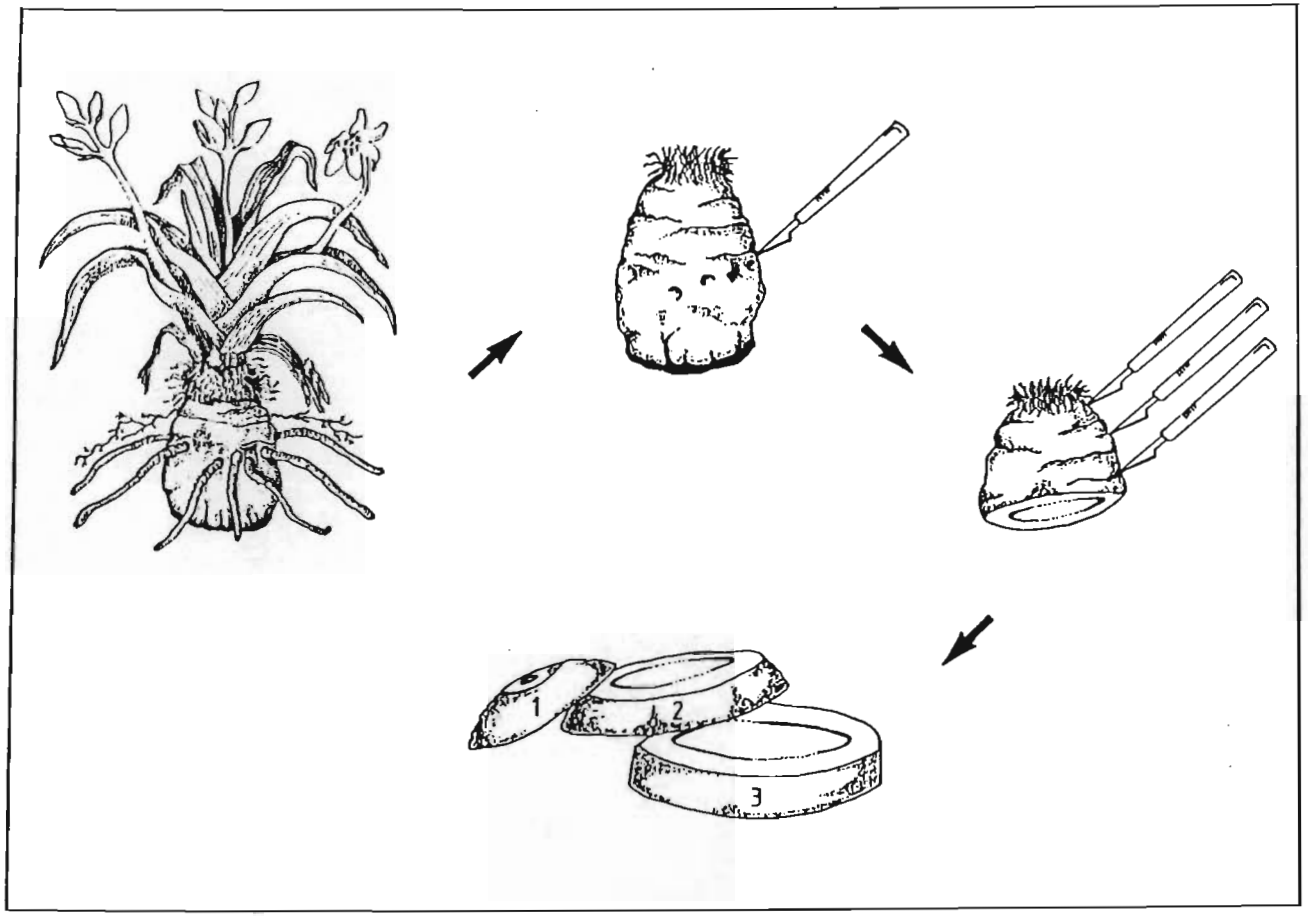


Figure 2.6: Diagrammatic representation of the regions from which *H. rooperi* corm explants were obtained.

These analyses were performed in a series of experiments the details of which are outlined in section 2.3.5. For each treatment, 14 replicates were used.

Results obtained from these experiments were recorded as a percentage of explants producing no organogenesis (NO), roots (R), shoots (S) or roots plus shoots (R+S). These responses were recorded at three different time periods (4 weeks, 8 weeks and 12 weeks), after inoculation. In addition to this information, the number of shoots produced on each replicate was recorded. Thus the total number of shoots produced per treatment could be calculated and the most prolific shoot producing treatment could be determined. Further information obtained from this experiment was, the rate at which shoots were produced *in vitro* from corm explants. These data were eventually used to calculate the theoretical number of plants which could be produced from corm explants, within a year.

To assist in this calculation, it was necessary to obtain information on the influence of the physiological status of the mother plant, upon *in vitro* plantlet formation. To achieve this, a standardised culture treatment was imposed upon corm explants at various times of the year (March, May, July and September). This treatment involved the culturing of corm explants, in a random position, on a culture medium supplemented with 30 $g\ell^{-1}$ sucrose, 1,0 $g\ell^{-1}$ casein hydrolysate and 0,001 $g\ell^{-1}$ BA the best shoot inducing hormonal treatment for corm explants). Observations were made 12 weeks after inoculation and the results obtained were recorded in a similar manner as the above experiment.

Finally, to achieve the rooting and "hardening-off" of *in vitro* developed shoots and plantlets, from corm explants, the following technique was found to be most successful:

1. Explants producing shoots only, were subcultured onto a hormone-free or low NAA (0,001 $g\ell^{-1}$) culture medium.
2. Once the shoots had rooted (usually after a period of 6 weeks), the plantlets were removed from the culture vessels and the agar washed from the roots. At this stage, fully developed plantlets, (i.e. those which had required no subculturing for rooting to be induced), were combined with the subcultured stock of plantlets.

3. All plants were then planted into small plastic pots containing river sand.
4. The pots were maintained in a 60 per cent shade-house for 2 weeks, during which period they were kept moist.
5. They were then transferred to a 10 per cent shade-house, for a further 2 week period. While under these conditions, they were watered weekly.
6. Following this procedure the plants were sufficiently hardy to be planted into their natural environment.

2.2.10 Anatomical investigations

In addition to the experimentation on the development of an *in vitro* regeneration technique for *H. rooperi*, various anatomical investigations were also undertaken. The purpose of these studies were;

1. to assist in the design of the afore mentioned experiments, and
2. to elucidate the results obtained in these experiments.

Various types of microtechniques were used to obtain material for viewing with the light and electron microscopes. Each of these techniques are briefly described below. The major reference source from which these techniques were adapted was O'BRIEN and McCULLY (1981).

Freezing microtechnique. Material sectioned using the freeze microtome, was affixed to the microtome chuck with a warm gelatinous mixture. This was then solidified and the material frozen with carbon dioxide (CO_2) gas. Sections were then taken, dehydrated and stained with Safranin and Fast Green (JENSEN, 1962). They were then mounted in 50 per cent glycerin and studied using an Olympus BH 2 light microscope.

Wax embedding microtechnique. For this routine material was initially fixed at room temperature (± 23 °C), in forma-acetic acid-alcohol (SASS, 1958). After 48 hours this solution was removed and the fixed tissue dehydrated in a tertiary butanol series, followed by a triple wash in pure tertiary butanol. Impregnation of the tissue with wax was achieved by adding increasing amounts of wax pellets to the final tertiary butanol wash. This procedure was performed at a temperature of 59 °C, over a

period of 24 hours. For final wax infiltration the tissues were immersed twice each for a period of 12 hours, in molten wax. Finally they were embedded in wax, in "paper boat" moulds. Sections were cut with a Jung hand-microtome. These were mounted on glass slides and stained with Safranin and Fast Green (JENSEN, 1962). They were then mounted in Haupt's adhesive before being examined on an Olympus BH 2 light microscope.

Resin embedding microtechnique. To obtain resin embedded material the tissues of interest were initially fixed at 4 °C in 6,0 per cent glutaraldehyde, buffered at pH 6,0 with 0,05 mol ℓ^{-1} sodium cacodylate. After at least 24 hours, this solution was removed and the fixed material rinsed in three 30 minute washes of 0,05 mol ℓ^{-1} sodium cacodylate buffer. The material was then post-fixed for 3 hours in 2,0 per cent osmium tetroxide, also buffered with 0,05 mol ℓ^{-1} sodium cacodylate. This was followed by a further three 30 minute washes in the buffer alone. Once all traces of osmium tetroxide were removed, the material was dehydrated in an alcohol series, followed by a double wash in pure propylene oxide. Impregnation of the tissues with epon resin was achieved by adding increasing amounts of resin to subsequent propylene oxide washes. For final impregnation, the tissues were placed in pure resin for 12 hours. Polymerization lasted 48 hours at 70 °C.

Sections for both light and electron microscopy were cut with glass and diamond knives respectively. The monitor sections cut for light microscopy were stained using toluidine blue. These sections were photographed after being examined with an Olympus BH 2 microscope. The sections for electron microscopy were stained with uranyl acetate and lead citrate, as described by REYNOLDS (1963). These sections were examined with a Joel 100 CX electron microscope, at an accelerating voltage of 80 kV, and then photographed.

Scanning electron microtechnique. Specimens for scanning electron microscopy were fixed in 6,0 per cent glutaraldehyde, buffered at pH 6,0 with 0,05 mol ℓ^{-1} sodium cacodylate, for 24 hours. They were then dehydrated in an alcohol series and critical point dried. The specimens were then mounted on specimen stubs and coated with a 800 nm thick layer of gold. These were then viewed with a Joel JSM-T200 scanning electron microscope.

2.3 RESULTS AND DISCUSSION

2.3.1 Sterilization investigations

Plants are inevitably contaminated with a wide range of micro-organisms. The retention of viable contaminants in association with plant material in contact with a nutrient medium, rapidly leads to the proliferation of these pathogens. This in turn seriously affects the growth of the explant. Therefore, the need for complete asepsis is a vital prerequisite for the successful *in vitro* culture of plant material. The first experiment undertaken in this investigation was thus, to establish a suitable sterilization technique for the various types of *H. rooperi* material to be cultured *in vitro*. The results of this experiment are presented in Tables 2.5a,b, c, and d. These data show that each type of explant requires a different sterilization procedure.

Of all the types of *H. rooperi* explants cultured, the leaves proved to be the most difficult to sterilize, as this material was readily damaged by the sterilant. Fifty-four per cent of the leaf explants were freed from contamination when rinsed in 1,75 per cent NaOCl for 10 minutes (Table 2.5a). Attempts to improve this percentage by increasing the sterilant concentration from 1,75 to 3,5 per cent NaOCl and 0,1 to 0,5 per cent $HgCl_2$, were unsuccessful (Table 2.5a and b). Maintenance of the explants in a lower concentration of sterilant for a longer period of time, was also unsuccessful. At 3,5 per cent NaOCl for 15 minutes and 0,5 per cent $HgCl_2$ for 0,25 minutes, all leaf explants although sterile, were "killed". For the inflorescence peduncle explants, the washing of the material in 3,5 per cent NaOCl for 15 minutes, was sufficient to free a relatively large percentage of explants from pathogen contamination (Table 2.5a). Higher asepsis could perhaps have been obtained if the material had been soaked in this solution for a longer period of time.

Although $HgCl_2$ is reputed to be difficult to remove from explants after sterilization (YEOMAN, 1973), this chemical proved to be a better sterilant than NaOCl, for *H. rooperi* flower bud explants (Table 2.5a and b). Eight-five per cent of the explants could be sterilized by rinsing them in 0,1 per cent $HgCl_2$ for 0,25 minutes. An increase in $HgCl_2$ concentration (from 0,1 to 0,5 per cent) proved to be counterproductive as this procedure, although resulting in a high percentage asepsis, destroyed all the explants. Similarly to flower buds, the sterilization of corm explants

Table 2.5: The percentage of *H. rooperi* explants surviving and sterile 4 weeks after inoculation. (Values in parentheses represent the number of successfully sterilized explants versus the number of replicates used per treatment).

Table 2.5a: The percentage of *H. rooperi* flower bud, inflorescence peduncle and leaf explants successfully sterilized with NaOCl.

STERILANT	STERILANT CONCENTRATION (per cent)	LENGTH OF STERILIZATION (minutes)	EXPLANT SOURCE		
			FLOWER BUD	INFLORESCENCE PEDUNCLE	LEAF
NaOCl	1,75	10	60 $(\frac{12}{20})$	21 $(\frac{5}{24})$	(54) $(\frac{13}{24})$
		15	75 $(\frac{15}{20})$	33 $(\frac{8}{24})$	33 $(\frac{8}{24})$
	3,5	10	75 $(\frac{15}{20})$	50 $(\frac{12}{24})$	46 $(\frac{11}{24})$
		15	50 $(\frac{10}{20})$	(66) $(\frac{16}{24})$	0 $(\frac{0}{24})$

Table 2.5b: The percentage of *H. rooperi* flower bud, inflorescence peduncle and leaf explants successfully sterilized with $HgCl_2$.

STERILANT	STERILANT CONCENTRATION (per cent)	LENGTH OF STERILIZATION (minutes)	EXPLANT SOURCE		
			FLOWER BUD	INFLORESCENCE PEDUNCLE	LEAF
$HgCl_2$	0,1	0,16	80 $(\frac{16}{20})$	42 $(\frac{10}{24})$	33 $(\frac{8}{24})$
		0,25	(85) $(\frac{17}{20})$	29 $(\frac{5}{24})$	33 $(\frac{8}{24})$
	0,5	0,16	0 $(\frac{0}{20})$	0 $(\frac{0}{24})$	25 $(\frac{6}{24})$
		0,25	0 $(\frac{0}{20})$	0 $(\frac{0}{24})$	0 $(\frac{0}{24})$

Table 2.5c: The percentage of *H. rooperi* corm explants successfully sterilized with NaOCl.

STERILANT	STERILANT CONCENTRATION (per cent)	LENGTH OF STERILIZATION (minutes)	EXPLANT SOURCE
			CORM
NaOCl	1,75	10	50 $(\frac{12}{24})$
		15	58 $(\frac{14}{24})$
	3,5	10	54 $(\frac{13}{24})$
		15	63 $(\frac{15}{24})$

Table 2.5d: The percentage of *H. rooperi* corm explants successfully sterilized with $HgCl_2$.

STERILANT	CONCEN-TRATION (per cent)	LENGTH OF STERILIZATION (minutes)	EXPLANT SOURCE
			CORM
$HgCl_2$	0,1	0,16	65 $(\frac{13}{20})$
		0,5	70 $(\frac{14}{20})$
		1,0	70 $(\frac{14}{20})$
		3,0	80 $(\frac{16}{20})$
		5,0	(95) $(\frac{19}{20})$
	0,5	0,16	60 $(\frac{12}{20})$
		0,5	40 $(\frac{8}{20})$

was more effective using $HgCl_2$ rather than NaOCl (Table 2.5c and d). However, unlike the flower bud sterilization procedure, corm explants required a much longer rinse in $HgCl_2$ for the sterilant to produce a high percentage asepsis. Ninety-five per cent of the explants could be freed from pathogens if the material was soaked in 0,1 per cent $HgCl_2$ for 5 minutes. Of all the procedures attempted to obtain aseptic cultures of *H. rooperi* explants, the most successful regimes are circled in Tables 2.5a, b, c and d. These regimes were routinely employed in subsequent *in vitro* treatments.

2.3.2 Hormonal investigations

The induction of morphogenesis from an explant of a plant occurs when the sterile material is brought into contact with a nutrient medium. The basis of all nutrient media is a mixture of mineral salts combining the essential macro-nutrients and micro-nutrients together with a source of carbon (usually sucrose). Very few plant tissues can however be established or maintained on such a simple medium. For the majority of tissues various additives are essential, particularly plant growth regulators (EVANS, SHARP and FLICK, 1981). There is considerable variability between taxa and between genotypes in the levels and types of plant growth hormones required for morphogenesis (HUSSEY, 1975a; HUGHES, 1981). Appropriate auxin and cytokinin levels must be determined for each species under study. Even different sources within the same plant may vary in their auxin and cytokinin requirements (PETRU and LANDA, 1974; HUGHES, 1981). For these reasons the next experiment conducted was to determine suitable hormone conditions for inducing morphogenesis (particularly shoot formation), from the various *H. rooperi* explants selected for analysis.

The first hormonal treatment was designed to investigate the response of flower bud, inflorescence peduncle and leaf explants on a nutrient medium supplemented with 2,4-D and BA. Results obtained in this experiment are listed in Tables 2.6a, b and c. From these results, it is clear that the flower buds were the most responsive type of explant material (Table 2.6a). The culturing of buds on a medium supplemented with 0 to 0,0005 $g\ell^{-1}$ 2,4-D and 0,002 to 0,005 $g\ell^{-1}$ BA resulted in callus formation. From this callus, adventitious shoots eventually developed.

Table 2.6: The morphogenetic response of *H. rooperi* flower bud explants (Table a), inflorescence peduncle explants (Table b) and leaf explants (Table c), 6 weeks after inoculation, as influenced by 2,4-D and/or BA. Each treatment comprised of 10 replicates.

Table a: Flower bud explant response.

BA CONCENTRATION ($g\ell^{-1}$)	2,4-D CONCENTRATION ($g\ell^{-1}$)				
	0	0,0005	0,001	0,002	0,004
0	NO	C	NO	NO	NO
0,002	C/S	C/S	C/R	C	NO
0,005	C/S	C/S	C	NO	NO
0,01	NO	NO	NO	NO	NO

NO = no organogenesis
C = callus formation
C/S = callus and shoot formation
C/R = callus and root formation

Table b: Inflorescence peduncle explant response.

BA CONCENTRATION ($g\ell^{-1}$)	2,4-D CONCENTRATION ($g\ell^{-1}$)				
	0	0,0005	0,001	0,002	0,004
0	NO	NO	NO	NO	NO
0,002	C	NO	C	NO	NO
0,005	NO	NO	NO	NO	NO
0,01	NO	NO	NO	NO	NO

Table c: Leaf explant response.

BA CONCENTRATION ($g\ell^{-1}$)	2,4-D CONCENTRATION ($g\ell^{-1}$)				
	0	0,0005	0,001	0,002	0,004
0	NO	NO	NO	NO	NO
0,002	NO	NO	NO	NO	NO
0,005	NO	NO	NO	NO	NO
0,01	NO	NO	NO	NO	NO

When the flower buds were incubated on a culture medium containing 0,001 $g\ell^{-1}$ 2,4-D and 0,002 $g\ell^{-1}$ BA, callus and indirect adventitious roots were induced to form. Supplementation of the culture medium with 0,004 $g\ell^{-1}$ 2,4-D and/or 0,01 $g\ell^{-1}$ BA inhibited morphogenesis of the buds, indicating that these hormone concentrations may have been supraoptimal. From the inflorescence peduncle explants, callus was induced to form (at the cut surface of the segments) in only two hormonal treatments (Table 2.6b). No *in vitro* response was obtained from the leaf explants (Table 2.6c). This very poor response may have been obtained due to the presence of 2,4-D in the culture medium.

Certain researchers have tended to avoid using the synthetic auxin 2,4-D because it is thought to cause chromosomal aberrations, polyploidy and severe suppression of organ differentiation (GRESSHOFF, 1978; STREET, 1979). MURASHIGE (1974) stated that 2,4-D stimulated callus production *in vitro* but antagonised organogenesis. This is particularly true amongst the Gramineae. In a number of species in this group of plants, callus could be induced to form if 2,4-D was added to the culture medium. However, for plant regeneration to occur, the 2,4-D had to be removed from the medium (EVANS, SHARP and FLICK, 1981). It has also been shown that in monocotyledonous tissues 2,4-D is predominantly metabolised into a physiologically inactive glucoside derivative, whereas in dicotyledonous tissues this is replaced by physiologically active amino acid conjugates (FEUNG, HAMILTON and MUMMA, 1976, 1977). For these reasons the use of 2,4-D in this study was discontinued.

In an attempt to find a suitable auxin to replace 2,4-D, an investigation was undertaken to determine the influence of BA on the *in vitro* response of flower bud, inflorescence peduncle and leaf explants. Tables 2.7a, b and c list the types of growth response obtained in this experiment. As with the previous experiment, this investigation indicated that the flower bud explants were the most responsive in culture (Table 2.7a). Callus and indirect adventitious shoots (which shall from now on be referred to as shoots) were induced to differentiate from this material, within 5 weeks of the explants being placed on a culture medium supplemented with 0,001 $g\ell^{-1}$ NAA and 0,001 or 0,005 $g\ell^{-1}$ BA. Although both these hormone treatments resulted in the same morphogenetic response, the combination of 0,001 $g\ell^{-1}$ NAA with 0,005 $g\ell^{-1}$ BA induced a larger proportion of the explants to respond *in vitro*. This response occurred from the flower buds (once the perianth segments had reflexed back slightly), at the junction between the segments and the inferior ovary (Plate 2.3b).

Table 2.7: The morphogenetic response of *H. rooperi* flower bud explants (Table a), inflorescence peduncle explants (Table b) and leaf explants (Table c), 6 weeks after inoculation, as influenced by NAA and/or BA. Each treatment comprised of 10 replicates.

Table a: Flower bud explants response.

BA CONCENTRATION ($g\ell^{-1}$)	NAA CONCENTRATION ($g\ell^{-1}$)				
	0	0,0005	0,001	0,005	0,01
0	NO	NO	C	NO	NO
0,001	NO	NO	C/S	C	NO
0,005	C	C	C/S	NO	NO
0,01	NO	NO	NO	NO	NO

Table b: Inflorescence peduncle explant response.

BA CONCENTRATION ($g\ell^{-1}$)	NAA CONCENTRATION ($g\ell^{-1}$)				
	0	0,0005	0,001	0,005	0,01
0	C	NO	NO	NO	NO
0,001	C	NO	C	NO	NO
0,005	C	NO	NO	NO	NO
0,01	NO	NO	NO	NO	NO

NO = no organogenesis

C = callus formation

C/S = callus and shoot formation

Table c: Leaf explant response.

BA CONCENTRATION ($g\ell^{-1}$)	NAA CONCENTRATION ($g\ell^{-1}$)				
	0	0,0005	0,001	0,005	0,01
0	NO	NO	NO	NO	NO
0,001	NO	NO	NO	NO	NO
0,005	NO	NO	NO	NO	NO
0,01	NO	NO	NO	NO	NO

In this investigation, although the number of treatments resulting in both callus and shoot production were less than in the 2,4-D and/or BA experiment, the amount of callus and shoots produced per explant was much higher. Also the concentration of auxin required to induce callusing and shooting was higher (0,001 $g\ell^{-1}$ NAA as opposed to 0,0005 $g\ell^{-1}$ 2,4-D. This was expected as 2,4-D is regarded as being a more potent auxin than NAA (SEABROOK, 1980). What was however most unusual, was the auxin to cytokinin ratio necessary for inducing callus formation from *H. rooperi* flower buds. In general most monocotyledonous plants require a high auxin to cytokinin ratio, or merely the presence of auxin for callus formation to be induced (EVANS, SHARP and FLICK, 1981). In contrast to this general trend however, a few examples can be cited in which an equivalent amount of auxin and cytokinin (HEUSER and APPS, 1976; YAKUWA, HARADA and TSUJI, 1982), or a higher cytokinin to auxin ratio (GROENEWALD, WESSELS and KOELEMAN, 1977; PIERIK, STEEGMANS and VAN DER MEYS, 1974) induces callus formation.

Once again a very poor morphogenetic response was obtained from the inflorescence peduncle explants (Table 2.7b) and no morphogenesis could be induced from leaf explants (Table 2.7c). These results are unusual as inflorescence stalks and leaves are frequently used as an explant source for regenerating monocotyledons *in vitro* (ZIV, HALEVY and SHILO, 1970; KAUL and SABHARWAL, 1972; BAJAJ and PIERIK, 1974; MEYER, FUCHIGAMI and ROBERTS, 1975; HUSSEY, 1975a; MATHEWS and RANGAN, 1979; HOSOKI and ASAHIRA, 1980a; HUSSEY, 1982a; NAKAMURA and KELLER, 1982b; BEYL and SHARMA, 1983), whilst the reversal of floral explants to the vegetative condition is extremely rare (KONAR and KITCHLUE, 1982). The decision made following these results was to discontinue using inflorescence peduncles and leaves as a source of explant material and to concentrate more on improving the *in vitro* regeneration technique for *H. rooperi*, from flower buds. Results of these investigations are recorded later on in the text (section 2.3.3).

Realizing that *H. rooperi* flower buds are seasonal in their availability, analyses were continued in search of a more readily available source from which to obtain explants. It was for this reason that investigations on the *in vitro* response of *H. rooperi* corm explants were embarked upon. Most *in vitro* studies utilizing the storage organ of monocotyledonous plants have involved plantlet propagation via bulb explants (HUSSEY, 1975a; SIMMONDS and CUMMING, 1976; HUSSEY, 1982a; STEINITZ and YAHEL, 1982). There have been very few reports in which corm material have been used. Therefore, our understanding of the requirements for corm culture is limited. According to HUSSEY (1975a), small pieces of *Freesia*, *Gladiolus* and *Sparaxis* corms could be induced to form adventitious plantlets in response to low concentrations of NAA (3×10^{-5} to $1{,}2 \times 10^{-4}$ $g\ell^{-1}$).

Bearing this information in mind, an experiment was performed in which the influence of NAA and/or BA upon *H. rooperi* corm explants was analysed. In this investigation and all further *in vitro* analyses using corm material, only explants excised from region 2 (Figure 2.3) were utilized. Explants obtained from regions 1 and 3 produced no response in culture. The types of morphogenetic response of region 2 explants once inoculated *in vitro*, are summarized in Table 2.8. These results indicate that

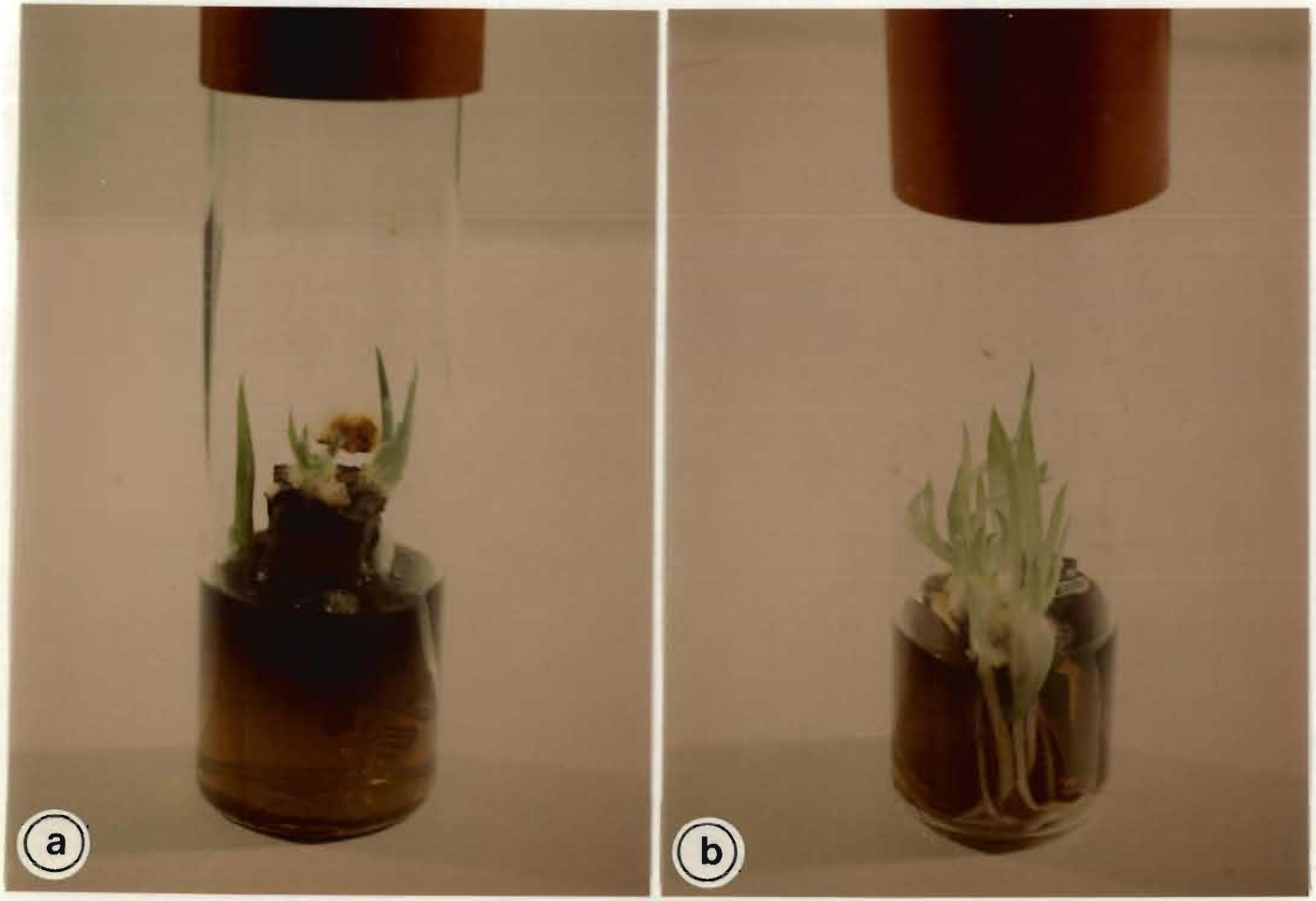


Plate 2.4: (a) Shoots initiated from a *H. rooperi* corm explant cultured on a medium supplemented with 0,0005 or 0,001 $g\ell^{-1}$ BA x 1,5.

(b) Shoot and root formation from a *H. rooperi* corm explant which has been maintained on a 0,0005 or 0,001 $g\ell^{-1}$ BA supplemented medium for a period of 10 weeks. x 1,5.

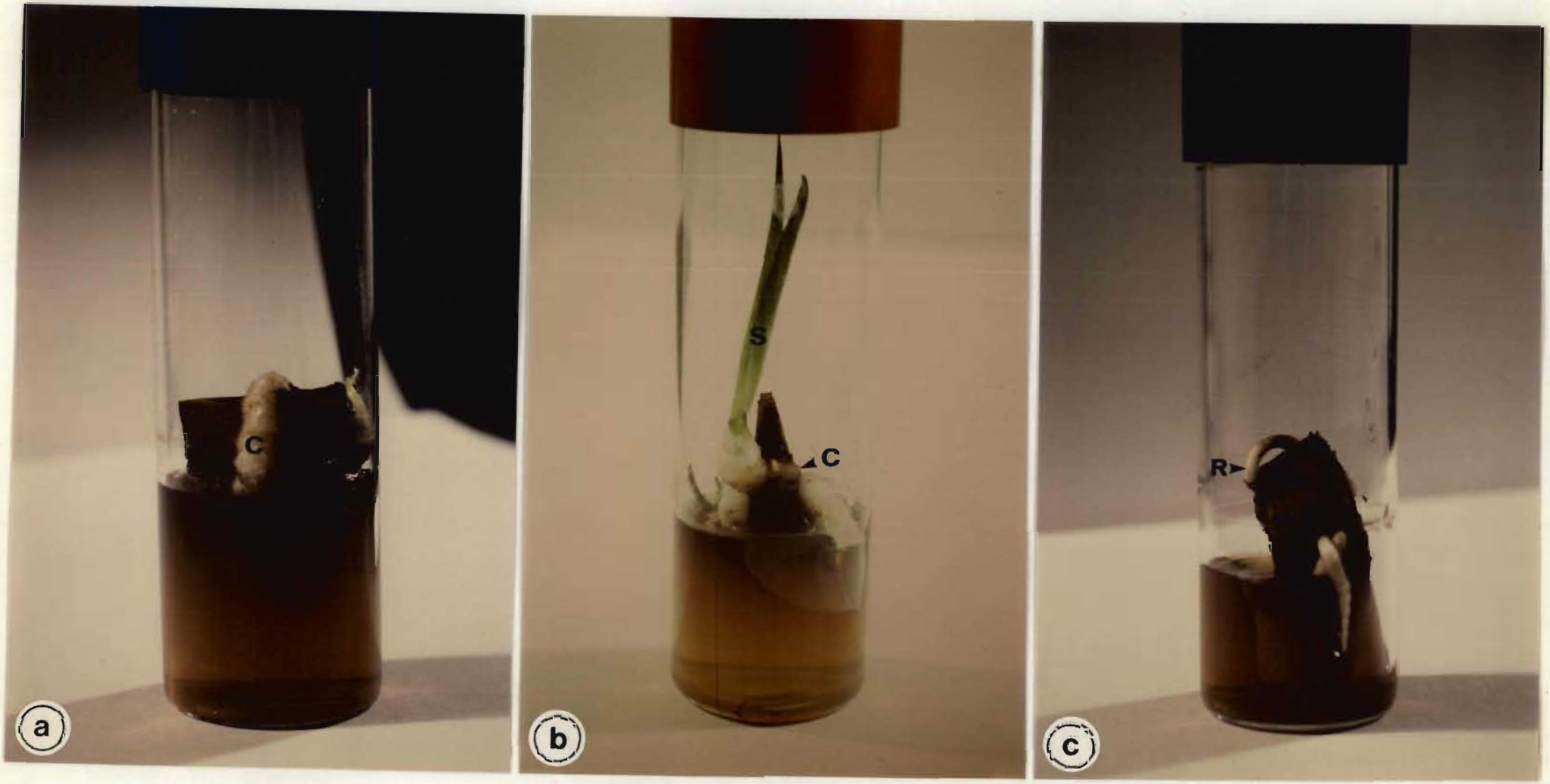


Plate 2.5: (a) Callus (C) proliferation from a *H. rooperi* corm explant. x 1,6.
(b) Shoot (S) differentiation from a *H. rooperi* corm explant. C = callus. x 1,4.
(c) Root (R) differentiation from a *H. rooperi* corm explant. x 1,4.

2.3.3 Flower investigations

The *in vitro* culture of flower buds and ovaries was introduced as early as 1942 by LARUE. These explants have been used mainly to study floral morphogenesis and *in vitro* flowering (KONAR and KITCHLUE, 1982). Buds in culture may not always follow a sequential development into flowers and occasionally they may revert to callus formation or a vegetative condition. In most circumstances callus, root and shoot formation occurs from the flower pedical or inflorescence peduncle. It is extremely unusual to find these morphogenetic responses occurring from the flower *per se*. Amongst monocotyledons the induction of callus, roots and shoots directly from flower buds, has been reported to occur in approximately 10 per cent of all genera cultured from this group of plants. These include: *Allium cepa* (DUNSTON and SHORT, 1979a) , *Haworthia* (MAJUMDAR, 1970; KAUL and SABHARWAL, 1972), *Hyacinthus* (HUSSEY, 1975a, 1975b), *Ipheion* Rafin (HUSSEY, 1975a), *Muscari botryoides* Mill. (HUSSEY, 1975a), *Narcissus* L. (HUSSEY, 1975a; SEABROOK, CUMMING and DIONNE, 1976; HOSOKI and ASAHIRA, 1980a), *Ornithogalum thrysoides* Jacq. (HUSSEY, 1975a, 1976c), *Scilla sibrica* Haw. (HUSSEY, 1975a) and *Tradescantia reflexa* Rafin and *T. paludosa* (KONAR and KITCHLUE, 1982). Reports have also been made on the proliferation of adventitious shoots from flower buds of *Freesia* (PIERIK and STEEGMANS, 1975b) and callus from denuded flowers of *Gladiolus* (BAJAJ, SIDHU and GILL, 1983). However, in the latter two investigations the precise location of the morphogenetic responses were not mentioned.

As a result of the rarity of the reversion of flower buds to the vegetative condition, the observation that *H. rooperi* flower buds respond in this manner *in vitro*, is academically significant. For this reason, further investigations were undertaken using this material to determine whether bud age influenced the *in vitro* response of this organ. Results obtained from this analysis indicated that of the three categories of buds cultured, only those buds which had not yet flowered but were more than 1,0 cm in length, produced callus and shoots once inoculated *in vitro*. In these buds, the ovary locules were found to contain very small, white ovules and the anthers which had not yet dehisced, contained mature pollen grains. The only response of young buds *in vitro* was a slight increase

in size, while older buds produced no response at all. Whether buds of these various ages usually react in this manner *in vitro*, is unknown.

Most of the reports on the culturing of monocotyledonous flower buds, do not specify the age of buds used. For example, HUSSEY (1976c) merely recorded that ovaries were dissected from unopened flower buds, while PIERIK and STEEGMANS (1975b) stated that young buds in which the corolla was not or just visible, were utilized in their investigation. DUNSTON and SHORT (1979a) were more precise, and reported that shoots could be produced from flower-heads of *Allium cepa* until the meiotic stage in pollen formation of their anthers. In *Haworthia turgida* Haw. cultures, MAJUMDAR (1970) noted that the cells of the ovary walls of flowers excised before anthesis, could be induced to develop shoots and callus. Whether ovary wall tissue of *Haworthia* flowers of other ages would have demonstrated such totipotency, is not recorded. Callusing obtained by KAUL and SABHARWAL (1972), from the base of the ovary wall of *Haworthia* were from gynoecia found to be empty in most cases. In two pollinated gynoecia of *Haworthia variegata* (L.) Bolus, fruit maturation and seed formation occurred. This information suggests that once pollination has taken place, flower bud tissue cannot revert back to the vegetative condition. The results obtained in the present investigation support this view, as old flower buds of *H. rooperi* produced no response *in vitro*.

In search of a reason as to why intermediate flower buds of *H. rooperi* had the ability to revert back to the vegetative condition, anatomical investigations were undertaken on cultured and uncultured buds of this age. The results obtained indicated that in cultured flower buds the region of callus production (Plate 2.3b), was closely related to the site of active cell division in uncultured buds (Plate 2.6). It is believed that in the latter buds, division was occurring in these cells in preparation for the reflexing back of the perianth segments. The only other region in uncultured buds in which such division was observed to be taking place, was in the ovules. It would therefore appear that the flower bud tissue located between the stamen filaments and the perianth segments, is the region which is induced to revert back to the callusing condition, once the buds are placed into culture. Anatomical investigation of cultured flower buds certainly indicated a close association between this region and the callus induced to develop from this organ (Plate 2.7).

Plate 2.6: (a) Longitudinal section through a *H. rooperi* flower bud which is more than 1,0 cm in length, and has not yet flowered. A = anther, L = ovary locule, O = ovary, o = ovule, P = perianth segment, s = style. x 13.

(b) Enlargement of the area encircled in 2.6a. x 175.

(c) Enlargement of the area encircled in 2.6b, showing the region of active cell division from which callus and shoot proliferation is believed to arise. DN = dividing nucleus, N = nucleus. x 1320.

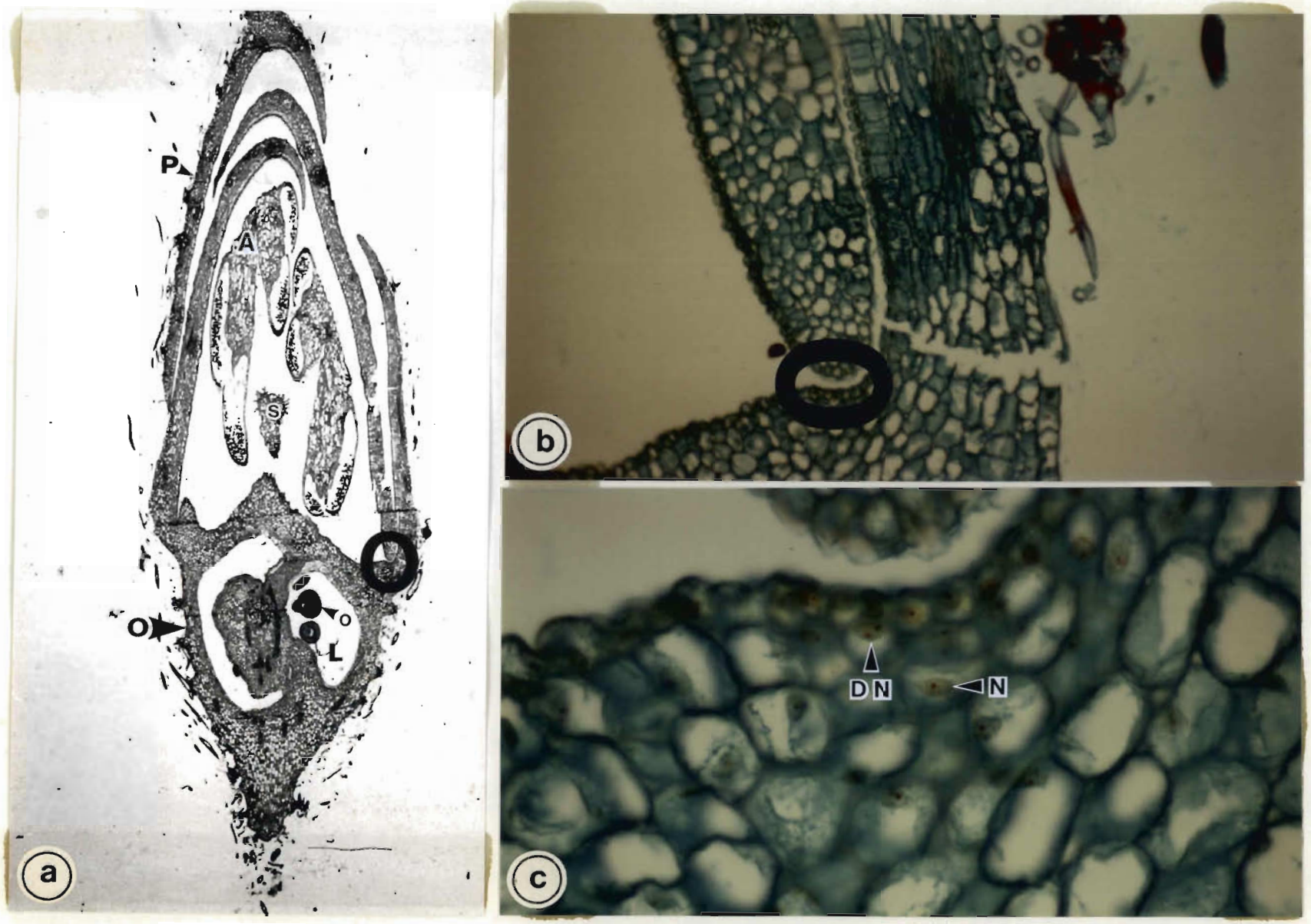
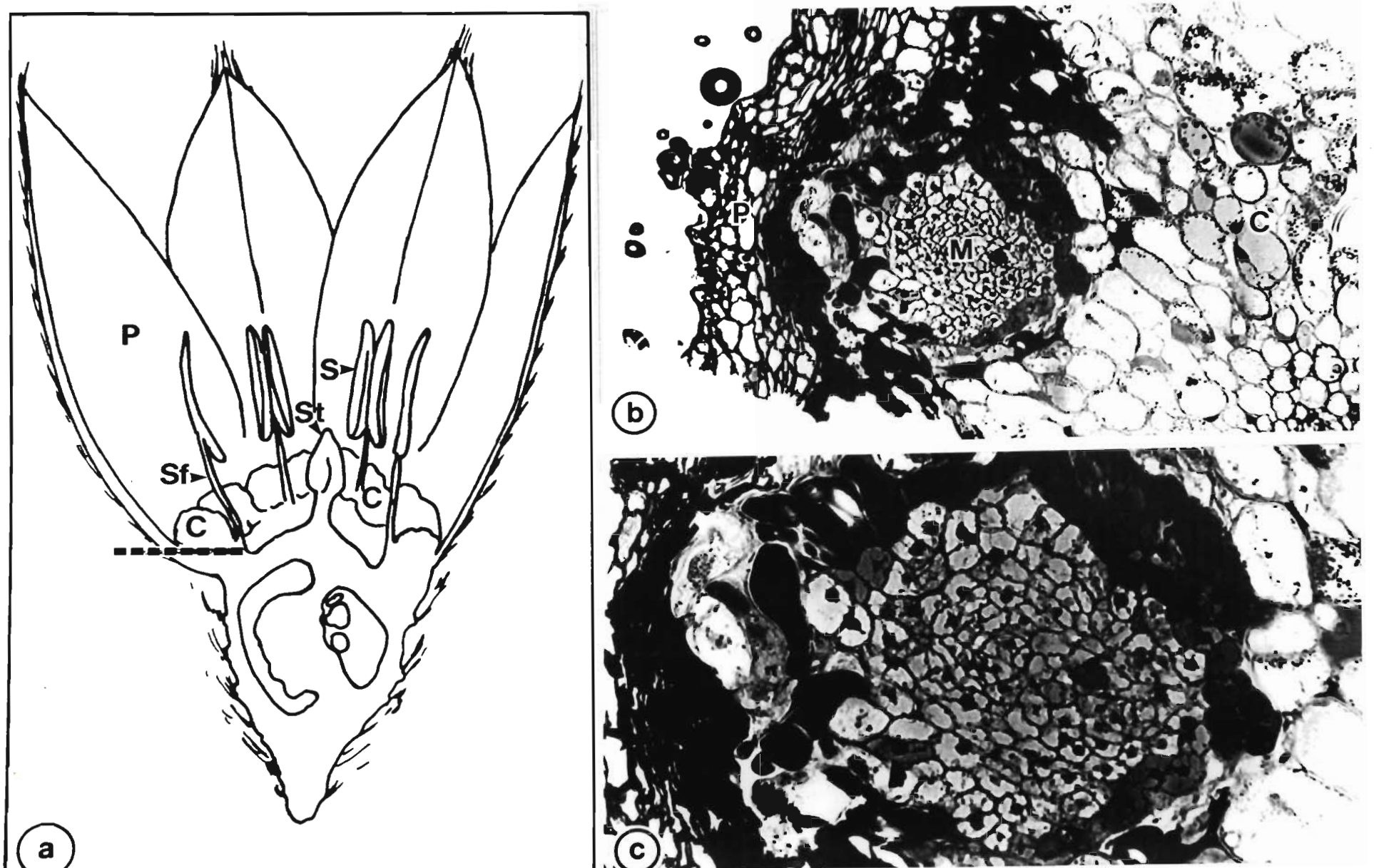

P
A
S
O
L
a
b
DN
N
c

Plate 2.7: (a) Diagram of a longitudinal section through a callusing cultured *H. rooperi* flower bud explant. C = callus, P = perianth segment, S = stamen, Sf = stamen filament, St = style. x 11,6.

(b) Transverse section through the region indicated by the broken line in 2.7a. C = callus, P = perianth segment, M = meristemoid. x 85.

(c) Enlargement of the meristematic region in 2.7b. x 170.


P
S
St
Sf
C
C
a
P
M
C
b
c

A number of workers have commented on the role of a wound reaction in the early stages of callus formation (YEOMAN, 1970; YEOMAN and AITCHISON, 1973; THOMAS and DAVEY, 1975). DUNSTON and SHORT (1979a) noted a predominance of shooting from wounded flower-head explants of *Allium cepa*. They suggested that a wound cambium may be responsible for the production of the shoots. A similar response to wounding was noted for *Gerbera* capitulum explants (PIERIK, STEEGMANS and MARELIS, 1973). In view of this information, an analysis of the effect of wounding upon flower buds of *H. rooperi* was undertaken. Results obtained in this investigation indicated that damaged buds (i.e. ones in which the perianth segments, stamens and style had been excised) (Plate 2.1b), responded more rapidly *in vitro*, than undamaged buds (Plate 2.1a). Callus and shoots were induced to form from damaged buds within 4 weeks, whereas whole buds only produced these morphogenetic responses 5 weeks after inoculation *in vitro*. The amount of callus produced from both bud types, was however not significantly different.

Following this investigation, an analysis was undertaken to determine whether bud orientation on the culture medium influenced the response obtained from *H. rooperi* flower buds. In this experiment, damaged flower buds (as described above), were utilized. These buds were placed either basipetally, acropetally or horizontally on the culture medium. From this investigation it was evident that the acropetal positioning of explants on the culture medium, was detrimental for flower bud callus and shoot formation. There was however no siginificant difference between the response obtained from the basipetally and horizontally positioned explants.

Noting that the flower buds of *H. rooperi* now being utilized *in vitro* were of a more specific age category than those initially used in this study, a re-analysis of the sterilization technique and hormone treatments was made. The results obtained for these two analyses are listed in Table 2.9 and 2.10. From the results recorded in Table 2.9 it is clear that the highest percentage asepsis was obtained when the buds were immersed in a 0,1 per cent $HgCl_2$ solution, for a period of 1 to 2 minutes. Under these conditions 90 per cent of the

Table 2.9: The percentage of *H. rooperi* flower bud explants surviving and sterile 4 weeks after inoculation. Each treatment comprised of 10 replicates.

STERILANT	CONCENTRATION (per cent)	LENGTH OF STERILIZATION (minutes)	EXPLANT SOURCE
			FLOWER BUD
$HgCl_2$	0,1	0,16	80
		0,25	80
		0,5	80
		1,0	90
		2,0	90
		5,0	40

explants remained sterile. This represents a 5 per cent increase over the number of explants sterilized in the initial sterilization experiments.

From the results obtained in an experiment on the re-analysis of the hormonal treatments (Table 2.10), it is obvious that the supplementation of the culture medium with BA, was necessary for the flower buds to be induced to form callus and shoots. It is also clear that an increase in BA concentration from 0,001 to 0,005 $g\ell^{-1}$, in combination with NAA (0,001 $g\ell^{-1}$) resulted in an increase in the percentage of explants producing this response. The retention of explants on the initial culture medium for a period of 12 weeks as opposed to 6 weeks, also resulted in an increase in the number of explants induced to callus and shoot. This number could theoretically have been improved upon, if a more precise method of categorizing buds could have been developed. Unfortunately, from a practical point of view this was not possible.

The influence of seasonal variation upon the morphogenetic response of explants, has been documented for a number of monocotyledonous plant cultures (ROBB, 1957; MII, MORI and IWASE, 1974; NARAYANA-SWAMY, 1977). Generally it is believed that the morphogenetic ability of an explant, is limited to periods of donor plant vegetative growth (ROBB, 1957; HUGHES, 1981). For this reason, an investigation of the influence of *H. rooperi* flower bud harvest time upon explant response, was undertaken. Results obtained from this experiment are listed in Table 2.11. These results indicate that there is a slight variation in the number of explants producing callus and shoots, during the flowering season. The highest percentage being recorded during mid-October. By the end of this month however, the numbers had decreased from 41 to 32 per cent. This decrease in percentage may be related to the overall status of the donor plants, in particular the levels of endogenous hormones within these plants. It has been indicated that seasonal changes in the cytokinin levels of *H. rooperi*, are closely related to the growth phases of this plant (PAGE and VAN STADEN, 1982). Finally, from the above experiment it is obvious that using the technique thus far developed for the regeneration

Table 2.10: The morphogenetic response of *H. rooperi* flower bud explants (category 2) as influenced by NAA and/or BA. Each treatment comprised of 72 replicates.

HORMONE CONCENTRATION ($g\ell^{-1}$)		NUMBER OF EXPLANTS STERILE AND SURVIVING (per cent)	NUMBER OF SURVIVING EXPLANTS PRODUCING CALLUS AND SHOOTS (per cent)	
BA	NAA		6 weeks after inoculation	12 weeks after inoculation
0	0,001	91	0	0
0,001	0,001	93	15	22
0,003	0,001	94	26	29
0,005	0,001	94	35	40

Table 2.11: The number of explants producing callus and shoots from *H. rooperi* flower buds (category 2), as influenced by explant harvest time. Each treatment comprised of at least 24 replicates.

EXPLANT HARVEST TIME (date)	NUMBER OF EXPLANTS STERILE AND SURVIVING (per cent)	NUMBER OF SURVIVING EXPLANTS PRODUCING CALLUS AND SHOOTS (per cent)	
		6 weeks after inoculation	12 weeks after inoculation
End-September (29/9/82)	95	35	40
Mid-October (16/10/82)	92	36	41
End-October (27/10/82)	90	27	32
Mid-November (13/11/82)	88	33	37

of *H. rooperi* from flower buds, an average of 37,5 (± 3,96) per cent of the explants can be induced to produce both callus and shoots during the entire flowering season.

2.3.4 Subculturing investigations

Following the development of a technique to induce *H. rooperi* flower buds to produce callus and shoots, the next objective of this study was to maintain the callus in a continuous shoot producing state. Much evidence is available which supports the principle that quantitative shifts in the ratio of plant growth hormones in the culture medium, induces organogenesis (GRESSHOFF, 1978; STREET, 1979). For this reason, it was decided that an analysis would be undertaken to determine whether the manipulation of phytohormones in the medium of *H. rooperi* callus cultures, would induce continuous shooting. Prior to analysing this aspect however, stocks of *H. rooperi* callus had to be increased. A standard method as outlined in section 2.2.8 was used to achieve this. A significant feature of this procedure is that the callus was cultured on a medium containing the same hormone levels as were used to initially induce callus and shoot formation. Once sufficient callus stocks had been established, the tissue was subcultured onto fresh media containing various levels of NAA and/or K or BA. The results obtained in this investigation are listed in Table 2.12. From these results a number of notable aspects were apparent. Firstly, the culturing of callus on the same medium as was used to induce and increase callus stocks (treatment 5), resulted in callus growth but was detrimental for shoot differentiation. The best culture medium for callus division and adventitious shoot formation was one containing 0,001 $g\ell^{-1}$ NAA and 0,001 $g\ell^{-1}$ BA (treatment 4) (Plate 2.8). To induce root formation from callus, it was necessary to transfer the callus onto a hormone-free culture medium (treatment 1). Finally, the supplementation of the culture medium with K (treatments 6, 7 and 8) rather than BA (treatments 9, 10 and 11), produced no marked visible difference in callus response.

Although in this experiment close correlations between hormone treatment and callus organogenesis were observed, it is not clear as to whether these hormones actually induced organogenesis in the

Table 2.12: The morphogenetic response of *H. rooperi* callus as influenced by NAA, BA and K. Each treatment comprised of 10 replicates.

TREATMENT	HORMONE CONCENTRATION ($g\ell^{-1}$)			MORPHOGENETIC RESPONSE
	NAA	K	BA	
1	0	0	0	Limited callus growth. Normal shoots and roots produced.
2	0,001	0	0	Callus growth. Short stubby roots produced.
3	0,001	0,005	0	Callus growth. Few normal shoots produced.
4	0,001	0	0,001	Much callus growth. Many normal shoots produced. Short stubby roots occasionally produced.
5	0,001	0	0,005	Callus growth. Shoots produced, mostly gnarled.
6	0	0,001	0	Limited callus growth. Few normal shoots produced.
7	0	0,003	0	Callus growth. Few normal shoots produced.
8	0	0,005	0	Callus growth. Few normal shoots produced.
9	0	0	0,001	Limited callus growth. Few normal shoots produced.
10	0	0	0,003	Callus growth. Few normal shoots produced.
11	0	0	0,005	Callus growth. Few gnarled shoots produced.

Plate 2.8: Adventitious shoot producing callus developed on a medium containing 0,001 $g\ell^{-1}$ NAA and 0,001 $g\ell^{-1}$ BA. x 1,6.

callus cultures *de novo*, or whether they merely permitted the expression of a process which had already begun during the previous subculture. The reason for expressing the latter view is because;

1. much of the callus used in the above analysis possessed microscopic adventitious shoots (Plate 2.9) prior to being utilized. These shoots were present, as the technique thus far developed in this study had been orientated towards the selection of a shoot producing callus, and

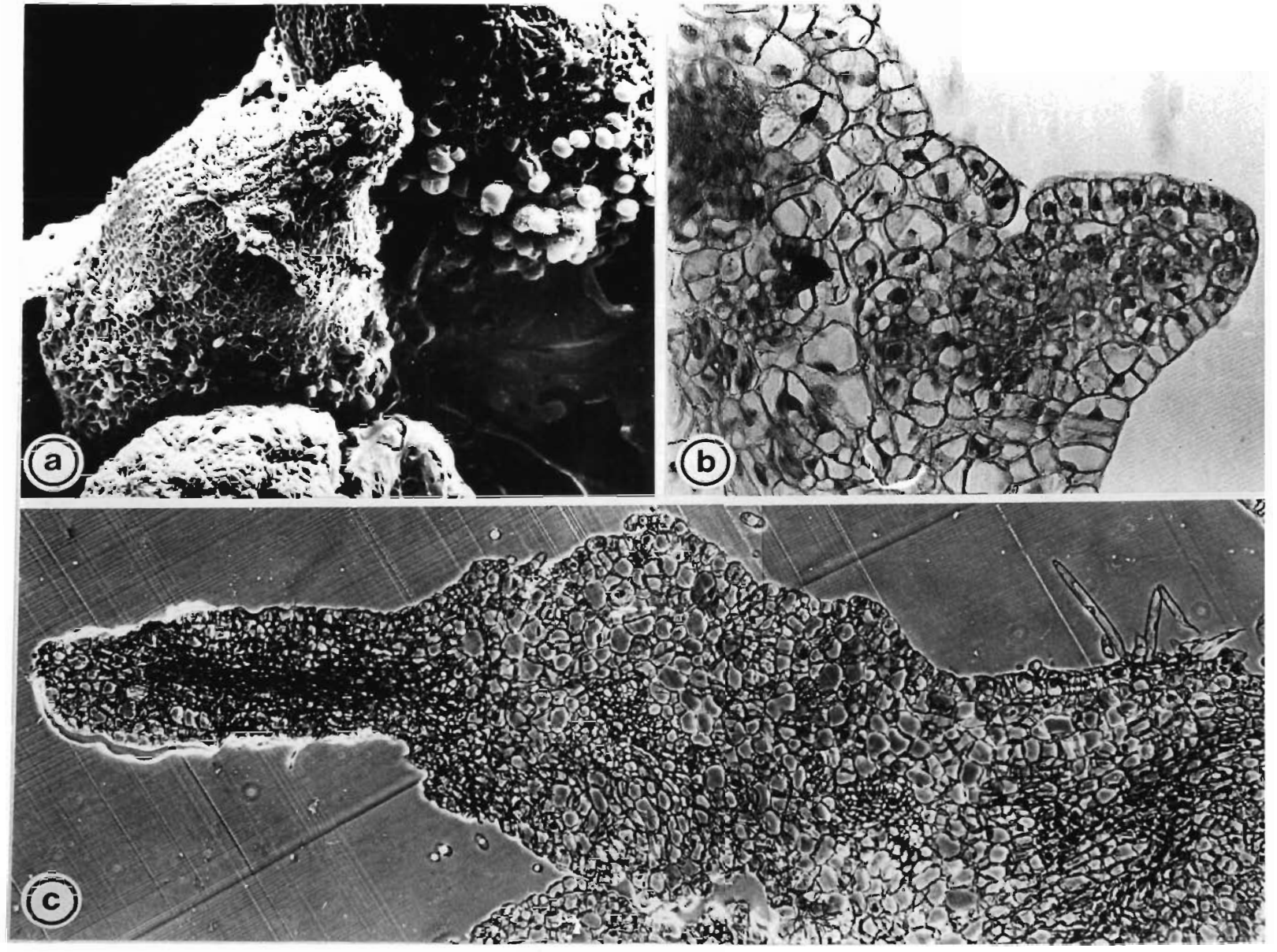
2. the danger of carry-over is especially important when dealing with sequential hormonal treatments. NITSCH (1968) for example found that tobacco pith callus was induced to grow when a cytokinin treatment preceded an auxin treatment. However, the reverse situation did not result in callus growth. This carry-over effect may be the reason why *H. rooperi* callus did not produce normal shoots, when maintained on the same medium as was used to induce and increase callus stocks from the initial flower bud explants. Possibly, by the time the callus was used in the above experiment the levels of cytokinin in the tissue had accumulated to supraoptimal levels from previous subcultures.

There is increasing evidence that extended subculturing of callus or cell suspension cultures can lead to a reduction in morphogenetic potential and an increase in the frequency of aberrant plants arising from the cultures (REINERT, 1973; YEOMAN, 1973; MURASHIGE, 1974; D'AMATO, 1977; GRESSHOFF, 1978; HUGHES, 1981). Loss of morphogenetic potential according to HUGHES (1981) can occur in several ways.

1. Reduced endogenous levels of growth regulators in the cultures may cause loss of potential.

2. Accumulation of chromosome abnormalities may inhibit developmental processes.

3. Shoot production from callus may be initiated from organised centers of cell division (meristemoids) which are derived from the initial explant. These organised centers may gradually be lost through repeated subculturing and rapid unorganised cell division.


a
b
c

In view of this information, and the fact that the main objective of this study was to develop an efficient *in vitro* technique for producing a continuous supply of *H. rooperi* plants for commercial and research activities, an investigation was embarked upon to determine whether *H. rooperi* callus maintained its growth and morphogenetic integrity (particularly its shoot forming ability), following numerous subcultures. As a result of the problems inherent in investigations on callus growth (YEOMAN and AITCHISON, 1973), a necessary prerequisite to this investigation was an analysis of the microscopic structure of *H. rooperi* callus. From this analysis it became apparent that as in most callus material, the area of active cell division was localised in the peripheral region of the callus (YEOMAN and AITCHISON, 1973). In this region the cells were very small and irregular in shape. Meristematic centers could be detected just below the upper callus surface (Plate 2.10a and b) and numerous adventitious shoot initials projected from the surface (Plate 2.10c). Cells towards the interior of the callus were mostly parenchymatous and tended to be more quiescent and much larger. Scattered throughout this central region, vascular differentiation was evident (Plate 2.10d).

Generally therefore, the structure of *H. rooperi* callus was not uniform. For this reason, only tissue from the periphery of the callus was utilized for analyses on the growth rate and maintenance potential of this tissue. Results of these analyses are illustrated in Figure 2.7 and recorded in Table 2.13. From Figure 2.7 it is evident that there is no significant difference between the growth rate of 30 week old and 66 week old callus. Over a six week inoculation period, both ages of callus demonstrated normal growth patterns. With respect to the shoot forming potential of *H. rooperi* callus, results listed in Table 2.13 indicate that both 30 week old and 66 week old callus produced similar total numbers of shoots. The mean number of shoots produced per flask of both ages of callus, was not significantly different. However, the 95 per cent confidence limit values indicate that the 30 week old callus was less variable in the number of shoots produced per flask, than the 66 week old callus. This suggests that the subculturing of *H. rooperi* callus results in an alteration of the shoot producing consistency of the callus. This fact may have been more conclusively proved, if the above investigation had been extended over a larger number of subculturing passages.

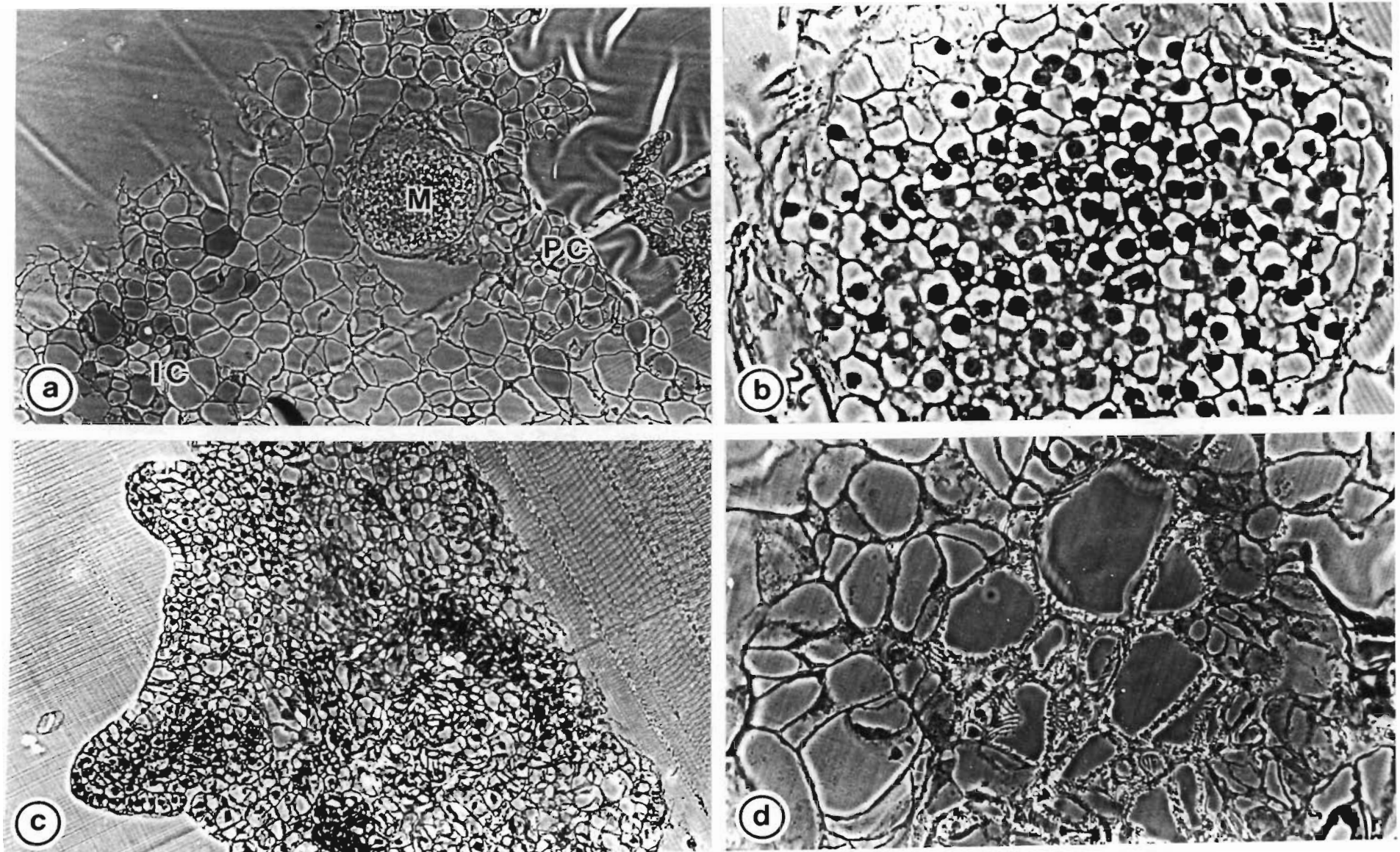
Plate 2.10: Microscopic structure of callus induced to differentiate from *H. rooperi* flower bud explants.

(a) Meristemoid (M) located in the peripheral region of the callus. IC = inner callus, PC = peripheral callus. x 85.

(b) Enlargement of the meristematic centre in 2.10a. x 340.

(c) Adventitious shoot initials projecting from the surface of the callus. x 85.

(d) Tracheal differentiation within the callus tissue. x 340.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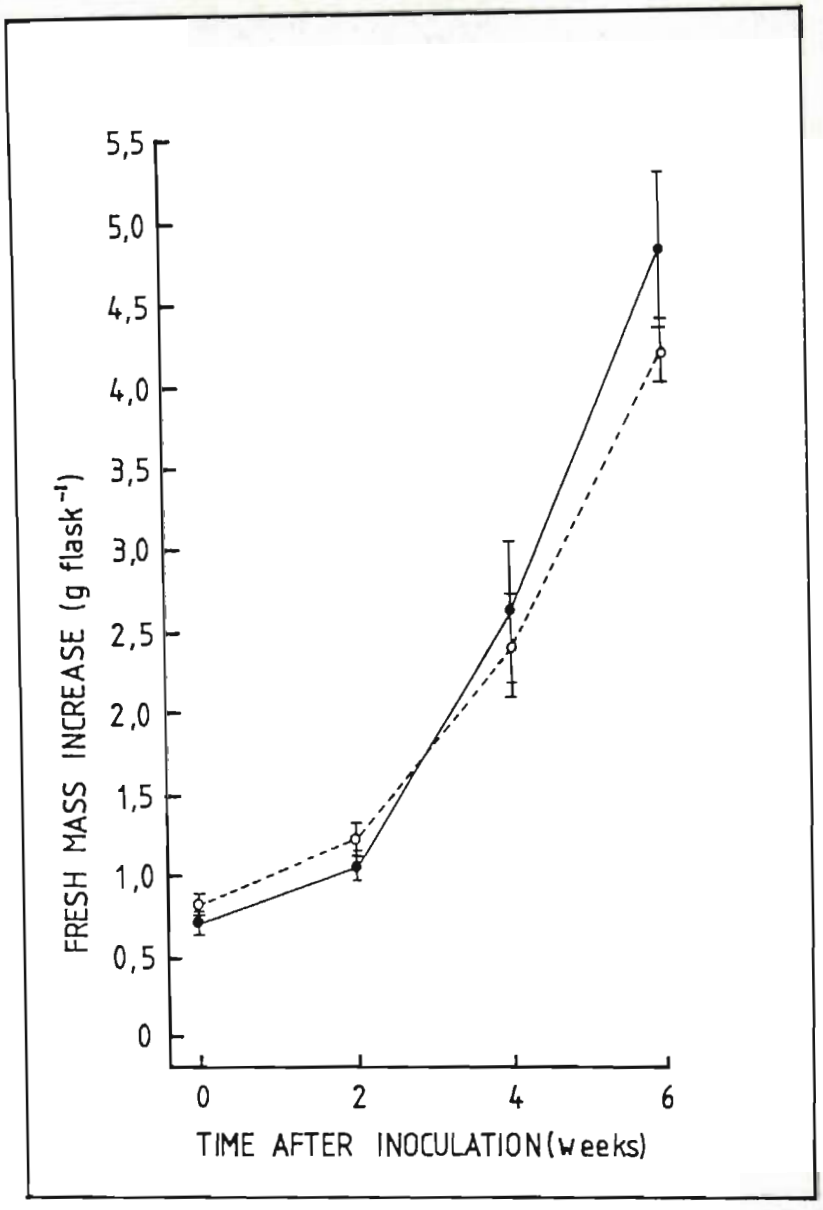


Figure 2.7: Growth rate of 30 week old (o----o) and 66 week old (●——●) callus established from *H. rooperi* flower bud explants.

Table 2.13: Number of shoots produced from 30 week old and 66 week old callus cultures, 6 weeks after inoculation. Each treatment comprised of 10 replicates.

CALLUS AGE (weeks)	TOTAL NUMBER OF SHOOTS PRODUCED	MEAN NUMBER OF SHOOTS PRODUCED PER REPLICATE (i.e. per flask of callus)*
30	279	28 (± 4,77)
66	260	26 (± 9,55)

* (± 95 per cent confidence limit values)

For a tissue culture method of propagation to be successful, a large number of the culture derived plants must be established *in vivo*. In this investigation the rooting of *in vitro* produced *H. rooperi* shoots, was achieved by the transfer of shoots onto a culture medium devoid of plant growth regulators. Roots were usually developed within 4 to 6 weeks. The fully differentiated plantlets were "hardened-off" as described in section 2.2.8. Following this technique, 75 per cent of the plantlets produced *in vitro*, were established *in vivo* (Plate 2.11). Theoretically therefore, using the *in vitro* and "hardening-off" techniques developed in this investigation, it should be possible to regenerate 80 994 plantlets from 100 *H. rooperi* flower buds, within a year.

2.3.5 Corm investigations

As a result of the discovery that *H. rooperi* corms offer an adequate source of material for the micropropagation of this plant (section 2.3.2), various anatomical investigations were undertaken. These were embarked upon to elucidate:

1. why only explants excised from region 2 (Figure 2.3), were responsive *in vitro*, and
2. why all shoots produced from these explants were preceded by callus formation, whilst for root differentiation callusing was not a necessary prerequisite.

Since monocotyledonous plants are distinguished by their lack of a vascular cambium, particularly in mature tissue (PHILIPSON, WARD and BUTTERFIELD, 1971; STEWARD and KRIKORIAN, 1979), this aspect was disregarded as being a reason for callus, root and shoot proliferation from corm explants of *H. rooperi*. Knowing the importance of a wound reaction to callus formation (LIPETZ, 1970; YEOMAN and AITCHISON, 1973; THOMAS and DAVEY, 1975), initial thoughts were that a wound cambium was responsible for the morphogenetic patterns observed to occur from corm explants of *H. rooperi*. However, these views were soon dispelled as anatomical investigations indicated that all corm explants, whether they were excised from region 1, 2 or 3, or whether they responded morphogenetically *in vitro* or not, formed a wound cambium once excised from the mother plant (Plate 2.12a to f).

Plate 2.11: "Hardened-off" plantlet cultured from a *H. rooperi* flower bud explant. x 0,3.

Plate 2.12: (a) Section through a callusing corm explant. C = region of callusing. x 16.

(b) Enlargement of area 1 of 2.12a, indicating more clearly the storied structure of the wound cambium cells. x 63.

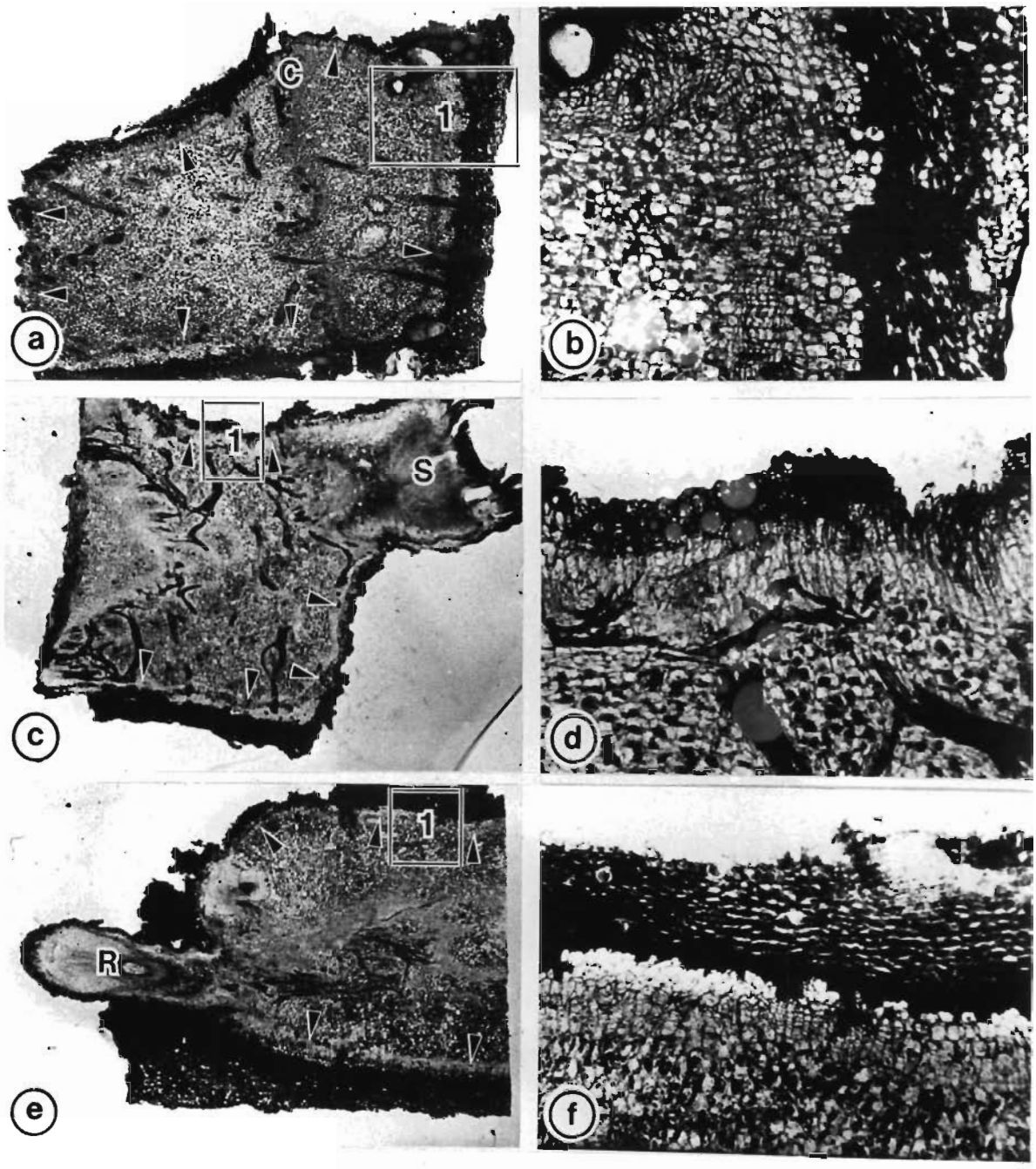
(c) Section through a shooting corm explant. S = shoot. x 16.

(d) Enlargement of area 1 of 2.12c. x 81.

(e) Section through a rooting corm explant. R = root. x 18.

(f) Enlargement of area 1 of 2.12e. x 81.

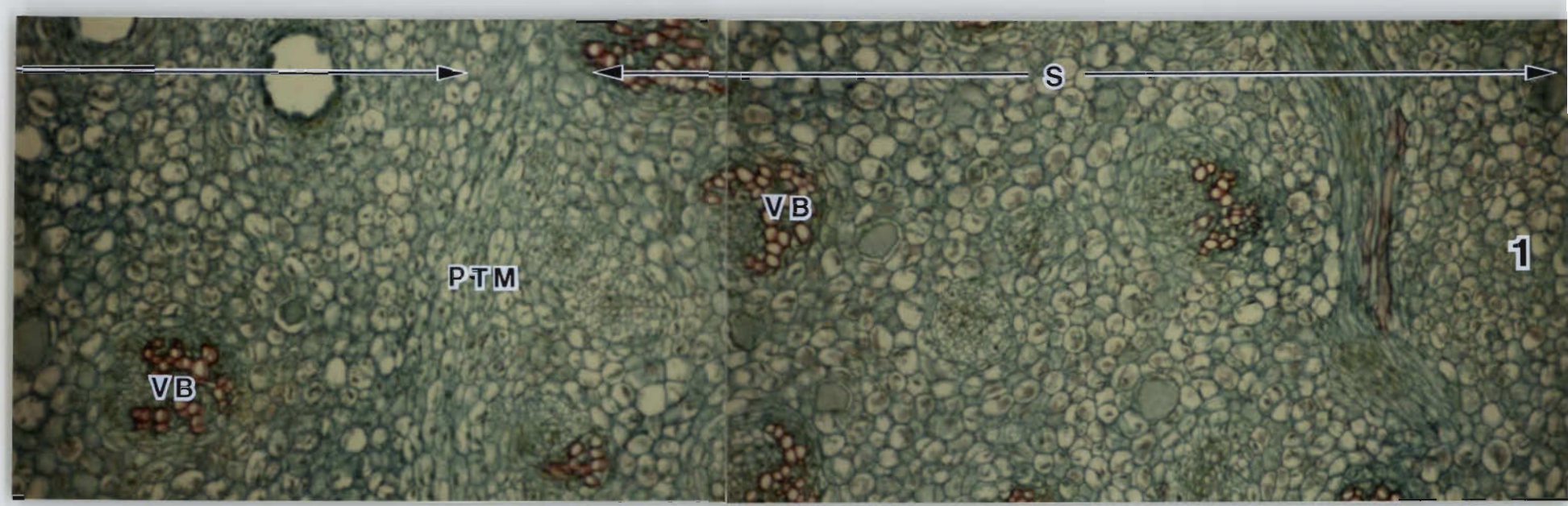
In 2.12a, c and e the arrows indicate the position of the wound cambium.


C
1
a
b
1
S
c
d
1
R
e
f

Following these results, the next analysis to be undertaken was to determine whether a cambium within the explants was responsible for the growth patterns observed. The anatomy of *Hypoxis* corms is an aspect of this plant which has received little attention. ARBER (1925) provided some illustrations on the structure of *H. setosa* Bak. corms, which indicated that the apical meristem is sunk into the corm and that the stele is centrally positioned. She also noted that a procambial mantle, or what has more recently become known as a primary thickening meristem (DE MASON, 1983), is present within the corm. In addition, the parenchyma-producing activity of this cambium was stated to be uniform throughout the length of this organ, except for the tip. This she regarded as being the reason for *H. setosa* corms being more or less cylindrical in shape. An overall plan of the structure of a mature corm of *H. obtusa* has also been presented by HEIDEMAN (1979). In agreement with ARBER (1925), it was indicated that the apical meristem is sunk into the corm and that the stele is centrally located. However no mention was made of the presence of a primary thickening meristem.

As a result of the lack of information concerning the anatomy of *Hypoxis* corms, investigations were undertaken on *H. rooperi* to determine the structure of corms of various ages (1 year, 2 years and mature corms). All material examined was obtained from the upper half of the corms, as this was the region from which explants were excised for *in vitro* experiments. In 1 year old corms (Plate 2.13), the epidermis consisted of a single layer of thin walled cells. Immediately below this a phellogen (or cork cambium) was present. The cortex of the corm was composed of densely packed parenchyma cells, containing starch. Scattered throughout this tissue, numerous mucilage canals, cells containing raphids and vascular bundles were present. Interior to the cortex parenchyma, a primary thickening meristem was present. This meristem completely encircled the centrally located vascular stele. Within the stele, numerous vascular bundles were irregularly dispersed throughout parenchyma tissue.

In 2 year old and mature corms, the overall anatomical plan was similar to 1 year old corms. Differences were however evident in the epidermis, the size of the ground parenchyma cells and their starch content, the size of the mucilage canals, the primary thickening meristem and the vascular bundle structure (Plates 2.14 and 2.15). Of these differences,


S
VB
PTM
VB
1

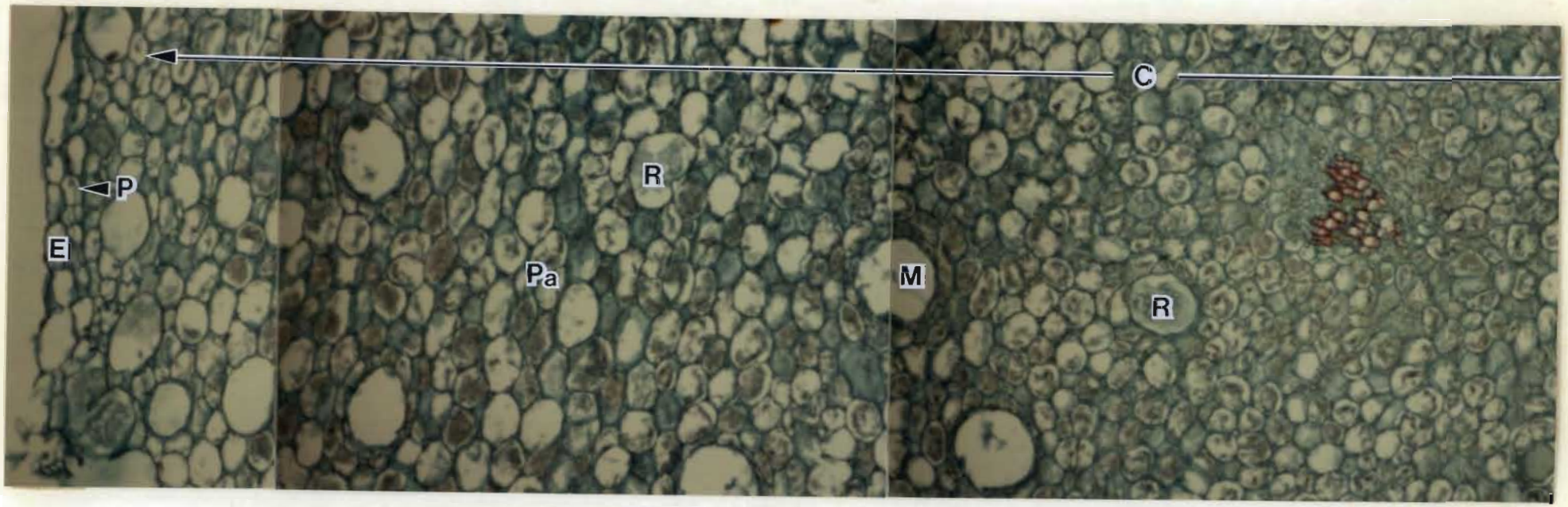


Plate 2.13: Transverse section through a 1 year old *H. rooperi* corm. E = epidermis, P = phellogen, C = cortex, R = cells containing raphids, Pa = parenchyma cells, M = mucilage canals, VB = vascular bundles, PTM = primary thickening meristem, S = central vascular stele containing vascular bundles (VB) and parenchyma cells (Pa). x 90. (1 = the centre of the corm).

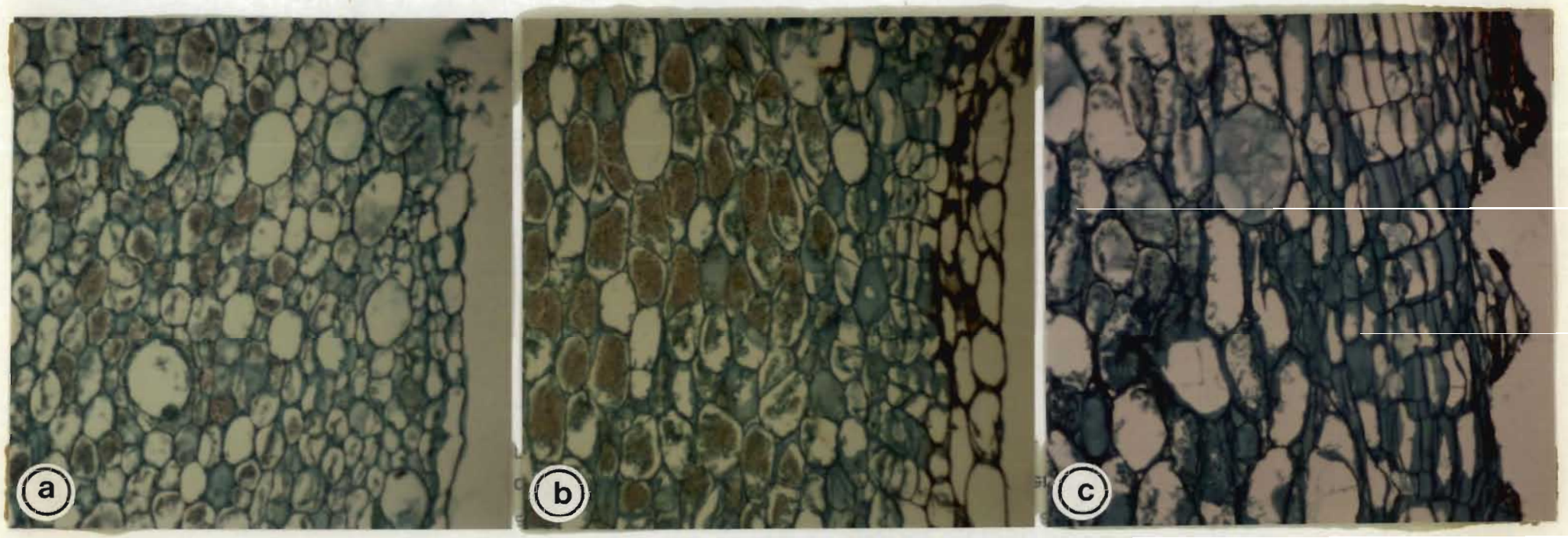
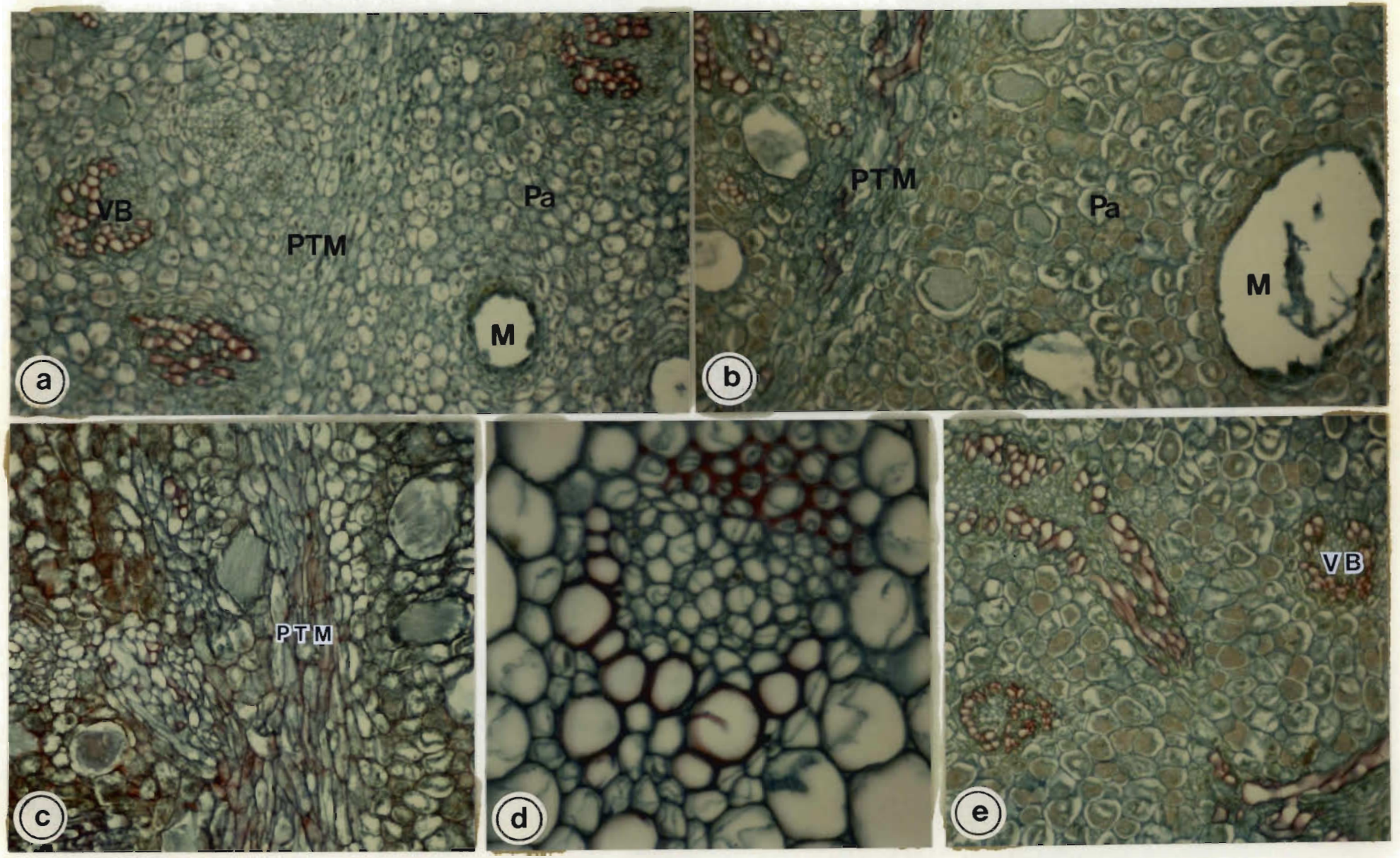


Plate 2.14: Transverse sections through the epidermal region of a 1 year old (a), 2 year old (b) and mature (c) *H. rooperi* corm. Note the difference in the epidermal cells, phellogen derived secondary growth, size of the ground parenchyma cells and amount of starch within these cells. x 90.

Plate 2.15: Transverse sections through the primary thickening meristem region of a 1 year old (a), 2 year old (b) and mature (c) *H. rooperi* corm; a vascular bundle of a mature corm (d) and the stelar region of a 2 year old corm (e). PTM = primary thickening meristem, Pa = parenchyma cells, M = mucilage canals, VB = vascular bundle. (a), (b), (c) and (e) x 90. (d) x 350.


VB
PTM
Pa
M
a
PTM
Pa
M
b
PTM
c
d
VB
e

the change in the structure of the primary thickening meristem was regarded as being particularly significant. The presence of vascular elements scattered amongst the cells of this meristem in older corms (Plate 2.16), implied that the function of this tissue altered as the corms aged. Whether this meristem remained active or acquired a different function in mature corms, could not be determined from the anatomical studies undertaken. From combined *in vitro* and anatomical studies however, it was evident that the primary thickening meristem was the tissue from which morphogenesis occurred (Plate 2.17). Therefore, whether or not the meristem had acquired a different function in older corms, when the tissue was placed into suitable culture conditions (section 2.3.2), it displayed meristematic activity. The reason why only region 2 corm explants responded morphogenetically *in vitro*, was thus obviously because this material contained the primary thickening meristem of the donor plant.

Another important aspect evident from anatomical analyses of uncultured corm material, was that root development occurred within this organ from the primary thickening meristem (Plate 2.18). Therefore, the reason why some corm explants produced roots without initially forming callus, is probably because the root primordia were present within these explants at the time of their excision. Root production may therefore not be directly related to the plant growth regulators present in the culture medium. Alternatively the production of shoots may, as these organs are produced *de novo* from the callus induced to form from the corm explants. Sections through explants of corm material which had produced callus (Plate 2.19a), indicated that the callusing bulge is composed mainly of irregularly shaped parenchyma cells. Scattered throughout this tissue cytodifferentiation was evident (Plate 2.19b and c) and regions of active cell division were present (Plate 2.19d). Analyses of explants at a later stage of development indicated that the callusing bulge eventually differentiated into an organ possessing a corm-like structure (Plate 2.20a) and that it was from this organ that shoots developed (Plate 2.20b). Whether these developmental processes as discussed above occur in other monocotyledonous cultures is unknown because, although numerous *in vitro* studies have been done on this group of plants, they are hardly ever accompanied by any detailed anatomical studies.

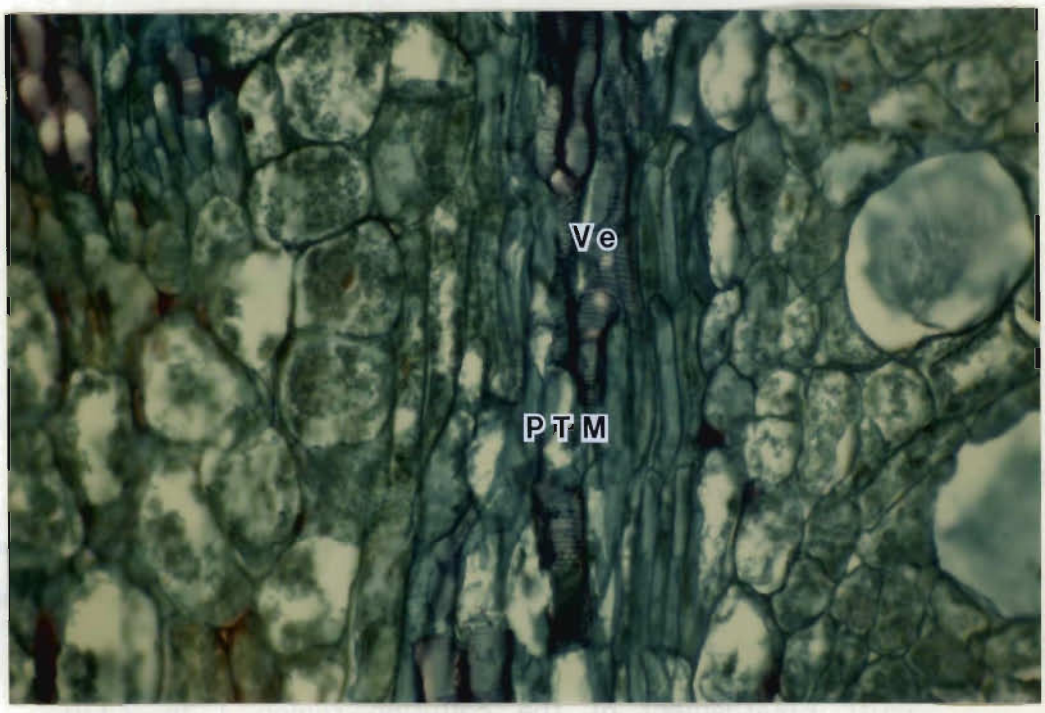
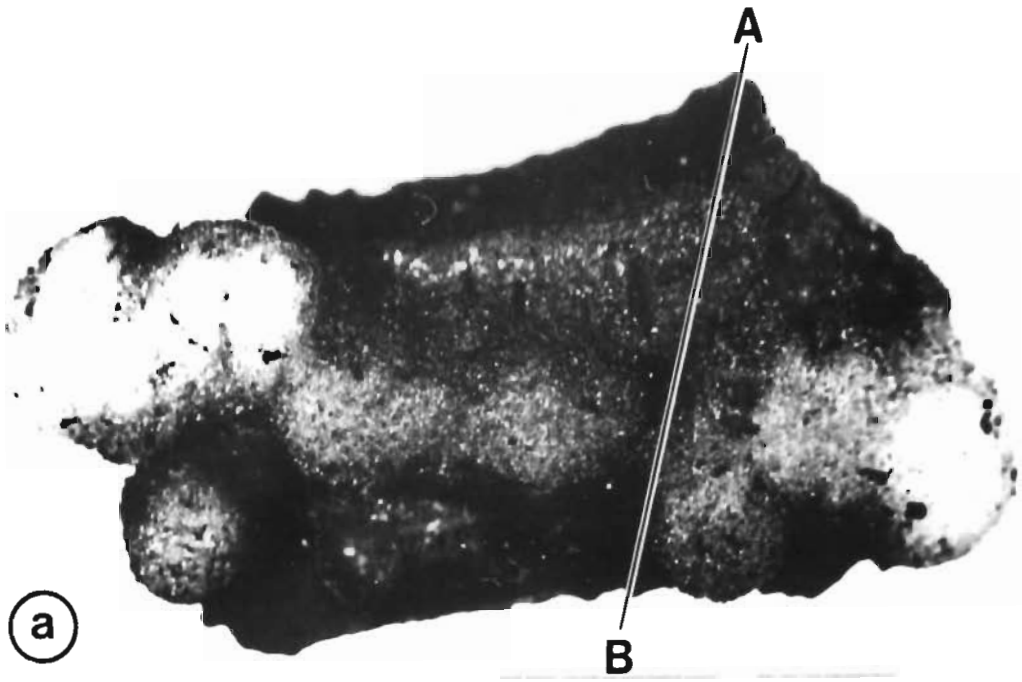


Plate 2.16: Transverse section through the primary thickening meristem (PTM) of a mature *H. rooperi* corm. Note the vascular elements (Ve) scattered throughout this meristem. x 175.

Plate 2.17: (a) Callusing *H. rooperi* corm explant. x 6.

(b) Section through the callusing corm explant along the line indicated in 2.17a. The arrows represent the primary thickening meristem. x 21.

(c) Enlargement of the callusing region 1 in 2.17b. x 110.


A
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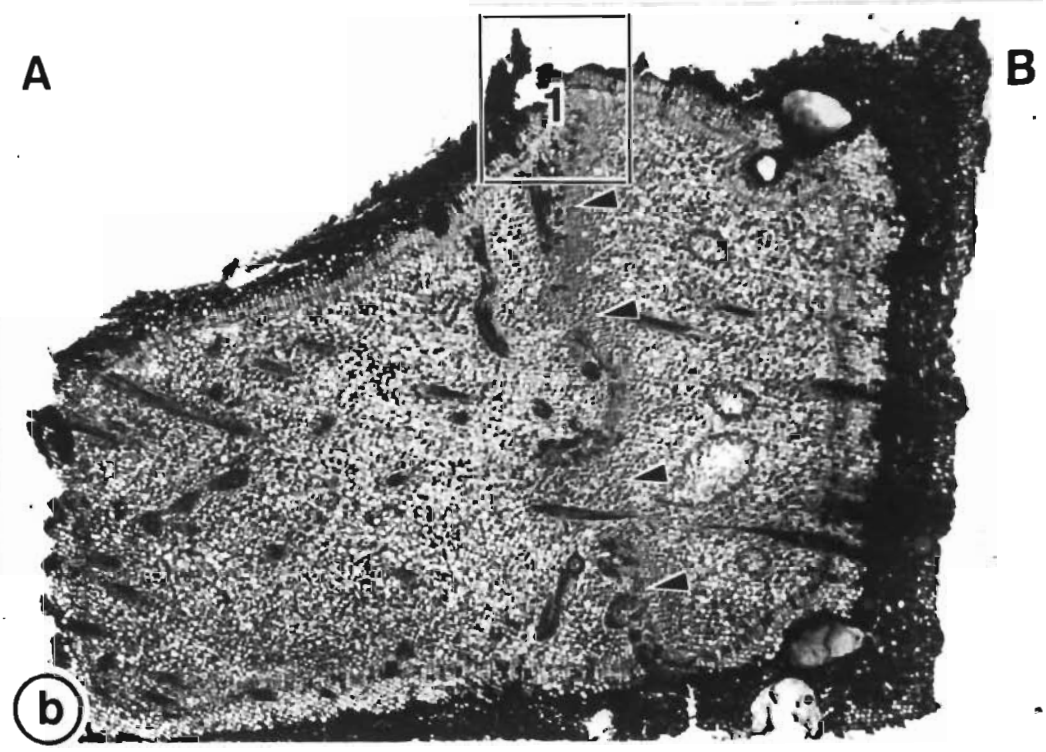
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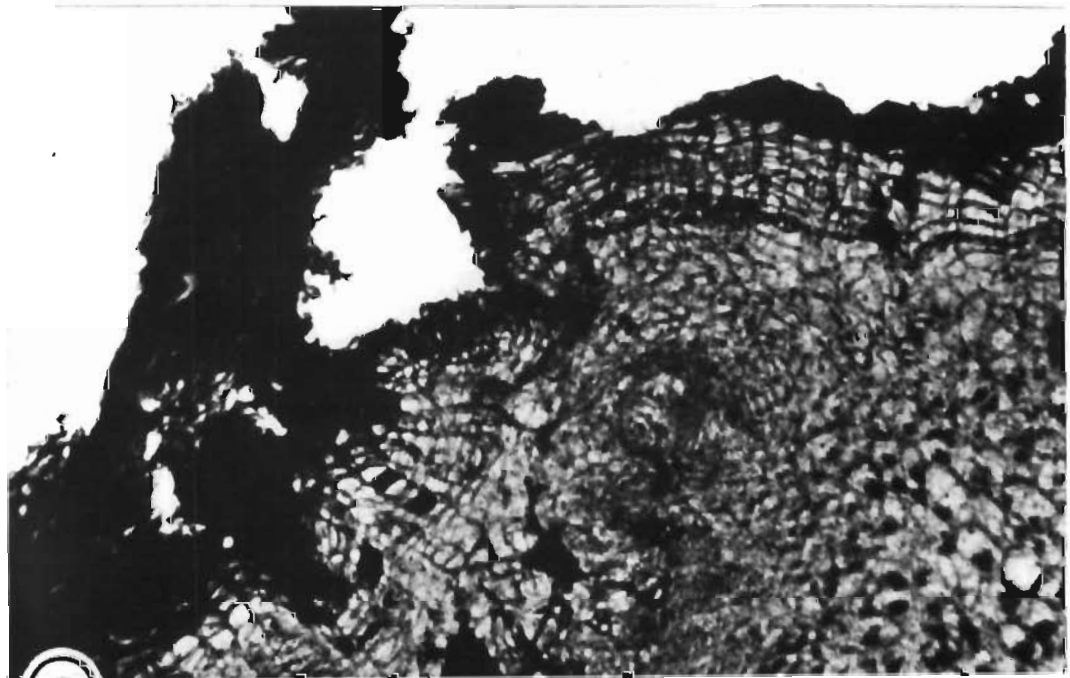


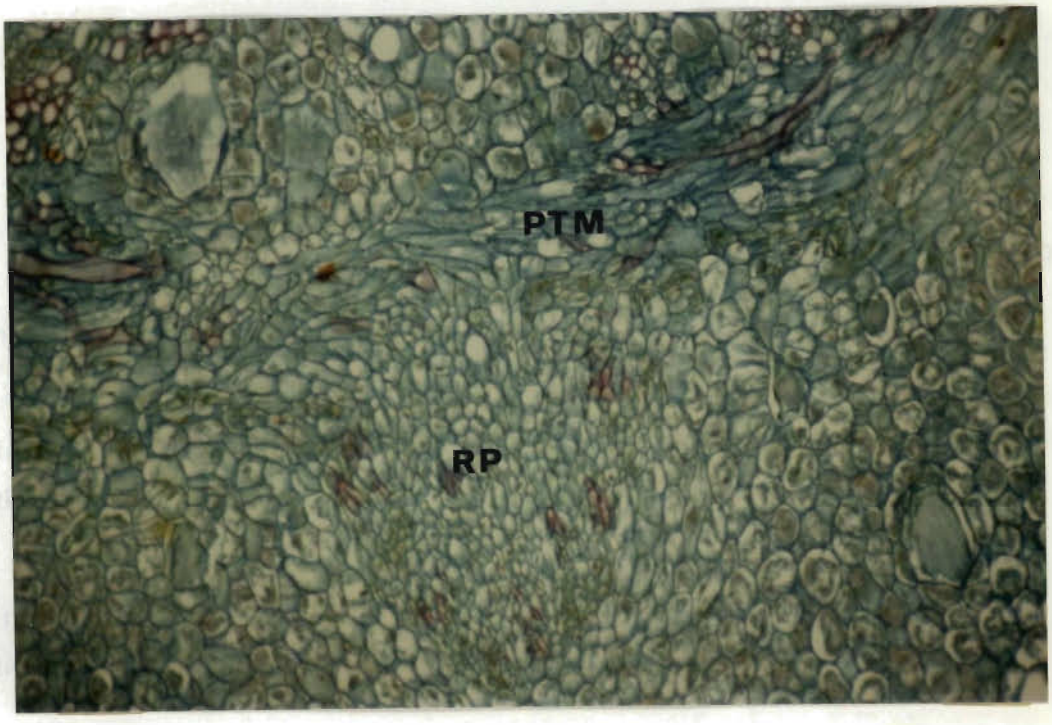


Plate 2.18: Transverse section through the primary thickening meristem (PTM) of a 1 year old *H. rooperi* corm. Note the root primordia (RP) emerging from the PTM. x 90.

Plate 2.19: Insert: Callusing *H. rooperi* corm explant. x 3.

(a) Section through the encircled callusing bulge represented in the insert photograph. x 40.

(b) and (c) Enlargement of areas 1 and 2 respectively of 2.19a, showing regions of vascular differentiation within the callusing bulge. x 170 and 340 respectively.

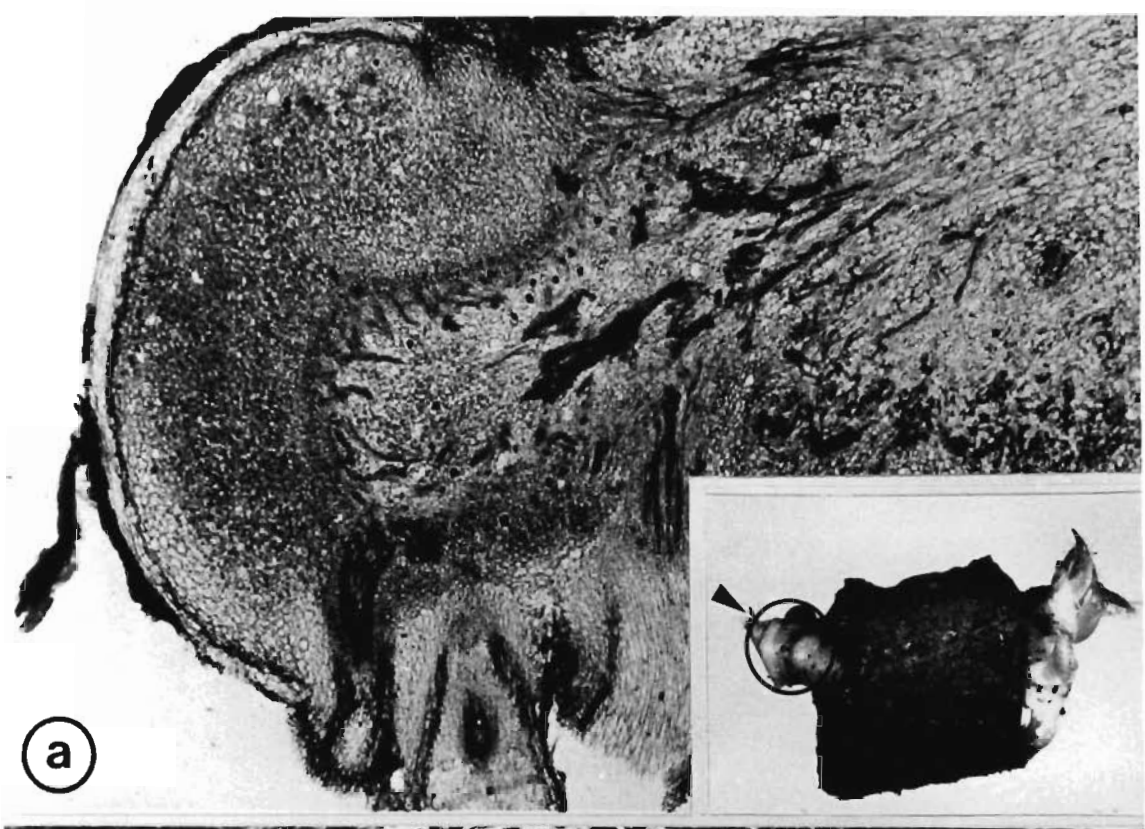
(d) Enlargement of area 3 of 2.19a, showing a region of meristematic activity within the callusing bulge. x 340.

3
2
1
a
b
c
d

Plate 2.20: Insert: Shooting *H. rooperi* corm explant. x 3.

(a) Section through the encircled shoot represented in the insert photograph, showing the differentiation of the callusing bulge into an organ possessing a corm-like structure. x 28.

(b) Scanning electron micrograph of the developing shoot at an angle as indicated by the arrow on the insert photograph. x 35.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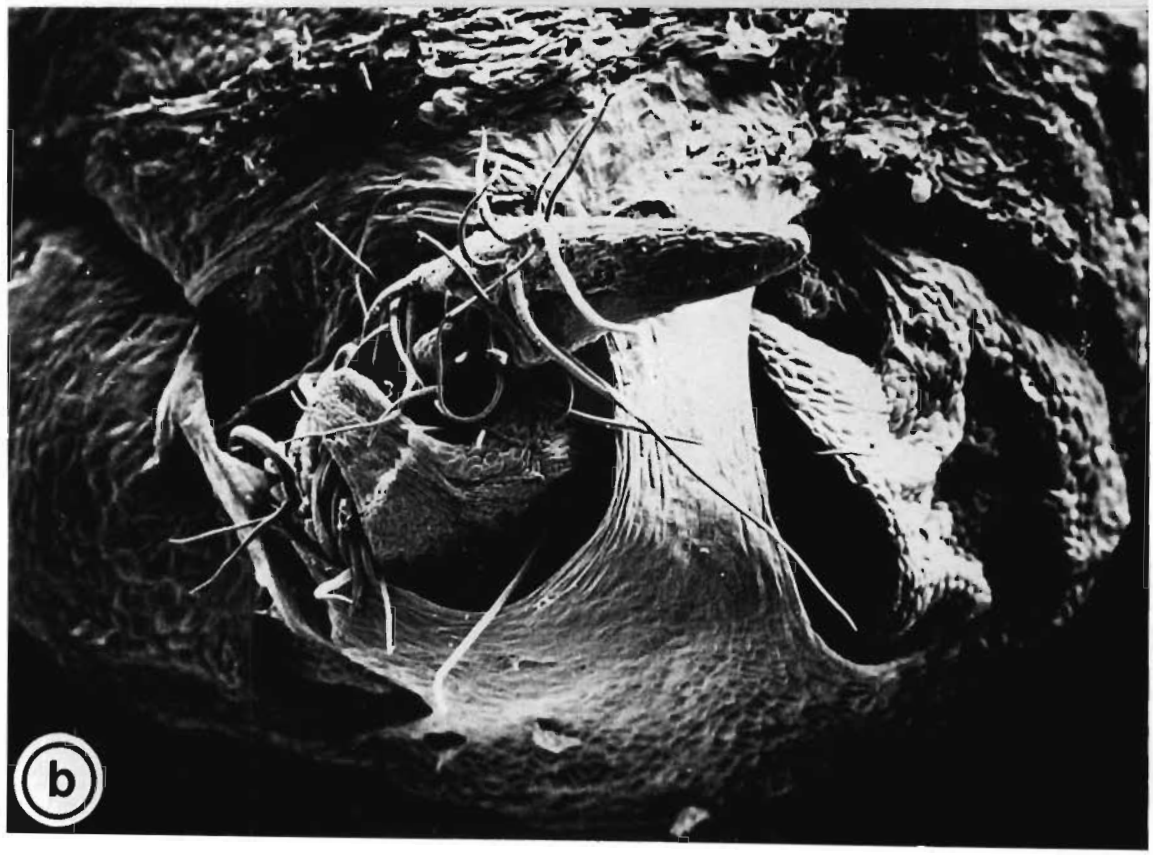
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Following the establishment of how corm explants produce shoots *in vitro*, further investigations were embarked upon to refine the technique of producing shoots from these explants. Aspects which were analysed included the influence of the following factors upon explant response;

1. explant orientation on the basal medium,
2. the presence of casein hydrolysate (1,0 $g\ell^{-1}$) in the culture medium,
3. various levels of sucrose in the culture medium, and
4. explant origin from within the donor plant.

From the explant orientation experiment (Figure 2.8), it is evident that the highest number of corm explants produced shoots (i.e. explants producing shoots and shoots plus roots), when the material was placed in a basal position on the culture medium (Figure 2.8a). The positioning of explants on their side (Figure 2.8b), markedly reduced the number of explants producing shoots, and instead, increased the percentage producing roots. As the rooting response was not of prime concern in this study, this treatment was regarded as being detrimental for shoot production. However, such a system (i.e. the difference between a basal and side orientated explant and their various morphogenetic responses), lends itself to research concerning the effect of endogenous explant factors upon explant response. The need for such research has been expressed by a number of authors (TRAN THANH VAN, 1977; GRESSHOFF, 1978; STREET, 1979). The orientation of explants randomly on the culture medium (Figure 2.8c), an aspect analysed merely from the expediency point of view of this technique, resulted in 65 per cent of the explants producing shoots. This percentage is almost intermediate to that obtained when the explants were orientated basally and on their side. Unfortunately it is 21 per cent lower than that obtained from explants placed in a basal position on the culture medium. Therefore, the use of this procedure merely to increase the production rate of the technique, would not be favourable.

Another important aspect evident from the above experimental results was the rate at which explants produced shoots. For example, when explants were placed in a basal position on the culture medium, the difference between the percentage of explants producing shoots after a 2 month and 3 month inoculation period, was only 10 per cent. These

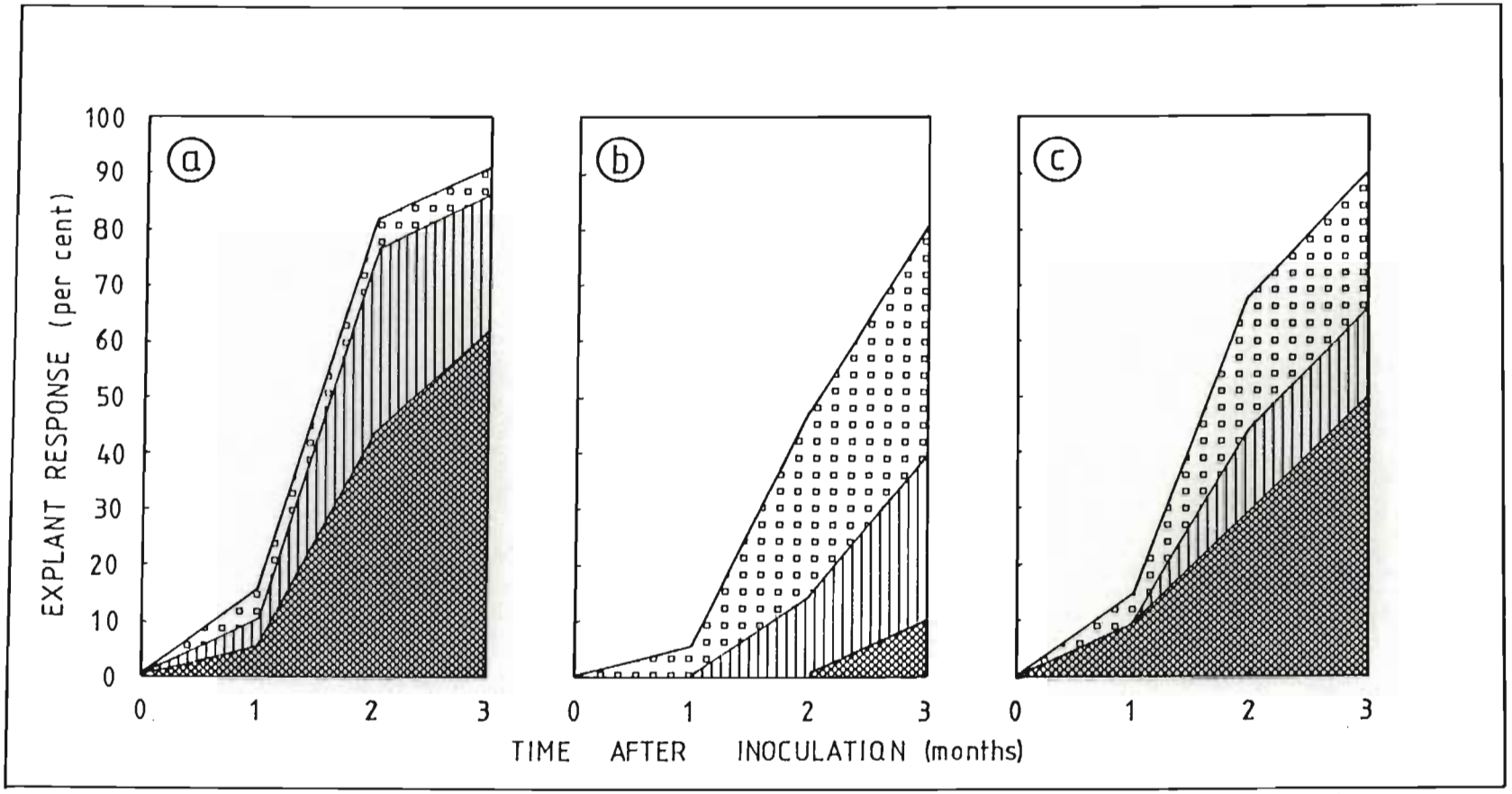


Figure 2.8: The response of *H. rooperi* corm material as influenced by basal (a), side (b) and random (c) orientation of the explants upon the culture medium. = Rooting explants. = Shooting explants. = Explants producing both roots and shoots.

differences were much greater in the other two orientation treatments. This implies that if this technique was commercialized, not much gain would be made in the total number of shoots produced from the explants if they were incubated for 3 months as opposed to 2 months. However, a longer incubation period of basally orientated explants on their initial culture medium, certainly would decrease the ratio of shooted explants which would need to be rooted. For example, after a 2 month incubation period of these explants, 56 per cent of the total number of shooted explants had produced both roots and shoots. Whereas, after a 3 month incubation period, 72 per cent had differentiated into complete plantlets.

Although a longer incubation period resulted in a higher number of fully differentiated plantlets (explants producing both roots and shoots), it decreased the ratio between production time and number of plantlets produced. Bearing in mind that shooted explants take approximately 6 weeks to develop roots once subcultured onto a rooting medium, 76 per cent of the initial number of explants used, can be developed into fully differentiated plantlets within 3,5 months, if the explants are harvested after 2 months. However, if explants are harvested after 3 months, 86 per cent of the initial number of explants used, can be developed into fully differentiated plantlets within approximately 4,5 months. These facts are particularly important if this technique is to be commercialized. The decision as to which procedure to follow, will undoubtedly depend upon the amount of incubation space available.

Following the above investigation, which indicated that the basal positioning of explants on the culture medium is best for producing the highest number of shooting explants, the next analysis undertaken was to determine whether the omission of casein hydrolysate from the culture medium would effect explant response. Because of the properties of this natural complex and other natural complexes, such as coconut milk and malt extract (HALPERIN and WETHERELL, 1964; GUHA and MAHESHWARI, 1967; BUTTON and BORNMAN, 1971; NATARAJA, 1971; VAN STADEN and DREWES, 1974, 1975a; DIX and VAN STADEN, 1982; DODDS and ROBERTS, 1982), the removal of casein hydrolysate was expected to decrease the morphogenetic response of the explants. However, results obtained in this investigation (Figure 2.9) proved that this natural complex had very little effect upon the total number of explants responding *in vitro*

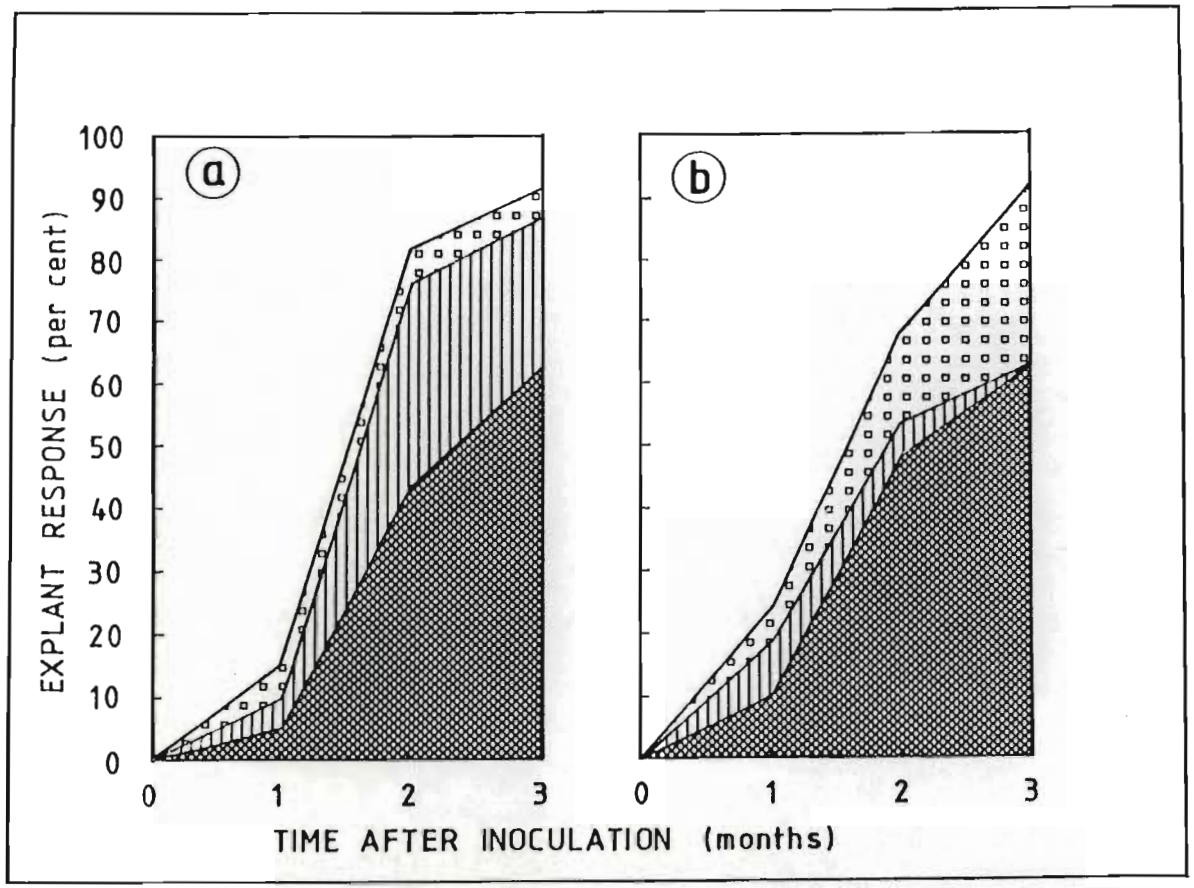


Figure 2.9: The response of *H. rooperi* corm explants as influenced by the presence (a) or absence (b) of casein hydrolysate in the culture medium. = Rooting explants. = Shooting explants. = Explants producing both roots and shoots.

and the rate of explant response. The proportion of explants producing roots, shoots or roots plus shoots, was however markedly influenced. On a culture medium supplemented with casein hydrolysate (Figure 2.9a), 86 per cent of the explants produced shoots after 3 months incubation. When no casein hydrolysate was present in the medium (Figure 2.9b), only 62 per cent of the explants produced shoots. Therefore, the removal of casein hydrolysate from the medium resulted in a decrease in the number of explants producing shoots and an increase in the number producing roots. These results suggest that casein hydrolysate possesses shoot promoting and/or root inhibiting properties. Possibly this natural complex contains growth substances as have been identified in coconut milk, malt and yeast extracts (LETHAM, 1974; VAN STADEN and DREWES, 1974, 1975a, 1975b; DIX and VAN STADEN, 1982).

To analyse the influence of sucrose upon explant morphogenesis, corm material of *H. rooperi* was cultured in a basipetal position, on a culture medium supplemented with casein hydrolysate and various levels of sucrose (20; 30 or 40 $g\ell^{-1}$). Results obtained in this investigation (Figure 2.10) indicated that the overall response of explants cultured on a medium supplemented with 20 $g\ell^{-1}$ sucrose (Figure 2.10a), is considerably lower than the response obtained from explants grown on a medium containing 30 or 40 $g\ell^{-1}$ sucrose (Figure 2.10b and c respectively). When comparing the response of the 2 latter treatments, it is evident that the total response of explants (i.e. the number of explants producing roots, shoots and roots plus shoots, 3 months after inoculation), was higher when explants were cultured on a medium supplemented with 40 $g\ell^{-1}$ sucrose. However, the number of explants producing shoots was higher when explants were cultured on a 30 $g\ell^{-1}$ sucrose supplemented medium. Of this total, 72 per cent of the explants produced shoots plus roots, while in the 40 $g\ell^{-1}$ sucrose treatment, 94 per cent of the total number of shooting explants, produced fully differentiated plantlets. Therefore, the ratio of explants producing shoots plus roots as opposed to shoots only, is much higher in the 40 $g\ell^{-1}$ sucrose treatment. It may thus be stated that an increase in the sucrose concentration from 30 to 40 $g\ell^{-1}$, promotes the formation of roots upon shooted corm explants of *H. rooperi*.

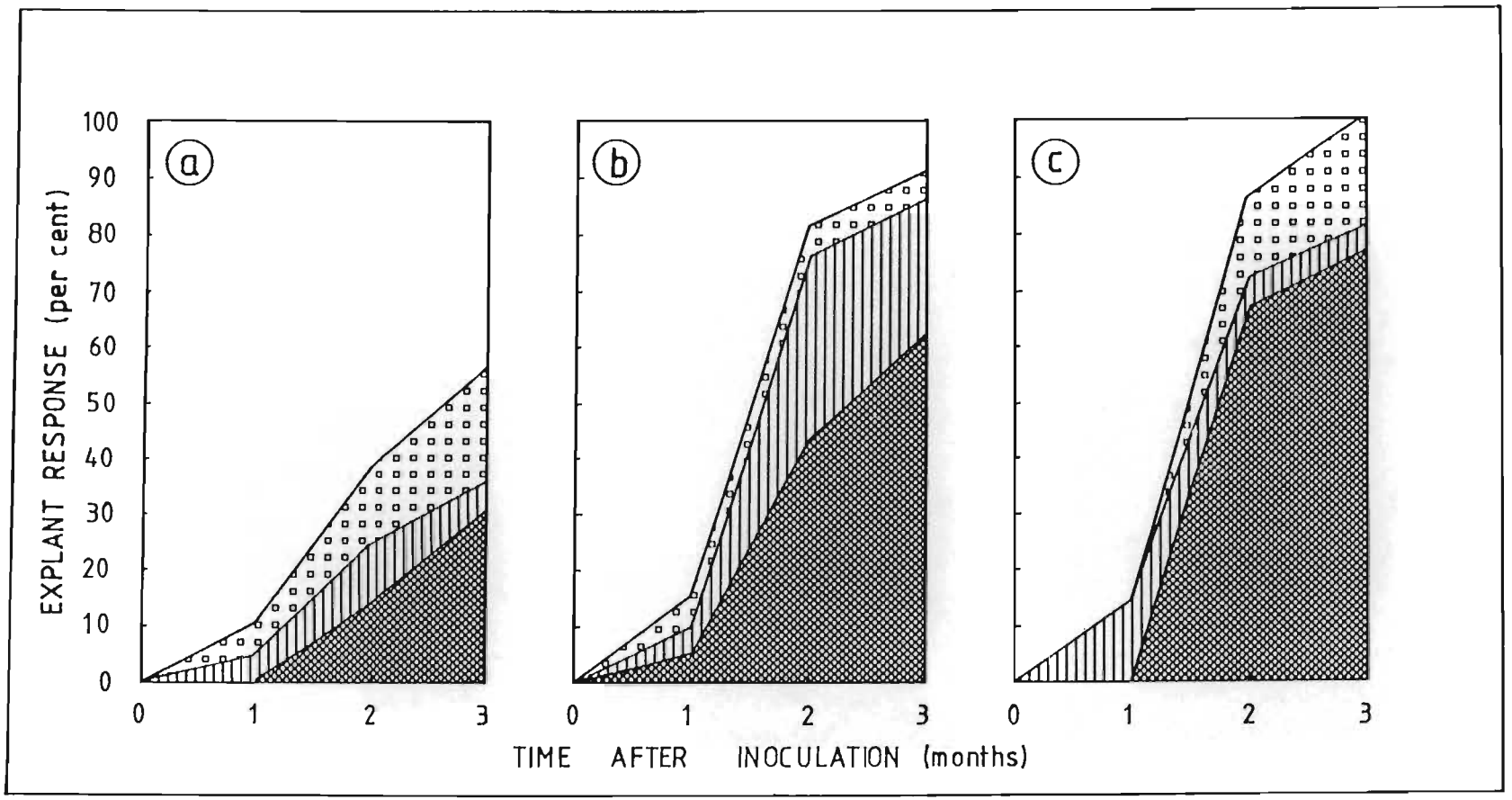


Figure 2.10: The response of *H. rooperi* corm explants as influenced by 20 (a), 30 (b) and 40 $g\ell^{-1}$ (c) sucrose in the culture medium. = Rooting explants. = Shooting explants. = Explants producing both roots and shoots.

This response is in accordance with the results obtained by WELANDER (1976) and TAKAYAMA and MISAWA (1979). In *Episcia* Mart. it has been shown that root formation is enhanced by adding sucrose at 30 $g\ell^{-1}$ to the rooting stage medium (HUGHES, 1981). Exactly how sucrose exercises this influence is unknown. According to TRAN THANH VAN (1977), sucrose probably mediates its effect on organ formation, through osmotic potential changes. BUTCHER and STREET (1960) however, suggest that sucrose probably affects root growth through the regulation of hormonal levels. FORSYTH and VAN STADEN (1984) state that it is possible that carbohydrates exercise their influence by changing the metabolism and distribution of endogenous and/or applied hormones. Galactose for example, has been reported to stimulate auxin synthesis (ANKER, 1974) but to inhibit auxin movement (KRUL and COLCLASURE, 1977) and to induce ethylene evolution (COLCLASURE and YOPP, 1976). Alternatively, glucose is reported to increase the efficiency of auxin movement (KRUL and COLCLASURE, 1977). These authors suggest that auxin-monosaccharide conjugates may be mobile forms of auxin. Although no mention is made in these experiments of the effect of sucrose, it is not unlikely according to FORSYTH and VAN STADEN (1984), that its effect could be exerted in a similar manner. If so, then the results obtained in the above experiment could be quite plausibly explained. It would mean that the rooting response resulting from corm explants cultured on a medium containing higher levels of sucrose, could be related to changes in endogenous hormone levels.

Finally, an analysis of the influence of explant origin upon the morphogenetic response of corm material, was performed by culturing explants obtained from region 2 (Figure 2.3) of layers 1; 2 and 3 (Figure 2.6), in a basal position on a culture medium supplemented with casein hydrolysate (1,0 $g\ell^{-1}$) and sucrose (30 $g\ell^{-1}$). In this investigation, results obtained (Figure 2.11) indicated that only explants obtained from layer 1 expressed all three forms of morphogenetic responses. Furthermore, this material produced these responses after an inoculation period of 2 months. It is believed that the reason why the rooting condition was not expressed by explants excised from layers 2 and 3 is because this material did not possess preformed root primordia, whereas layer 1 probably did. Therefore, the culture medium cannot be regarded as being inductive to root formation, but merely permissive to the expression of preformed root primordia.

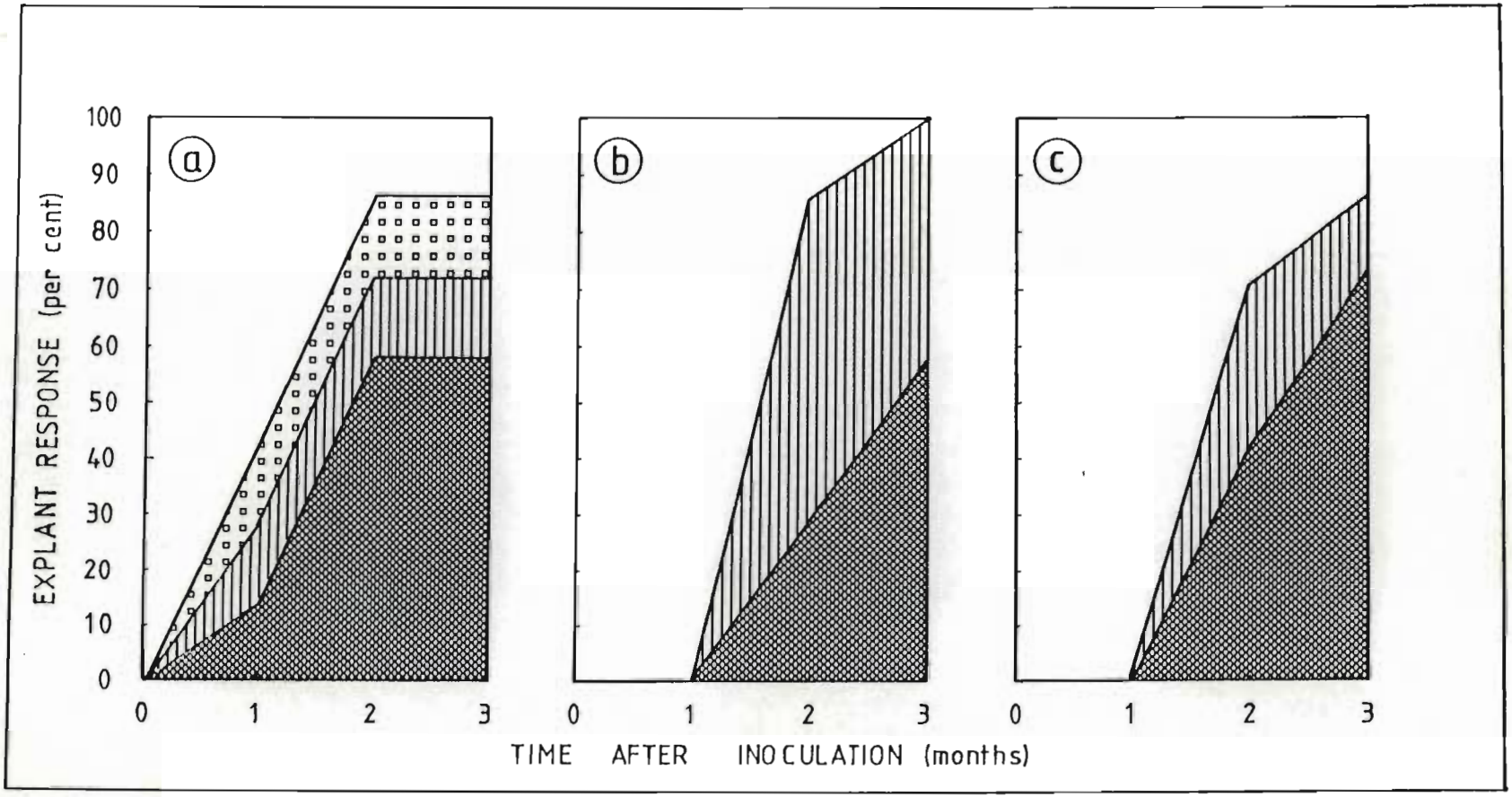


Figure 2.11: The response of *H. rooperi* corm explants as influenced by explant origin, namely from layers 1 (a), 2 (b) and 3 (c) within the host plant corm. = Rooting explants. = Shooting explants. = Explants producing both roots and shoots.

With regard to the production of shoots by the explants of various origins, it is evident from the results that all explants obtained from layer 2 of the donor plant, produced shoots. Of this total, 57 per cent produced roots as well. Where explants were obtained from layer 3 of the donor plant, 87 per cent differentiated shoots, 83 per cent of which also produced roots. Similarly, 72 per cent of the explants excised from layer 1 produced shoots and of this total, 81 per cent produced roots as well. Therefore, it may be stated that although all explants excised from layer 2 produced shoots, a relatively large proportion (by comparison to those obtained from layers 1 and 3), required subculturing to induce root formation.

In summary therefore, results obtained from the above series of experiments indicated that the percentage of explants induced to shoot are highest if;

1. explants are orientated in a basal position on the culture medium,
2. the culture medium is supplemented with 1,0 $g\ell^{-1}$ casein hydrolysate,
3. 30 $g\ell^{-1}$ sucrose is added to the culture medium rather than 20 or 40 $g\ell^{-1}$ sucrose, and
4. the explants are obtained from layer 2 within the donor plant, rather than layers 1 or 3.

Although the fourth statement is of academic significance, from a practical point of view it would be wasteful to utilize only those explants obtained from layer 2 for regenerating plantlets. Particularly seeing as all three layers of the donor plant produce shoots. In fact, it would mean that the ratio of plantlets produced per donor plant would be very low. Thus in the event of this technique being commercialized, the use of all three layers for the *in vitro* regeneration of *H. rooperi* would be advocated.

In the above series of corm experiments, in addition to the type of morphogenetic response of the explants being recorded, the number of shoots produced in each treatment was also documented. These figures are presented in Table 2.14. From these results it is evident that the highest number of shoots were produced, when explants were randomly positioned on a culture medium supplemented with 1,0 $g\ell^{-1}$ casein hydrolysate and 30 $g\ell^{-1}$ sucrose (treatment 3). In this treatment, only 65 per cent of the total number of explants used underwent shooting. In contrast

Table 2.14: The influence of explant orientation on the culture medium, casein hydrolysate, sucrose concentration and explant origin, upon shoot production from corm explants of *H. rooperi*. Each treatment comprised of 14 replicates.

TREATMENT					PERCENTAGE OF EXPLANTS PRODUCING SHOOTS	TOTAL NUMBER OF SHOOTS PRODUCED PER TREATMENT
NUMBER	EXPLANT ORIENT-ATION	CASEIN HYDROLYSATE (+ present) (- absent)	SUCROSE CONCEN-TRATION ($g\ell^{-1}$)	EXPLANT ORIGIN		
1	BASAL	+	30	1+2+3	86	14
2	SIDE	+	30	1+2+3	39	10
3	RANDOM	+	30	1+2+3	65	20
4	BASAL	-	30	1+2+3	62	14
5	BASAL	+	20	1+2+3	35	8
6	BASAL	+	40	1+2+3	81	17
7	BASAL	+	30	1	72	10
8	BASAL	+	30	2	100	15
9	BASAL	+	30	3	87	18

to this, in treatment 8 where 100 per cent of the explants produced shoots, only 15 shoots were developed. Therefore, it may be stated that it is incorrect to assume that treatments producing high percentages of shooting explants yield high numbers of shoots. The use of the parameter "percentage of explants responding in a certain morphogenetic manner", is particularly misleading.

In view of this fact, the influence of explant orientation, casein hydrolysate, sucrose concentration and explant origin, upon the number of shoots produced per treatment, were re-analysed. The results obtained from these experiments are recorded in Table 2.15 and Figure 2.12. Once again, alteration in the orientation of explants on the culture medium (treatments 1, 2 and 3), proved that the positioning of explants randomly on the culture medium, yielded the highest number of shoots. The removal of casein hydrolysate from the culture medium (treatment 4), by comparison to treatment 3, markedly reduced the number of shoots produced per treatment. A combined analysis of sucrose concentration and explant origin upon the number of shoots produced per treatment, generated the following results (Figure 2.12). Firstly, that a higher number of shoots were produced from explants cultured on a medium supplemented with 30 $g\ell^{-1}$ sucrose, as opposed to 20 or 40 $g\ell^{-1}$ sucrose. Further, that explants excised from layer 3 of the corm, yielded higher numbers of shoots than those excised from layers 1 and 2. Therefore overall, the highest number of shoots were produced from the treatment in which explants were excised from layer 3, and cultured on a medium supplemented with 30 $g\ell^{-1}$ sucrose. As mentioned earlier, the influence of explant origin upon the numbers of shoots produced, is mainly of academic significance. Having established that all three layers produce shoots *in vitro*, from a commercial point of view, it would be more productive to utilize explants excised from all three layers rather than a single layer within a donor plant.

In conclusion therefore, from the above series of experiments, it is evident that the culturing of corm explants from layers 1, 2 and 3, in a random position on a medium supplemented with 1,0 $g\ell^{-1}$ casein hydrolysate and 30 $g\ell^{-1}$ sucrose, is the best procedure for producing the highest number of shoots per donor plant. Using this treatment, within a 3 month inoculation period 64 per cent of the explants can be induced to produce 2,3 shoots each.

Table 2.15: The influence of explant orientation and casein hydrolysate upon shoot production from corm explants of *H. rooperi*. Each treatment comprised of 14 replicates.

TREATMENT					PERCENTAGE OF EXPLANTS PRODUCING SHOOTS	TOTAL NUMBER OF SHOOTS PRODUCED PER TREATMENT
NUMBER	EXPLANT ORIENT-TION	CASEIN HYDROLYSATE (+ present) (- absent)	SUCROSE CONCEN-TRATION ($g\ell^{-1}$)	EXPLANT ORIGIN		
1	BASAL	+	30	1+2+3	85	13
2	SIDE	+	30	1+2+3	40	10
3	RANDOM	+	30	1+2+3	64	21
4	RANDOM	-	30	1+2+3	29	4

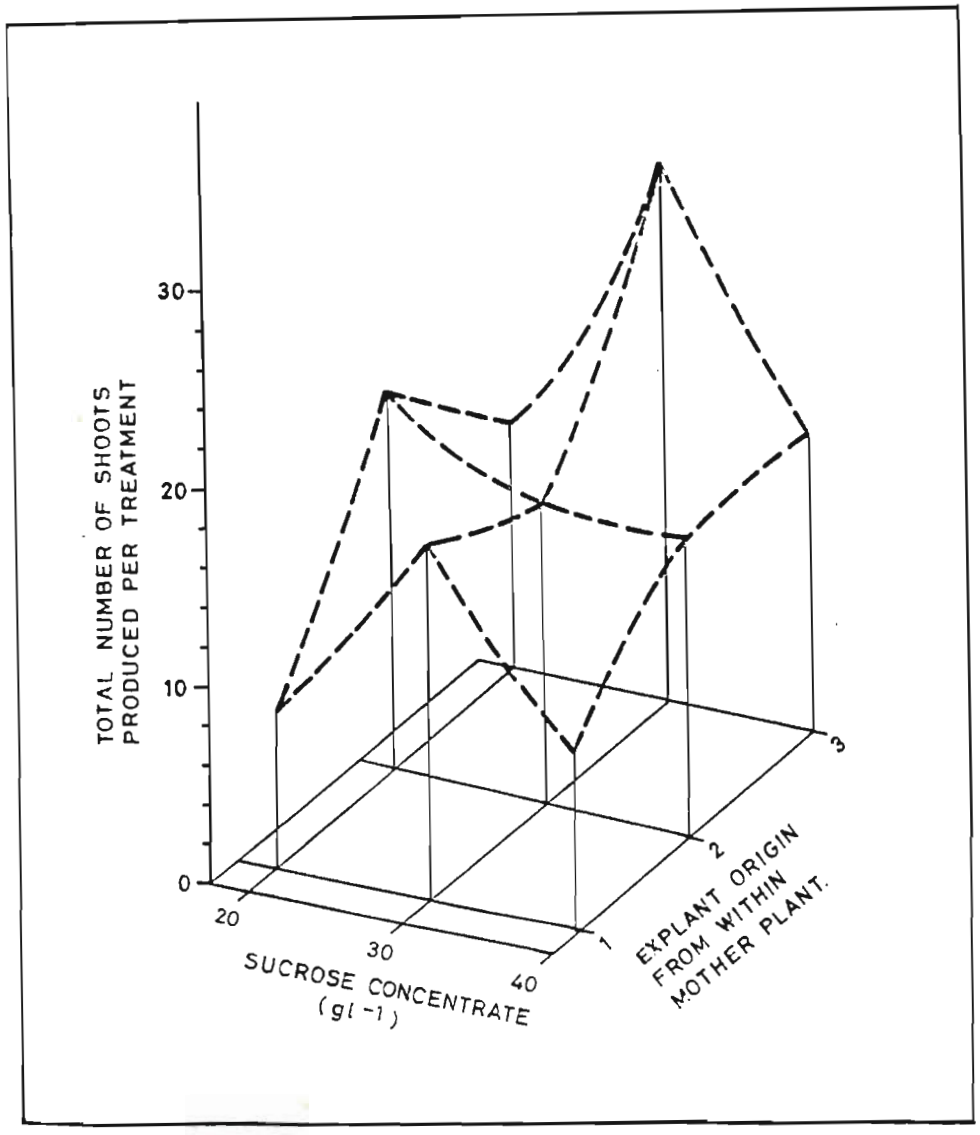


Figure 2.12: The effects of sucrose concentration and explant origin upon the numbers of shoots produced from *H. rooperi* corm explants.

A final experiment performed in the study of the development of an *in vitro* technique for propagating *H. rooperi* plantlets from corm material, was an analysis of the influence of explant harvest time upon the number of shoots produced per explant. This analysis was initiated in an attempt to determine whether the technique thus far developed, if practiced throughout the year, would be seasonally influenced. From the results obtained in this experiment (Figure 2.13), it is evident that similar numbers of shoots were produced per explant, when the material was harvested during March, May and September. Material harvested in July however, produced significantly higher numbers of shoots per explant. Although these differences occurred, because of the fluctuation in the percentage of explants producing shoots at the various time intervals, the total number of shoots produced per treatment was not significantly different (Table 2.16).

On average, 1,94 shoots were developed from each explant and 71,3 per cent of the sterile explants produced this shooting response throughout the period of this investigation. Of the number of explants expressing shoot morphogenesis, 93 per cent produced roots as well. Only 7 per cent of the shooted explants required subculturing for root differentiation to be induced. Assuming that the above determined shoot production rate occurs throughout the year, then using the technique developed in this study, without undertaking any subculturing experiments, an average of 116 fully differentiated plantlets can be established from 100 corm explants within an inoculation period of 3 months. If shooted explants are subcultured and thereby rooted, bearing in mind the rooting process takes approximately 6 weeks (section 2.2.9), an additional 8 plantlets would be produced. Their development would however take 4,5 months. In view of the small gain in the number of shoots produced per 100 corm explants, the performance of this subculturing process would probably not be cost productive in the commercial practice of this technique.

Upon completion of the development of an *in vitro* technique for propagating *H. rooperi* plants from corm material, the final investigation to be undertaken was the "hardening-off" of *in vitro* developed plantlets (Plate 2.21). This aspect presented few problems and a technique as outlined in section 2.2.9 was developed. Following this procedure, 90 per cent of the cultured plantlets could be established *in vivo* . In total

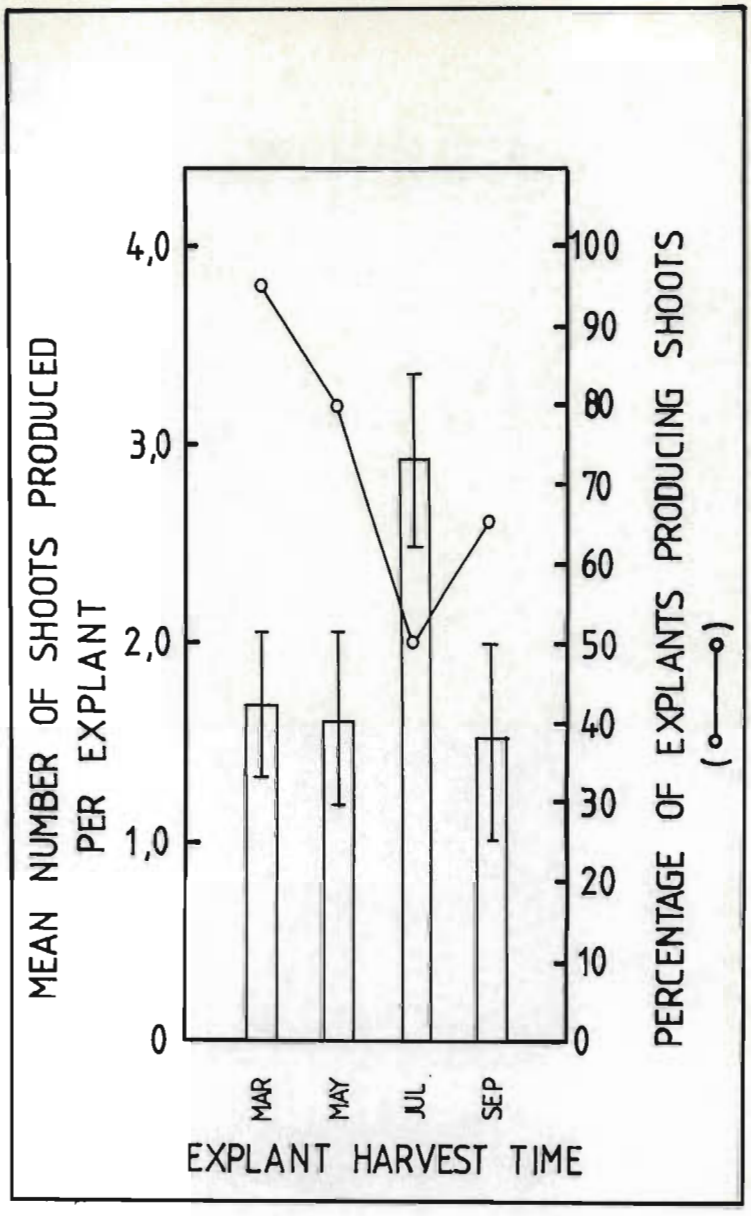


Figure 2.13: The effect of explant harvest time upon *in vitro* shoot production from *H. rooperi* corm explants.

Table 2.16: The influence of explant harvest time upon shoot production from *H. rooperi* corm explants. Each treatment comprised of 14 replicates.

PARAMETER MEASURED	EXPLANT HARVEST TIME			
	MARCH	MAY	JULY	SEPTEMBER
Number of shoots produced per explant*	1,68 (± 0,37)	1,61 (± 0,43)	2,92 (± 0,43)	1,54 (± 0,48)
Percentage of explants producing shoots	95	80	45	65
Total number of shoots produced per treatment*	159 (± 35,15)	129,85 (± 34,4)	131,4 (19,35)	100,1 (± 31,2)

* (± 95 per cent confidence limit values)

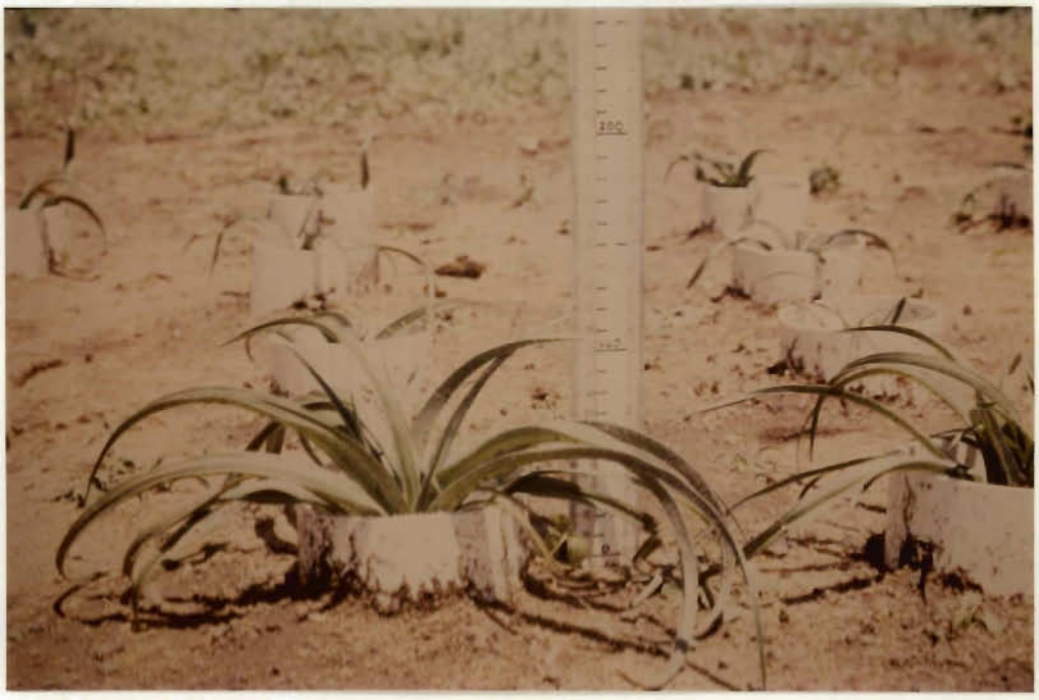


Plate 2.21: "Hardened-off" plantlet cultured from a *H. rooperi* corm explant. x 0,3.

therefore, using the *in vitro* and "hardening-off" techniques developed in this investigation, theoretically 104 or 112 plantlets can be regenerated from 100 corm explants within 3 or 4,5 months respectively. As only an average of 40 explants can be excised from the upper half of a mature corm, 2,5 corms would have to be destroyed in the production of these plantlets.

This propagation procedure and rate, although destructive and considerably lower than that calculated for *H. rooperi* regeneration from flower buds, possesses numerous advantages. For example, corm explants are available throughout the year, whereas flower bud explants are only obtainable from September to November. Therefore, the propagation procedure using corm material is not seasonally controlled, whilst that from flower buds is. Further, plantlet regeneration from corm explants occurs via a more direct and rapid pathway to that developed for flower buds. A minimal amount of callus is formed during this procedure, and very little subculturing is necessary to produce plants from corm material. Hence the organization of this technique is quite simple. Alternatively, the propagation of plantlets from flower bud explants occurs following the production of relatively large amounts of callus. Therefore, for this procedure to run smoothly, a more rigid schedule is required. Finally, as the regeneration of plantlets from corm explants occurs following minimal callus formation, problems such as the loss of morphogenetic potential and aberrant plantlet production are avoided. Hence, the cloning of plants can probably be achieved using corm material as an explant source.

2.4 CONCLUSIONS

More than 80 years have passed since HABERLANDT's (1902) publication of his first attempt to culture isolated plant cells. Today worldwide research activities in the field of plant tissue culture, have confirmed virtually universal applicability of the principle to a large number of ferns, gymnosperms and angiosperms. The most substantial use of this technique has been in the rapid clonal multiplication of these plants. Currently, sufficient information is available to provide a basic guide for the development of *in vitro* propagation procedures for most plant species.

From the present study it has been shown that it is possible to propagate *H. rooperi* using *in vitro* techniques. To achieve this it was found that the flower and corm tissue of this species provided a suitable explant source. In retrospect, it is fortunate that analyses were undertaken to develop propagation procedures using both these explant types, as the flower bud tissue cultures ultimately provided a means of producing a continuous supply of shoots (and hence plantlets), and the corm tissue a method of regenerating plantlets via a more direct and rapid procedure. The leaf and inflorescence stalk material of *H. rooperi* did not respond favourably in culture. This was rather surprising as these organs are often used for the aseptic regeneration of monocotyledonous plants.

To induce the regeneration of plantlets from flower bud explants of *H. rooperi*, investigations indicated that flower bud age plays a significant role. It was established that only those buds which were at least 1,0 cm in length, but which had not yet flowered, would respond *in vitro*. Neither smaller, younger nor older buds would revert back to the vegetative condition once inoculated *in vitro*. Although not unequivocally proven in this study, the morphogenetic response obtained from *H. rooperi* flower buds appeared to originate from the tissue located between the perianth segments and the stamen filaments, and only occurred once the former organs had reflexed back. The most successful procedure established for decontaminating intermediate age flower buds, involved the initial washing of the explants in running tap water for 30 minutes, followed by a 2 minute rinsing of the material in 95 per cent ethanol. The final sterilization of this tissue was achieved by the immersion of the buds in a 0,1 per cent mercuric chloride solution for 1 to 2 minutes (Figure 2.14). Using this procedure 90 per cent of the buds cultured could be sterilized.

In addition to flower bud age, the hormonal levels within the culture medium were also found to influence the explant response. Investigations on this aspect proved that the greatest proliferation of callus and adventitious shoots from *H. rooperi* flower buds, could be induced by inoculating the buds in either a basipetal or horizontal position, onto a basic MURASHIGE and SKOOG (1962) medium supplemented with 0,001 $g\ell^{-1}$ NAA and 0,005 $g\ell^{-1}$ BA. Upon this medium an average of 37,5 per cent of the explants cultured at anytime throughout the flowering season, produced callus and shoots, within a period of 12 weeks. The excision of the

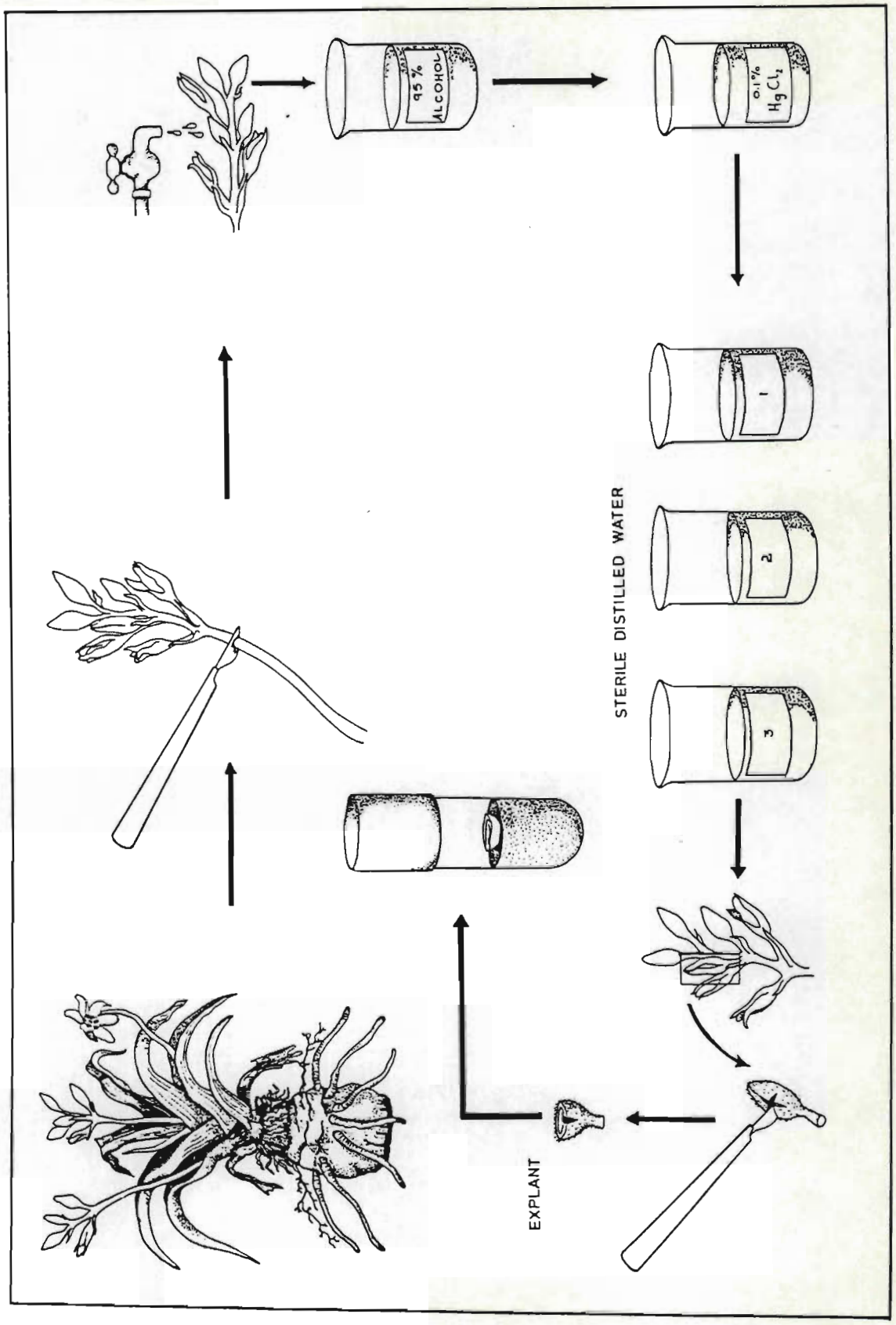


Figure 2.14: Procedure utilized to sterilize *H. rooperi* flower bud explants.

perianth segments, stamens and style from the flower bud explants, was found to enhance the rate of this callusing response.

Following the establishment of shoot forming callus from flower buds of *H. rooperi*, investigations undertaken proved that it was possible to manipulate callus morphogenesis in such a manner, so as to produce a continuous supply of shoots. This was achieved by regularly subculturing the callus on a MURASHIGE and SKOOG (1962) medium supplemented with 0,001 $g\ell^{-1}$ NAA and 0,001 $g\ell^{-1}$ BA. The monitoring of the shoot producing potential of this callus over a 30 week and 66 week period, indicated that the callus does not lose the ability to produce shoots over this period of time. Hence the procedure developed can be regarded as being a reliable means of producing a regular supply of *H. rooperi* plantlets.

To fulfil the objective of rooting and "hardening-off" of shoots produced from flower bud cultures, an effective method (i.e. one with a 75 per cent success rate) was developed in this study. The procedure although relatively simple, is more lengthy than that developed for the "hardening-off" of plantlets propagated from *H. rooperi* corm explants. Finally, from analyses undertaken using *H. rooperi* flower bud cultures, it was theoretically calculated that approximately 81 000 plantlets can be regenerated from 100 flower buds within a period of 1 year. From these figures it is tempting to state that the regeneration rate of this procedure is high. However, whether it is high enough or economically feasible to justify the practical application of the technique on a commercial scale, remains to be established.

With regard to the propagation of plantlets from *H. rooperi* corm explants, it was established that the anatomical structure of the explant plays a significant role in its response. Only corm explants excised from the primary thickening meristem region of the donor plant, were responsive *in vitro*. The decontamination of this material was most effectively achieved by sterilizing the tissue in a 0,1 per cent mercuric chloride solution for a period of 5 minutes (Figure 2.15). To induce a shooting response from corm explants the best culture medium was a solidified MURASHIGE and SKOOG (1962) salt mixture supplemented with 0,0005 or 0,001 $g\ell^{-1}$ BA; 1,0 $g\ell^{-1}$ casein hydrolysate and 30 $g\ell^{-1}$ sucrose. It

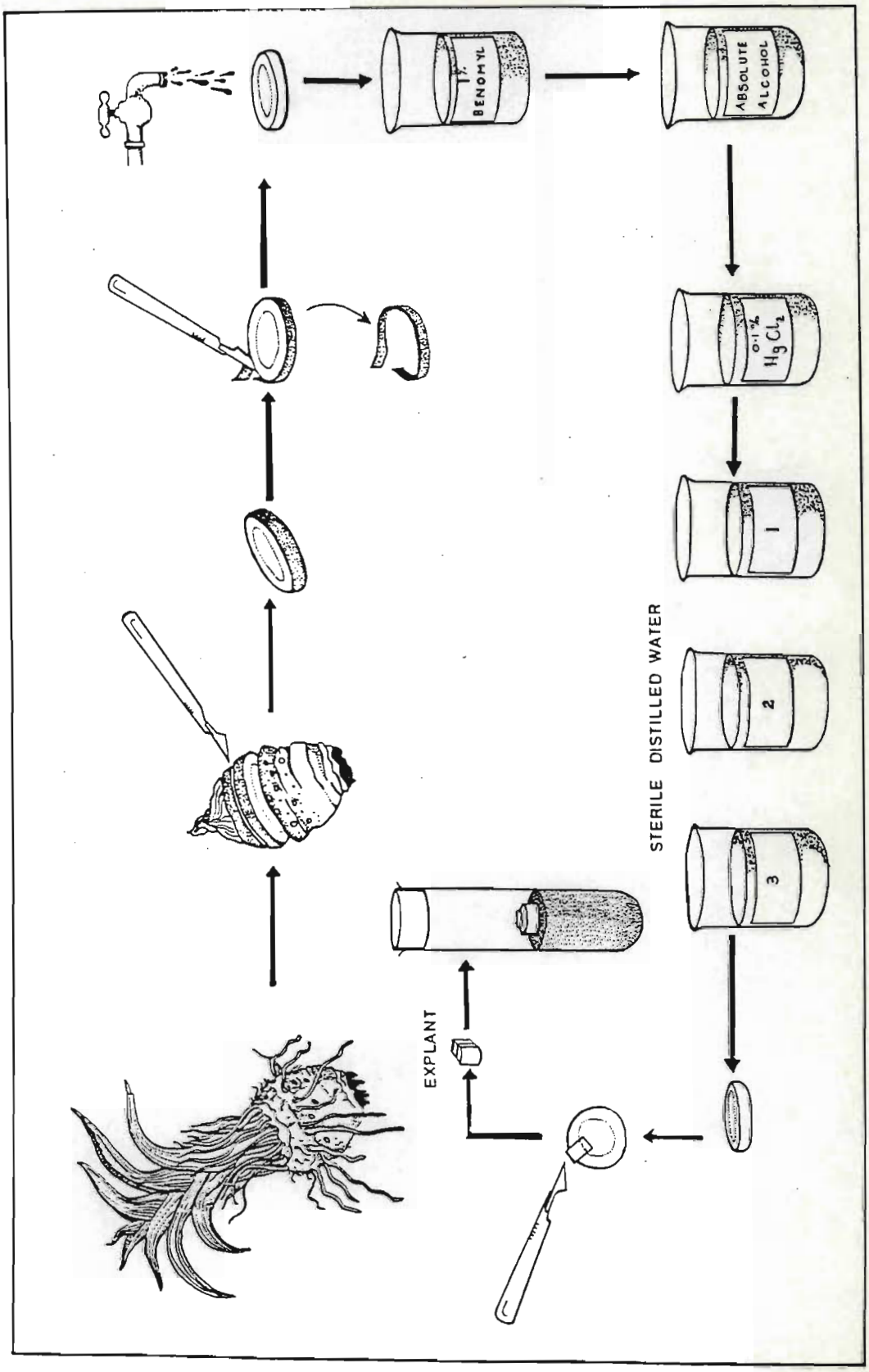


Figure 2.15: Procedure utilized to sterilize *H. rooperi* corm explants.

was also established that the specific positioning of corm explants on the culture medium, does not enhance the numbers of shoots produced from the material. Analyses regarding the precise position of explant excision from within the donor plant, indicated that this aspect was influential in altering the shooting response of the tissue. However, it was acknowledged that from a practical viewpoint, the refinement of the procedure to this degree would merely reduce the productivity of the technique, as fewer numbers of plantlets would ultimately be regenerated from each donor plant.

Investigations on the fluctuations in the numbers of shoots produced from corm explants cultured at various time intervals throughout the year, demonstrated that on average 1,94 shoots can be regenerated from 71,3 per cent of the explants cultured. As a large percentage of these shooted explants also formed roots, the rooting and "hardening-off" of plantlets produced from corm explants was relatively easy. Following the technique devised and outlined in section 2.2.9, 90 per cent of the cultured plantlets were established *in vivo*. Finally, it was estimated that using the technique developed, approximately 100 plantlets can be proliferated from 100 corm explants within a period of 3 months. This clearly represents a much lower regeneration rate than that calculated for plantlet propagation from *H. rooperi* flower buds. However, as mentioned previously, the procedure of propagating plantlets from corm explants does possess various valuable qualities which the flower bud procedure lacks.

In conclusion, it can be stated that at present the only methods available for regenerating *H. rooperi* are those which have been established in this study. Since this aspect, as noted in the literature review, is primarily of commercial significance there are a few important facts which should be emphasised. Firstly, in order to achieve the greatest level of plantlet production following the methods as they presently exist, it would be best to utilize both procedures in conjunction with each other. Secondly, as there are an almost infinite number of parameters which influence tissue culture systems, much scope exists for further refinement and improvement of the propagation techniques developed. Further, if the procedures developed are to be commercially utilized, it is necessary that an assessment be made of the genetic stability of the progeny

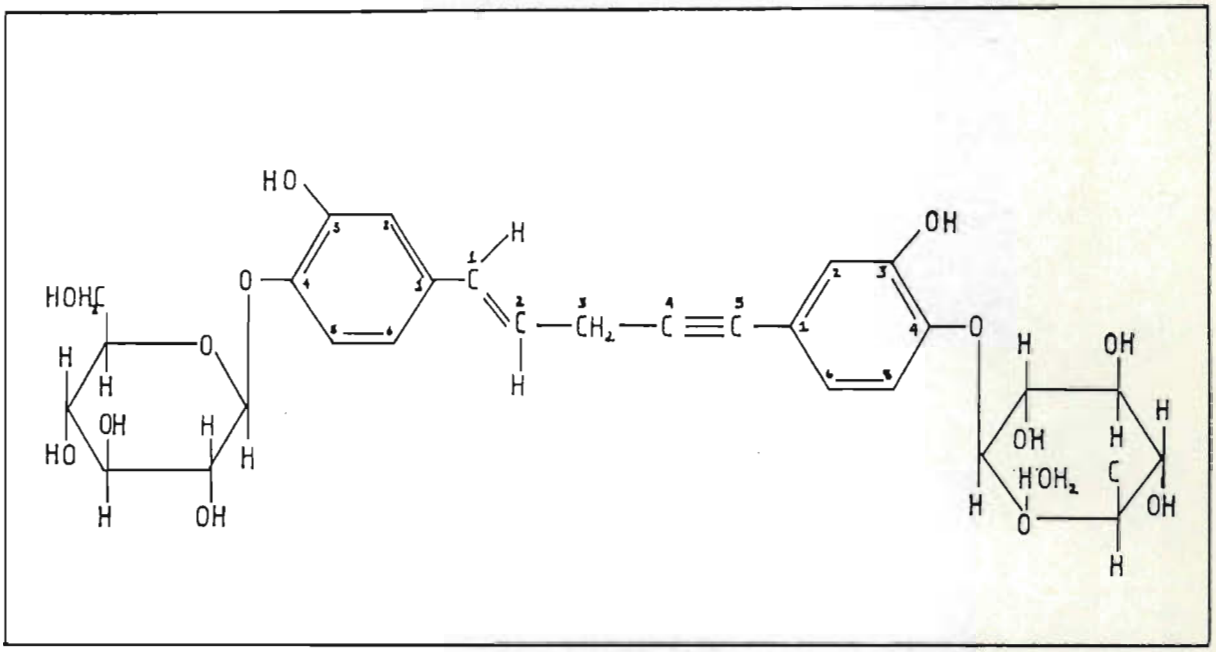
produced from *in vitro* grown *H. rooperi* material, prior to such a venture. Finally, as reports on the commercial and economic aspects of tissue culture systems (ANDERSON, MEAGHER and NELSON, 1977; HOLDGATE, 1977; OGLESBY, 1978; HOLDGATE, 1980; JACOB , DENEE and COUMANS, 1980; LEVY, 1981; OKI, 1981; EVANS and SHARP, 1982; HARNEY, 1982) have estimated variable costs for plants derived via *in vitro* procedures, and have noted that often these techniques are to labour intensive to be practical, it would be valuable to consider these aspects with respect to *H. rooperi* culture prior to the commercialization of the procedures described.

CHAPTER 3

PRODUCTION OF HYPOXOSIDE *IN VITRO*

3.1 INTRODUCTION

Hypoxoside ($C_{29}H_{34}O_{14}$) the believed active pharmaceutical consituent of *Hypoxis* corm extracts, is an unusual phenolic glucoside possessing the following molecular structure:



BETTOLO, PATAMIA, NICOLETTI, GALEFFI and MESSANA (1982) were the first group of scientists to report upon the physico-chemical nature and chemical behaviour of this compound. They noted that to isolate hypoxoside from rhizomes of *H. obtusa*, methanolic extracts of this material had to be submitted to a countercurrent distribution between water : ethyl acetate : n-butanol (10 : 8 : 2). Using this technique hypoxoside crystals totalling 3,7 per cent of the rhizome were obtained. These crystals possessed a melting point of 149 to 151 °C, were found to be very soluble in water and methanol, showed UV maxima (when dissolved in methanol) at 258, 291, 298 and 310 nm (log ε 3,46; 3,00; 3,00; 2,39), and gave a positive reaction for phenolic groups with ferric ferricyanide. As yet no information is available concerning the synthesis of hypoxoside. It has been suggested that this compound may be formed from two phenylalanine units (BETTOLO, PATAMIA, NICOLETTI, GALEFFI and MESSANA, 1982).

Plant tissue cultures are valuable systems for studying the biosynthesis of secondary metabolites. They also provide an alternative means for producing commercially important plant products (BUTCHER, 1977). In comparison to the conventional cultivation of whole plants for producing secondary metabolites, plant cultures offer major advantages (BUTCHER, 1977; TABATA, 1977);

1. they are not subject to the limitations of soil, season and environmental conditions;
2. compounds can be produced year round under controlled laboratory conditions; and
3. their metabolic processes can be regulated and hence their productivity maximized.

For these reasons and the fact that *H. rooperi* plantlets require a number of years to reach maturity, investigations into the possible production of hypoxoside *in vitro* were started.

3.2 MATERIALS AND METHODS

3.2.1 Material

The phenolic repertoire of cell cultures has rarely been compared in detail with that of the corresponding intact plant. Uncertainty exists as to whether the differences that have been noted in culture are real or apparent. In this study therefore, the material used included both mature *H. rooperi* corm tissue collected from the environs of Pietermaritzburg, and cultured tissue developed using the techniques described in Chapter 2. Authentic hypoxoside was obtained from Professor S.E. DREWES, Chemistry Department, University of Natal, Pietermaritzburg, South Africa.

3.2.2 Cultured material

The analysis of *in vitro* cultured material was conducted on 5 different morphogenetic forms of material;

1. undifferentiated callus,
2. root forming callus,

3. shoot forming callus,

4. malformed root tissue developed from flower bud explants, and

5. malformed root tissue developed from corm explants.

The first 3 types of tissue were obtained by culturing flower buds (more than 1,0 cm long which had not yet flowered), on a culture medium (section 2.2.3) supplemented with 0,001 $g\ell^{-1}$ NAA and 0,003 $g\ell^{-1}$ BA (medium A). After a period of 3 months the callus proliferating from these buds was transferred onto a fresh medium containing 0,001 $g\ell^{-1}$ NAA and 0,001 $g\ell^{-1}$ BA (medium B). This callus was maintained in a proliferating state by subculturing at 6 weekly intervals. Eventually it was divided into root forming callus, shoot forming callus and undifferentiated callus (Plate 3.1a). These three types of callus were maintained separately in culture on medium B (above), until required.

Malformed root tissue obtained from flower buds (Plate 3.1b) was induced to develop by culturing buds (more than 1,0 cm long which had not yet flowered), on a medium (section 2.2.3) supplemented with 0,001 $g\ell^{-1}$ NAA and 0,001 $g\ell^{-1}$ BA. Although the majority of the buds produced callus when inoculated onto this medium, 5 per cent produced malformed roots. These roots usually differentiated directly from the flower buds. Stocks of this rooting material were established by serial subculturing of the tissue, onto fresh medium containing the hormone levels initially used (0,001 $g\ell^{-1}$ NAA and 0,001 $g\ell^{-1}$ BA) to induce the rooting response. All subculturing was performed at 6 weekly intervals.

The culturing of corm explants excised from region 2 (Figure 2.3), on a culture medium (section 2.2.3) supplemented with 1,0 $g\ell^{-1}$ casein hydrolysate, 0,001 $g\ell^{-1}$ NAA and 0,001 $g\ell^{-1}$ BA, resulted in the formation of malformed root tissue. On average 54 per cent of the explants produced these malformed roots (Plate 3.2). Stocks of this material were produced following the same procedure used to increase the amount of malformed root tissue developed from flower buds.

3.2.3 Procedures used for the qualitative and quantitative analyses of hypoxoside

Qualitative analyses. Of the number of extraction procedures tested for the qualitative analysis of corm and cultured tissue material, the

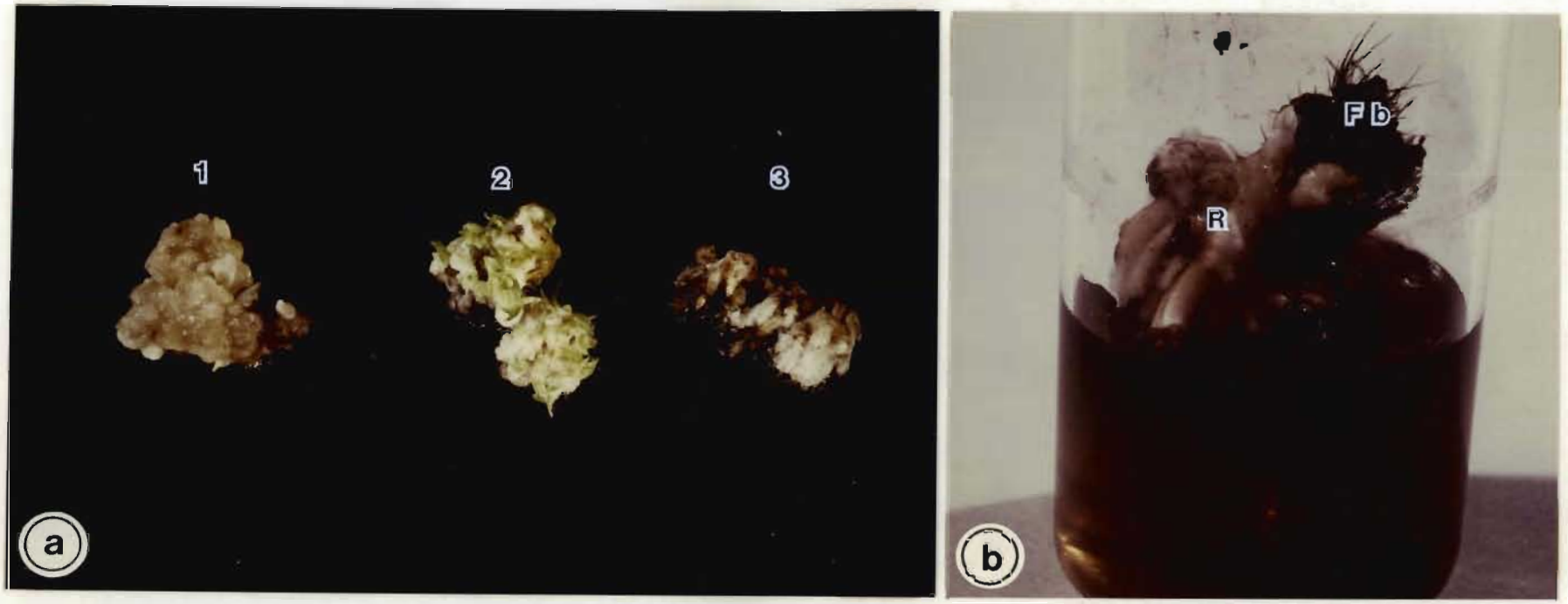


Plate 3.1: (a) Types of callus produced from *H. rooperi* flower bud explants. 1 = undifferentiated callus, 2 = shoot forming callus, 3 = root forming callus. x 1,0.

(b) Malformed root tissue (R) differentiated from a *H. rooperi* flower bud explant (Fb). x 2,0.

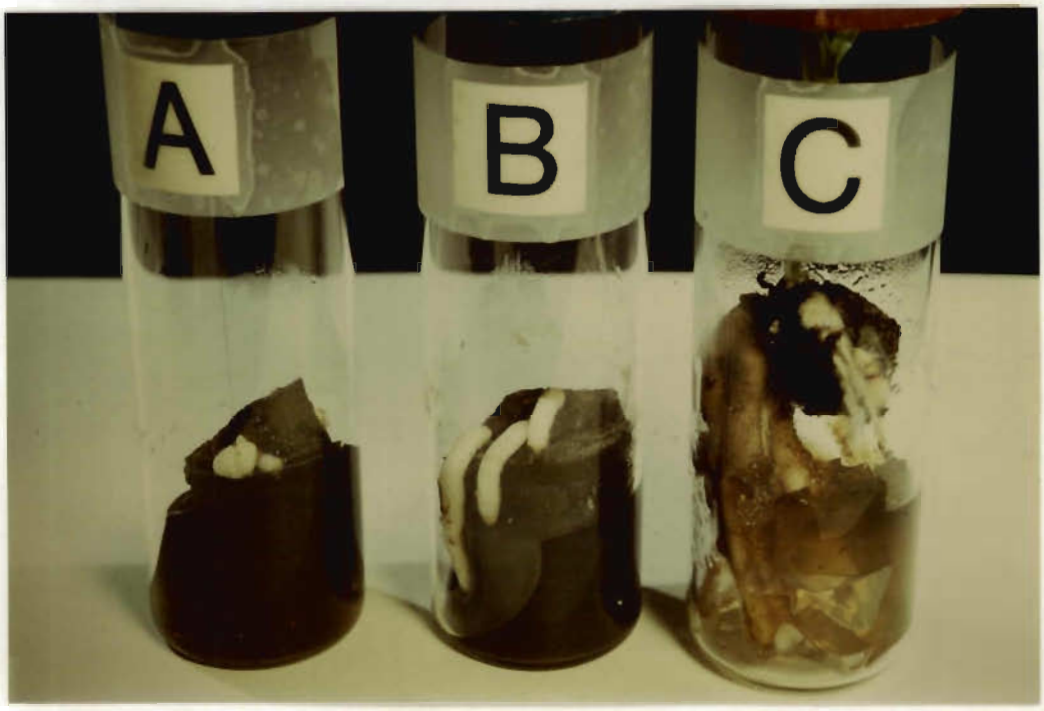


Plate 3.2: Malformed root tissue developed two (A), three (B) and four (C) months after *H. rooperi* corm explant inoculation *in vitro*. x 1,2.

method outlined below was found to be most successful and was subsequently used throughout this study:

1. Material to be analysed was homogenised with 80 per cent ethanol. After 3 hours at room temperature the extract was filtered through Whatman No. 1 filter paper and the residue washed with 80 per cent ethanol.

2. The ethanolic extract was taken to dryness *in vacuo* at 40 °C and the residue dissolved in 0,1 ml 80 per cent ethanol.

3. This extract was then applied to a Merck Kieselgel 60 F_{254} thin layer chromalography (TLC) plate and partitioned using the upper phase of butan-2-ol : benzene : distilled water : methanol (4 : 3: 2 : 1) (solvent I). Once the solvent front had run for a distance of 12 cm, the TLC plate was dried in a stream of air.

4. Under the ultra-violet (UV) light, the compound which co-chromatographed with hypoxoside was recorded. The desired band was removed from the TLC plate and eluted from the silica-gel with 80 per cent ethanol. A small portion of the desired band was not removed in order to tentatively identify the presence of hypoxoside. This was achieved by spraying with a phenolic detector namely; concentrated sulphuric acid and formaldehyde (10 : 0,05).*

5. The removed eluant was subsequently filtered through Whatman No. 1 filter paper with 80 per cent ethanol and then taken to dryness *in vacuo* at 40 °C. The residue was redissolved in 0,1 ml 80 per cent ethanol and applied to a second TLC plate for further purification and tentative identification with an authentic hypoxoside standard.

6. The extract was partitioned in n-butanol : acetic acid : diethyl-ether : water (9 : 6 : 3 : 1) (solvent II). Once the solvent front had run for 12 cm, (after approximately 3 hours) the TLC plate was removed and dried in a stream of air.

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* Personal communication Professor S.E. DREWES, Chemistry Department, University of Natal, Pietermaritzburg, South Africa.

7. After detection of the phenolic glucoside (hypoxoside) under UV light, the desired band which co-chromatographed with authentic hypoxoside, was again eluted from the TLC plate with 80 per cent ethanol. The eluant obtained was subsequently taken to dryness *in vacuo* at 40 °C and then redissolved in 0,1 ml 100 per cent methanol.

8. This extract was then subjected to high performance liquid chromatography (HPLC) using a Varian 5000 HPLC. After numerous analyses in which the solvent components and ratios, sensitivity and detector conditions were altered, the most successful liquid chromatography conditions determined were:

Column:	MCH 5 µ cap
Solvent:	Acetonitrile : Water (20 : 80)
Flow rate:	1,0 ml $minute^{-1}$
Room temperature:	± 22 °C
Pressure:	264 atmospheres
Sensitivity:	0,05
Detector:	265 nm
Chart speed:	20 cm $hour^{-1}$

A typical chromatogram for authentic hypoxoside subjected to these conditions, is illustrated in Figure 3.1.

Quantitative analyses. Two techniques were utilized for the quantitative analysis of hypoxoside within corm and tissue cultured material. The first technique involved the isolation of hypoxoside from a known mass of tissue (usually 1,0 g fresh mass), using the extraction procedure described above. This was followed by the comparison of the purified extracts peak height against a calibration curve. From this the percentage hypoxoside within the tissue was estimated. With each series of analyses a new calibration curve was determined. An example of such a curve is illustrated in Figure 3.2. This technique although very accurate, was extremely tedious and labour intensive.

For this reason, a second technique based upon a similar principle to the one above, was employed. This technique was developed by Dr C.B. ROGERS*. To quantify the amount of hypoxoside present within

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* Department of Chemistry, University of Durban-Westville, Durban, South Africa.

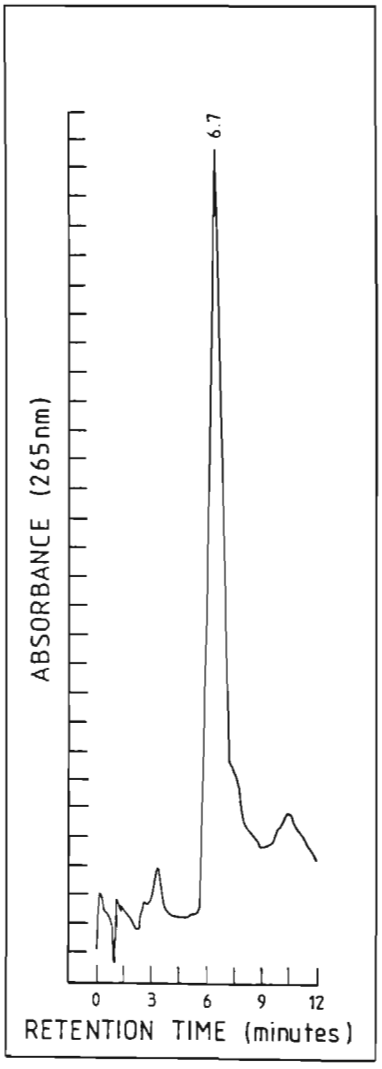


Figure 3.1: High pressure liquid chromatogram of authentic hypoxoside as partitioned using acetonitrile : water (20 : 80), on a Varian 5000 HPLC instrument.

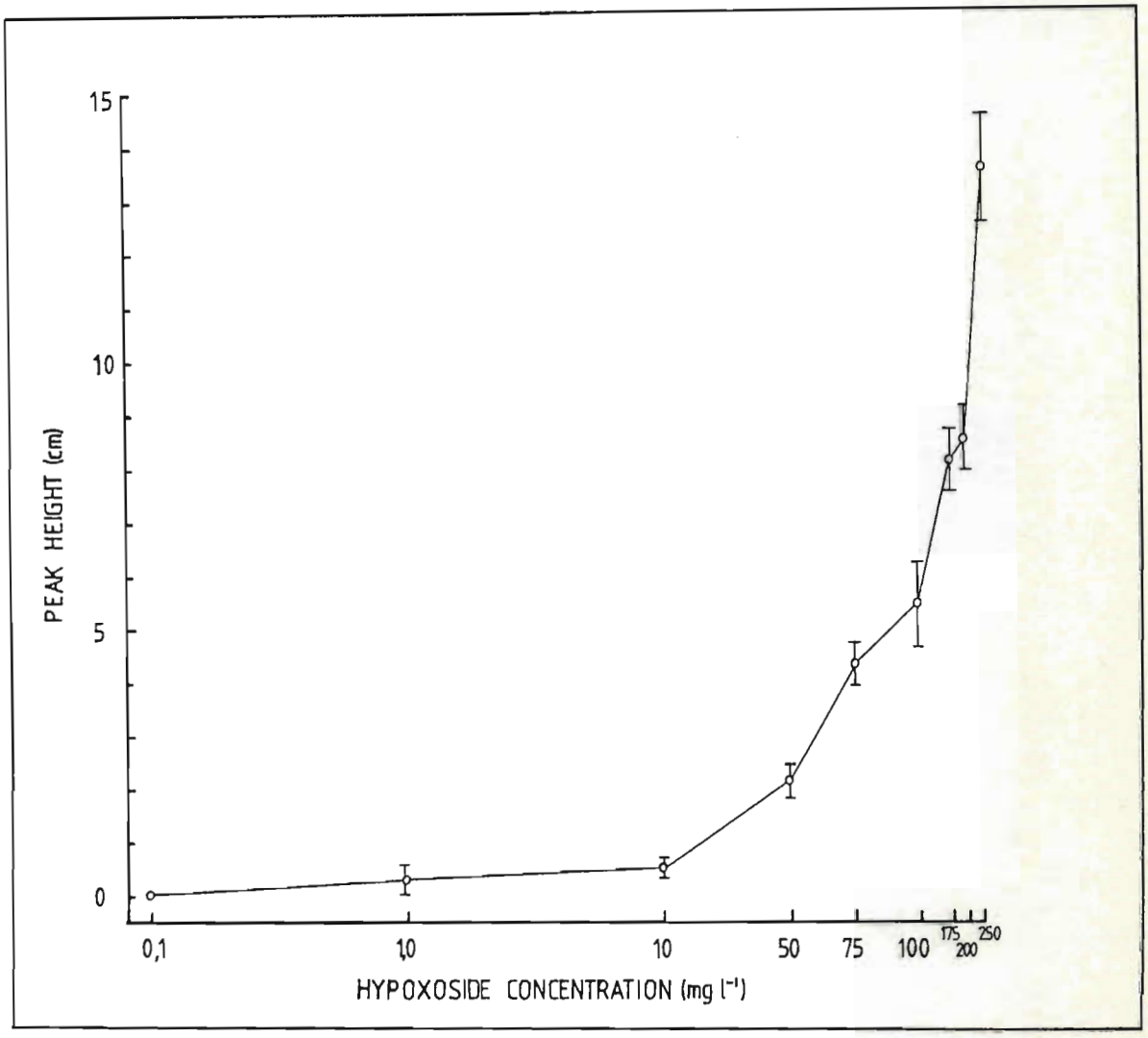


Figure 3.2: Calibration curve of hypoxoside concentration as determined using a Varian 5000 HPLC instrument.

tissue, extracts of a known mass of tissue (usually 5 g fresh mass) were obtained using the procedure described, in stages 1 and 2 of section 3.2.3. These extracts were then analysed using a calibrated and automated Waters M6000 HPLC. The chromatographic conditions used were:

Column:	C_{18} Reverse Phase Microbondpack
Solvent:	Acetonitrile : Water (20 : 80)
Flow rate:	1,0 ml $minute^{-1}$
Room temperature:	$\pm$ 25 °C
Sensitivity:	0,4
Detector:	260 nm
Chart speed:	15 cm $hour^{-1}$

A typical chromatogram for authentic hypoxoside subject to these conditions is illustrated in Figure 3.3. Using this technique the percentage hypoxoside detected within the tissues of interest were found to be very similar to the percentages determined using the first quantitative procedure. As the latter procedure was not as time consuming, it was routinely used throughout this study.

3.2.4 Anatomical investigations

In addition to the isolation and quantification of hypoxoside using the techniques described in section 3.2.3, various anatomical investigations were undertaken in an attempt to locate hypoxoside within the cells of corm and cultured tissue. The antomical structure of the cultured tissue yielding the highest quantity of hypoxoside was also determined. For these investigations wax and resin embedded material were analysed. The techniques utilized to embed, section, stain and view this material are described in section 2.2.10.

3.2.5 Corm and cultured tissue investigations

All experiments undertaken in the following series of investigations were repeated at least twice.

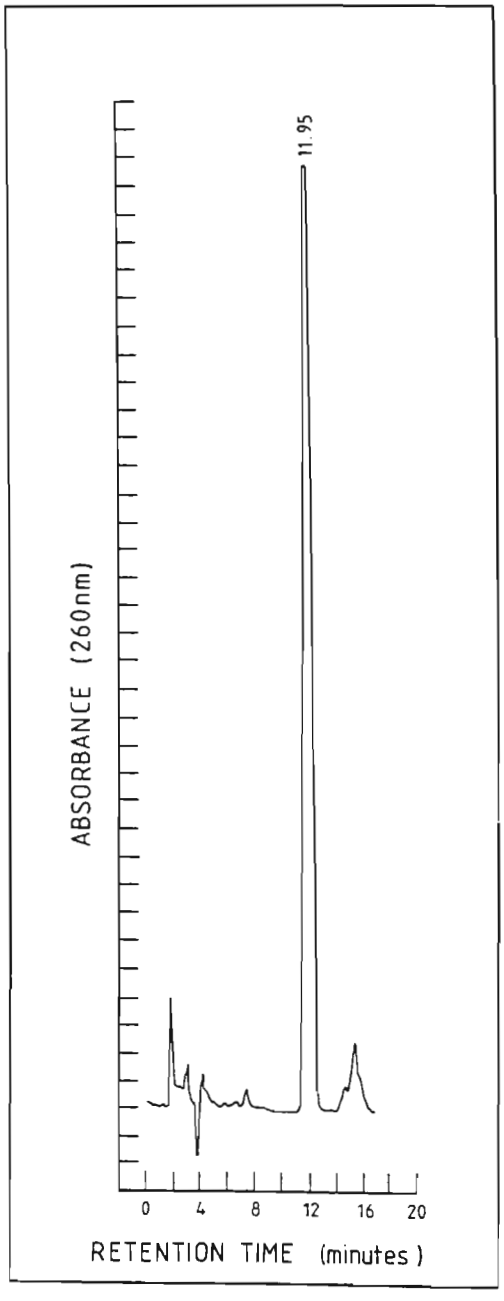


Figure 3.3: High pressure liquid chromatogram of authentic hypoxoside as partitioned using aceto - nitrile : water (20 : 80), on a Waters M6000 HPLC instrument.

3.2.5.1 Corm analyses

Qualitative investigations. The first investigation undertaken using corm material of *H. rooperi*, was to determine whether any qualitative disparity occurred in the hypoxoside content of the following material;

1. tissue excised from regions 1, 2 and 3 (Figure 2.3) of the upper half (above the row of functional contractile roots) of corms harvested in February,
2. tissue randomly excised from the upper half and lower half of corms harvested in February, and
3. tissue randomly excised from the upper half of corms harvested in May, July and October.

Following this investigation, corm material harvested in February was analysed using the entire qualitative procedure described in section 3.2.3. This analysis was undertaken;

1. to illustrate that hypoxoside could be extracted from corm material using the technique developed, and
2. to establish a standard set of data with which tissue culture extracts could be compared.

Quantitative investigations. Employing the quantitative technique developed by Dr C. B. ROGERS, estimations of the percentage hypoxoside present within the 8 different corm extracts (as outlined in the qualitative section above) were determined. These figures were ultimately used for calculating whether the production of hypoxoside *in vitro*, was commercially feasible.

3.2.5.2 Cultured tissue analyses

Qualitative investigations. Using cultured tissue, two qualitative investigations were undertaken. The first was to determine whether any of the 5 morphogenetic forms of tissue (section 3.2.2) contained hypoxoside, and the second was to extract hypoxoside from the tissue containing the highest amount of this chemical. To complete these analyses all forms of cultured tissue were treated as outlined in stages 1, 2 and 3 of section 3.2.3. Subsequently the tissue yielding the highest

amount of hypoxoside was subjected to the entire extraction and purification procedure, described in section 3.2.3 (*Qualitative analyses*).

Quantitative investigations. These investigations were initiated to determine which of the 5 morphogenetic forms of cultured tissue (section 3.2.2), contained the highest amount of hypoxoside, and whether the hypoxoside content within this tissue varied:

1. with time after being induced *in vitro*, and
2. upon subculturing and in relation to tissue growth.

To obtain information concerning the fluctuation of hypoxoside within the cultures yiedling the highest amount of this chemical, fresh tissue was initiated *in vitro* and treated as illustrated in Figure 3.4. For each treatment 10 replicates were used. As the results obtained in this experiment caused uncertainty regarding the synthetic ability of hypoxoside containing cultures, a further analysis was undertaken. The main objective of this experiment was to establish whether the cultured tissue could synthesise hypoxoside. To determine this, the highest yielding hypoxoside tissue (which at this stage had already been subcultured 4 times), was once again subcultured and placed under conditions which have previously been found to influence phenolic synthesis. These conditions were;

1. various levels of sucrose (20; 30 and 40 $g\ell^{-1}$) in the culture medium, and
2. either full (165 $g\ell^{-1}$ NH_4NO_3 and 95 $g\ell^{-1}$ KNO_3) or half the strength (82,5 $g\ell^{-1}$ NH_4NO_3 and 47,5 $g\ell^{-1}$ KNO_3) of the MURASHIGE and SKOOG (1962) nitrogen containing stocks (i.e. stocks 1 and 2 Table 1.4).

As light plays a significant role in the production of many phenolics (BUTCHER, 1977; McCLURE, 1979), some of the hypoxoside containing tissue was also subjected to continuous dark conditions, while other was placed under a 16 hour light : 8 hour dark, light regime. For each of the above treatments 20 replicates were used, half of which were harvested after 1 month culture and the remaining half after 3 months culture.

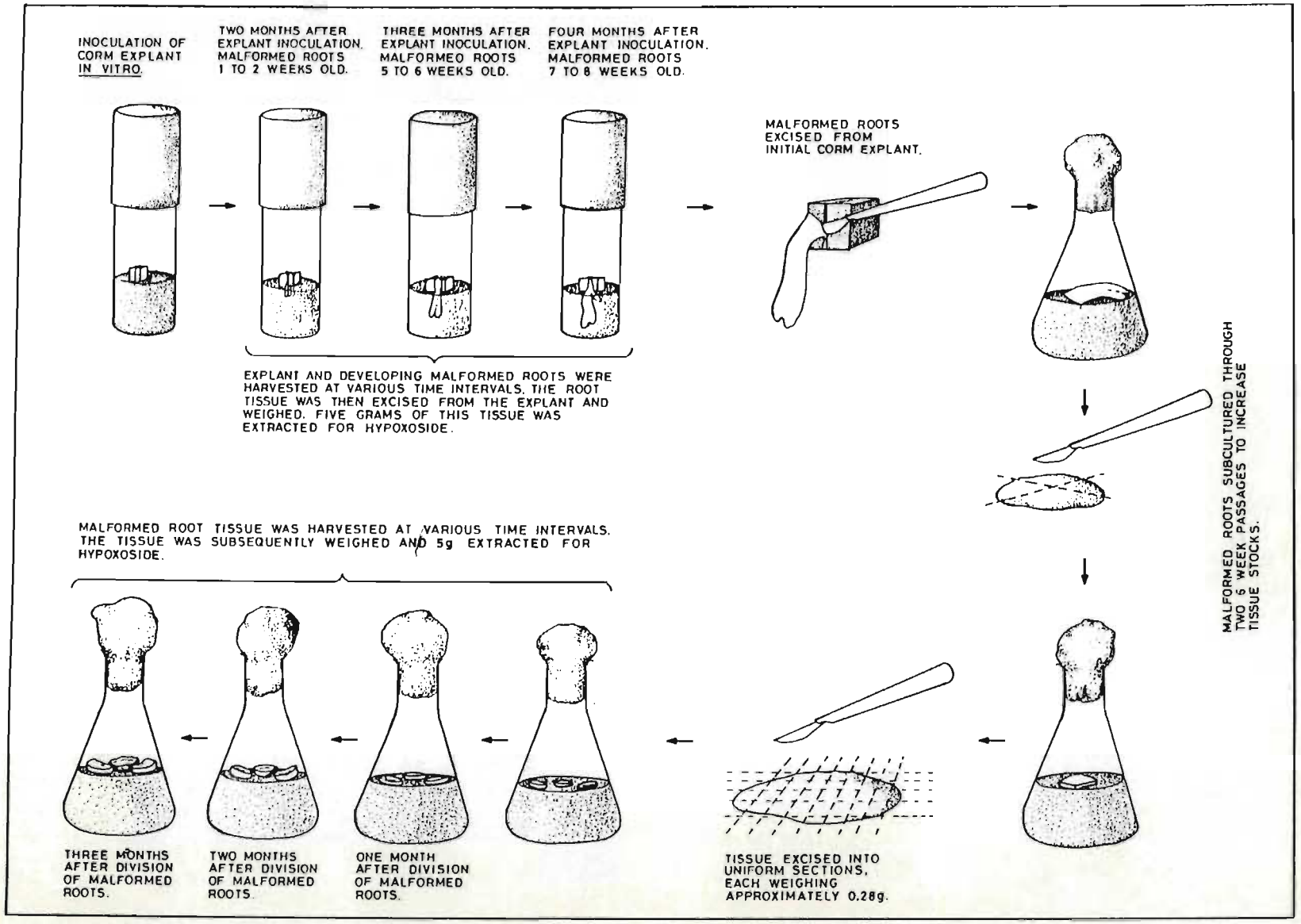


Figure 3.4: Culture procedure utilized for establishing malformed root tissue from *H. rooperi* corm

3.3 RESULTS AND DISCUSSION

3.3.1 Corm analyses

The qualitative analyses of corm tissue excised from different regions within a single corm, and corms harvested at various times of the year (section 3.2.5.1), indicated that hypoxoside could be detected in all corm material (Figure 3.5). Although the presence of hypoxoside was consistent, data obtained from quantitative analyses of the different corm extracts (Table 3.1) proved that:

1. the hypoxoside content of regions 1, 2 and 3 (Figure 2.3) of the upper half of corms are similar,
2. the lower half of a corm contains less hypoxoside than the upper half,
3. fluctuations do occur in the amount of hypoxoside present within corms harvested at various times of the year. Of the months analysed, corms harvested in August contained the lowest amount of hypoxoside, and
4. on average 6,78 per cent hypoxoside can be extracted from corm material harvested throughout the year.

Table 3.1: Hypoxoside content in 5 g (fresh mass) of *H. rooperi* corm material.

STATUS OF CORM MATERIAL ANALYSED	HYPOXOSIDE CONTENT (percentage of the fresh mass)
REGION 1	7,63
REGION 2 (material used for producing malformed root tissue *in vitro*)	7,71
REGION 3	7,56
FEBRUARY (upper half of corm)	7,61
FEBRUARY (lower half of corm)	6,48
MAY (upper half of corm)	6,14
JULY (upper half of corm)	4,97
OCTOBER (upper half of corm)	8,67

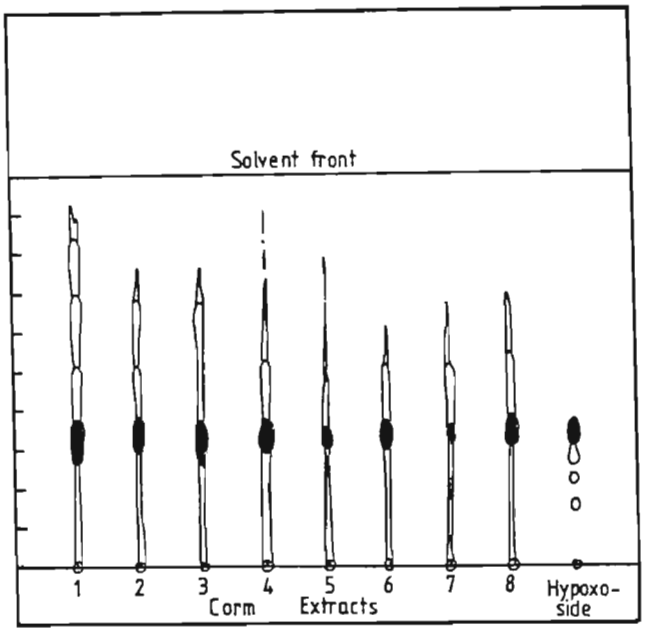


Figure 3.5: *H. rooperi* corm extracts partitioned on Kieselgel 60 F_{254} TLC plates, using butan-2-ol : benzene : distilled water : methanol (4:3:2:1) (solvent I). 1, 2 and 3 = tissue excised from regions 1, 2 and 3 respectively (Figure 2.3), of the upper half of corms harvested in February. 4 = tissue randomly excised from the upper half of corms harvested in February. 5 = tissue randomly excised from the lower half of corms harvested in February. 6, 7 and 8 = tissue randomly excised from the upper half of corms harvested in May, July and October respectively.

A detailed qualitative analysis of corm material also yielded much information. Firstly, it proved that the technique developed for extracting and purifying hypoxoside (section 3.2.3) was suitable for isolating this chemical from corm tissue. Secondly, a TLC trace of a corm extract partitioned in solvent I (Figure 3.6) and subsequently in solvent II (Figure 3.7), indicated that the Rf values for the compound which co-eluted with hypoxoside, as detected under the UV light, occurred at 0,19 to 0,29 and 0,56 to 0,69 respectively. Further, identification of this compound after purification in solvent II and using HPLC, provided an HPLC trace for the corm extract (Figure 3.8a) similar to that obtained for authentic hypoxoside (Figure 3.8b). A few spurious peaks were detected in the corm extract, within the first 3 minutes after injection of the compound into the HPLC. However, these were found to be silica-gel contaminants (Figure 3.8c). The retention time for hypoxoside was 6,7 minutes.

Phenolics are mainly studied in plants by chemical techniques (RIBÉREAU-GAYON, 1972; HARBORNE, 1973). At the transmission electron microscope level precise phenolic identification is not easily made. Post-fixation of material with osmic acid gives a similar electron dense appearance to lipids and phenolics. Therefore, although phenolics are stainable (BRISSON, ROBB and PETERSON, 1976; McCLURE, 1979; HAYAT, 1981), the problem is mainly to distinguish these compounds from other osmiophilic material. According to BRISSON, ROBB and PETERSON (1976), phenolics generally have a more granular appearance than lipids. Furthermore, tissues impregnated with phenolics are not easily sectioned and require a longer fixation period or the use of a very low viscosity embedding medium.

Investigations on the ultrastructure of *H. rooperi* corm material harvested in February, indicated that large round and/or amoeboid-shaped osmiophilic globules are present within the parenchyma cells of the cortex of the corm (Plate 3.3a and b). These globules are located in the vacuoles of cells containing relatively little cytoplasm and starch. Whether these are hypoxoside inclusions or not could not be unequivocally proven. Staining of freeze microtome sections (section 2.2.10) of corm material, with ferric chloride (a common phenolic stain), did not confirm

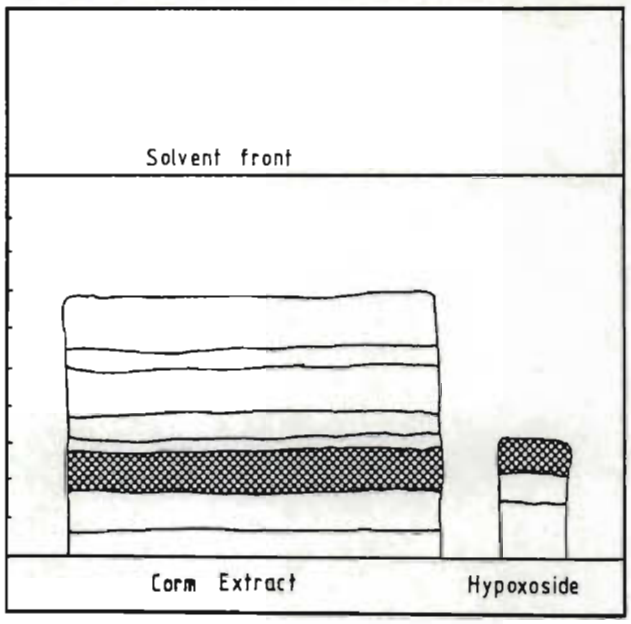


Figure 3.6: *H. rooperi* corm extract partitioned on Kieselgel 60 F_{254} TLC plates, using butan-2-ol : benzene : distilled water : : methanol (4 : 3 : 2 : 1) (solvent I).

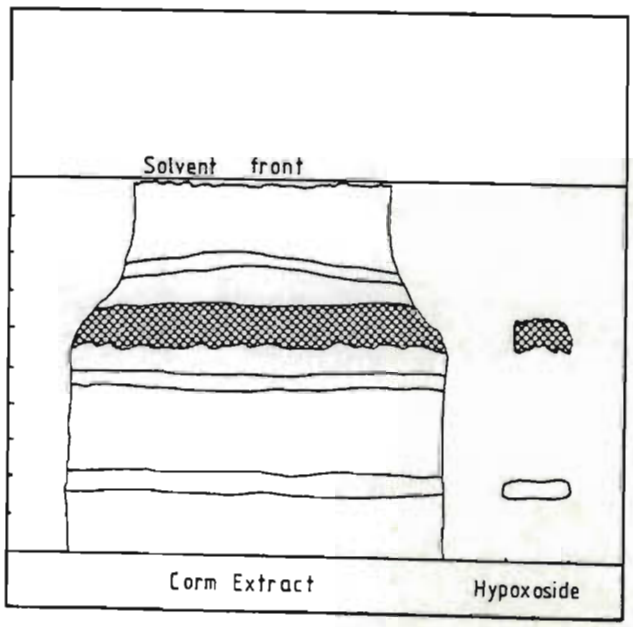


Figure 3.7: *H. rooperi* corm extract partitioned on Kieselgel 60 F_{254} TLC plates, using n-butanol : acetic acid : diethylether : water (9 : 6 : 3 : 1) (solvent II).

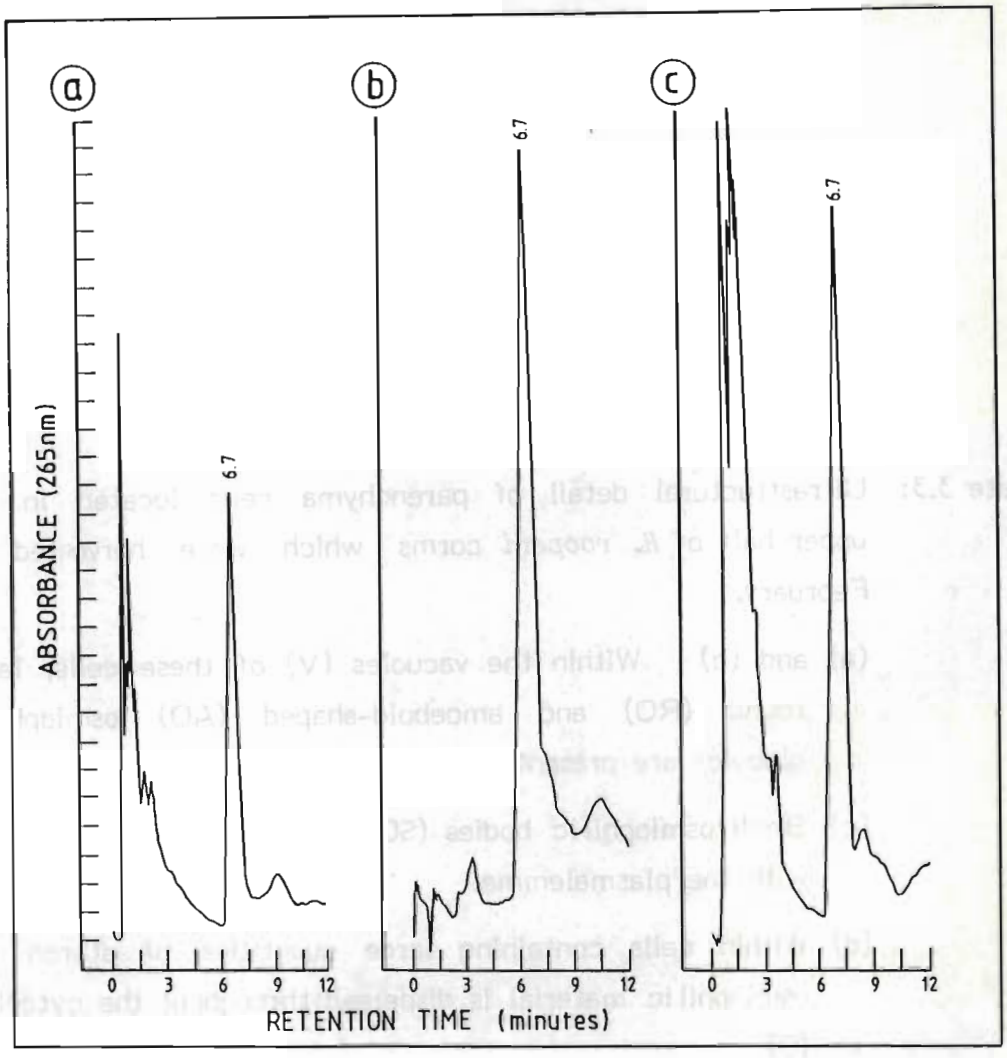


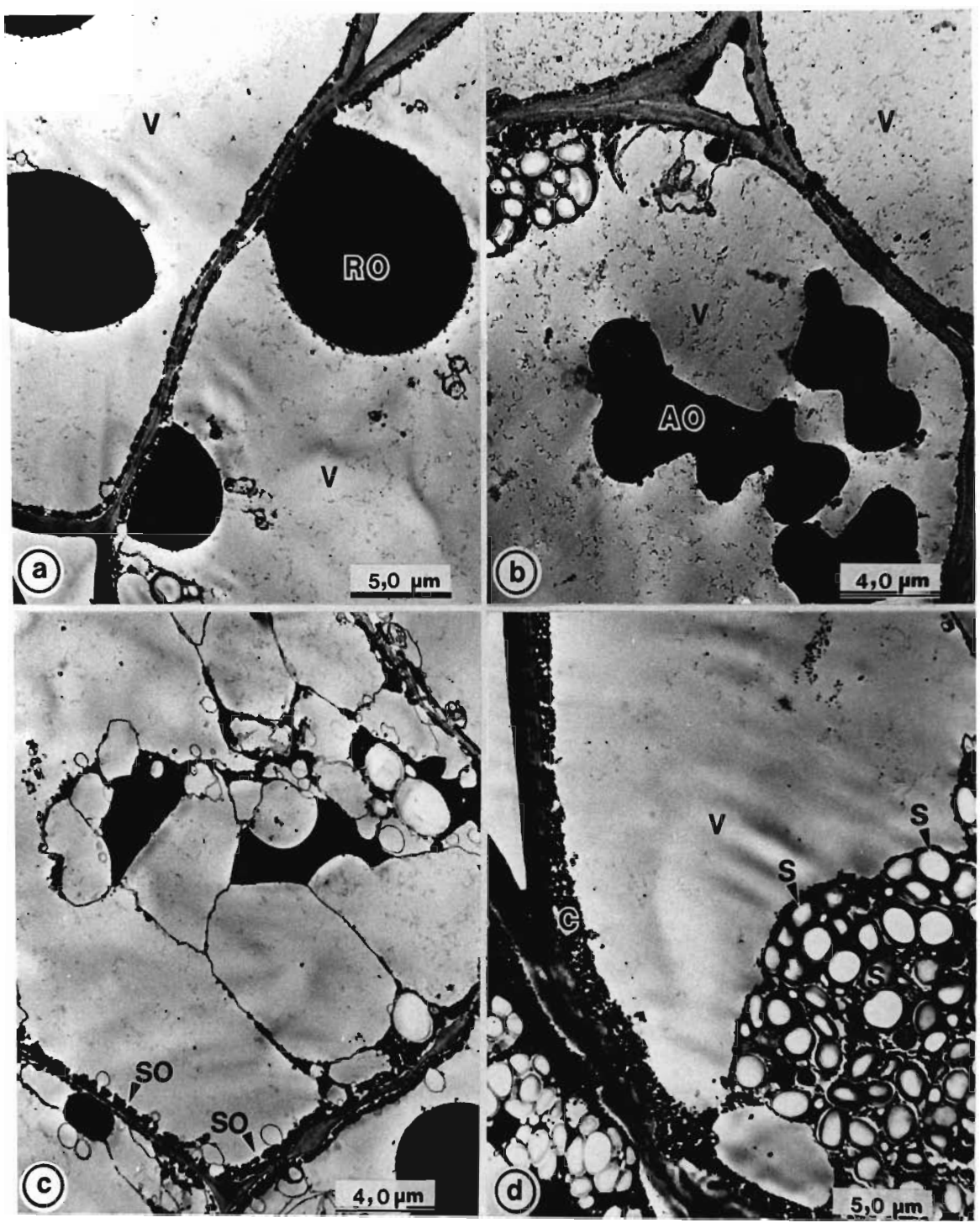
Figure 3.8: High pressure liquid chromatograms of (a) *H. rooperi* corm tissue extract, (b) authentic hypoxoside and (c) authentic hypoxoside after separation on Kieselgel 60 F_{254} TLC plates.

Plate 3.3: Ultrastructural detail of parenchyma cells located in the upper half of *H. rooperi* corms which were harvested in February.

(a) and (b) Within the vacuoles (V) of these cells, large round (RO) and amoeboid-shaped (AO) osmiophilic globules are present.

(c) Small osmiophilic bodies (SO) are also closely associated with the plasmalemma.

(d) Within cells containing large quantities of starch (S) osmiophilic material is dispersed throughout the cytoplasm (C).


V
RO
V
a
5,0 µm
V
V
AO
b
4,0 µm
SO
SO
c
4,0 µm
V
S
S
S
C
d
5,0 µm

the presence of hypoxoside. Furthermore, attempts to determine whether these globules were phenolic in nature using ultraviolet microscopy, were unsuccessful. Therefore, based upon the fact that the globules reacted with osmium tetroxide and were located within the vacuole of the cells, as are many other phenolics (BAUR and WALKINSHAW, 1974; MUELLER and BECKMAN, 1974, 1976; McCLURE, 1979), the observed globules were assumed to be phenolic in nature. Since no phenolic, other than hypoxoside has been isolated from *H. rooperi* corms, the globules are most likely hypoxoside.

From investigations presently being undertaken on the biological properties of hypoxoside (which are not reported upon in this study), evidence has been found which supports the view that the osmiophilic deposits are probably hypoxoside. For example, soybean callus cells cultured for 1 month on MILLER's medium (Table 3.2) supplemented with 0,4 $g\ell^{-1}$ authentic hypoxoside, when viewed using the transmission electron microscope, were found to contain amoeboid-shaped osmiophilic globules in their vacuoles (Plate 3.4a). These were primarily deposited near the tonoplast (Plate 3.4b). In untreated soybean callus however, no osmiophilic deposits were detected in the cell vacuoles (Plate 3.4c and d).

Returning to the ultrastructure of corm cells, in addition to the large round and amoeboid-shaped globules present within cells possessing large vacuoles, small osmiophilic bodies were also detected. These small globules were usually found in close association with the plasmalemma (Plate 3.3c). Alternatively, in cells containing large quantities of starch, the osmiophilic material was dispersed throughout the cytoplasm (Plate 3.3d). This type of phenolic distribution is commonly associated with the leaching of these compounds from the vacuoles into the cytoplasm and is hence regarded as an artifact associated with poor fixation (MUELLER and BECKMAN, 1974). Whether or not this is true for the material analysed in this investigation could not be established. Further transmission electron microscope studies would be necessary to determine this fact. It does however seem unlikely that the material was poorly fixed, as cells with large vacuoles and well preserved round or amoeboid-shaped osmiophilic globules, were often located adjacent to starch containing cells with osmiophilic cytoplasm .

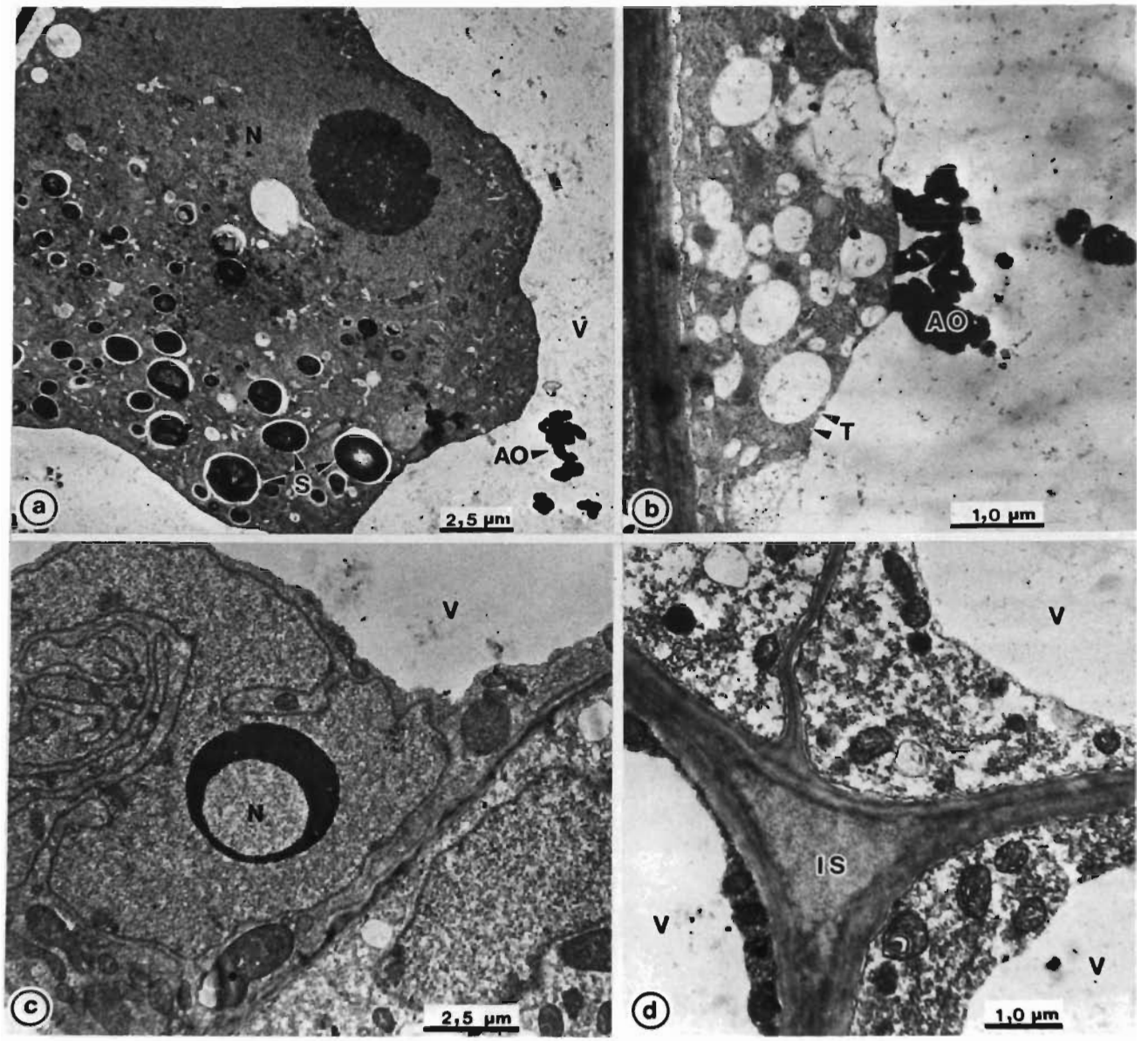
Table 3.2: Basal medium used for the soybean callus bioassay (MILLER, 1963, 1965).

STOCK SOLUTION	CHEMICAL	MASS $g\ell^{-1}$ STOCK SOLUTION	ml STOCK SOLUTION USED ℓ^{-1} MEDIUM
1	KH_2PO_4	3,0	100
	KNO_3	10,0	
	NH_4NO_3	10,0	
	$Ca(NO_3)_2.4H_2O$	5,0	
	$MgSO_4.7H_2O$	0,715	
	KCl	0,65	
	$MnSO_4.4H_2O$	0,14	
2	NaFeEDTA	1,32	10
	$ZnSO_4.7H_2O$	0,38	
	H_3BO_3	0,16	
	KI	0,08	
	$Cu(NO_3)_2.3H_2O$	0,035	
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0,01	
3	myo-inositol	10,0	10
	nicotinic acid	0,2	
	pyridoxine. HCl	0,08	
	thiamine. HCl	0,08	
4	NAA	0,02	10
Additional	Sucrose		30 $g\ell^{-1}$ medium
	Agar		10 $g\ell^{-1}$ medium

pH adjusted to 5,8 with NaOH

Plate 3.4: (a) and (b) Ultrastructural detail of soybean callus tissue treated with hypoxoside, indicating that amoeboid-shaped osmiophilic globules (AO) are present within the vacuoles (V) or in close association with the tonoplast (T) of these cells. N = nucleus, S = starch granules.

(c) and (d) Within untreated soybean callus tissue, osmiophilic deposits are absent. IS = intracellular space, N = nucleus, V = vacuole.


N
V
AO
S
a
2,5 µm
AO
T
b
1,0 µm
V
N
c
2,5 µm
V
IS
V
V
d
1,0 µm

3.3.2 Cultured tissue analyses

Of the 5 forms of cultured tissue analysed, only 3 possessed compounds which co-chromatographed with authentic hypoxoside (Figure 3.9), namely, root forming callus and malformed root tissue differentiated from flower bud and corm explants. The quantitative assessment of hypoxoside within these tissues (Table 3.3), proved that the root forming callus contained the lowest level of hypoxoside. Very similar levels of hypoxoside were present in the malformed root tissues of flower bud and corm origin. Since only root-type tissue contained hypoxoside, it appears that root differentiation is necessary for hypoxoside to be produced in cultured tissue. These results suggest that the root may be the site of hypoxoside synthesis. A number of ultrastructural studies have revealed the presence of phenolics in root cells of various plants (MACE, 1963; GINZBURG, 1967; BECKMAN and MUELLER, 1970; MUELLER and BECKMAN, 1974, MACE and HOWELL, 1974; MUELLER and BECKMAN, 1976). Evidence of the synthesis of these compounds in this tissue, has however not been established. To determine this, it has been stated that a more co-ordinated effort using biochemical, histochemical and autoradiographic techniques in conjunction with electron microscopy is necessary (MUELLER and BECKMAN, 1974).

Table 3.3: Hypoxoside content in 5 g (fresh mass) of cultured tissue, obtained from *H. rooperi* flower bud and corm explants.

CULTURED TISSUE ANALYSED	HYPOXOSIDE CONTENT (percentage of the fresh mass)
Root forming callus	0,004
Malformed root tissue developed from flower buds	0,017
Malformed root tissue developed from corm explants	0,015

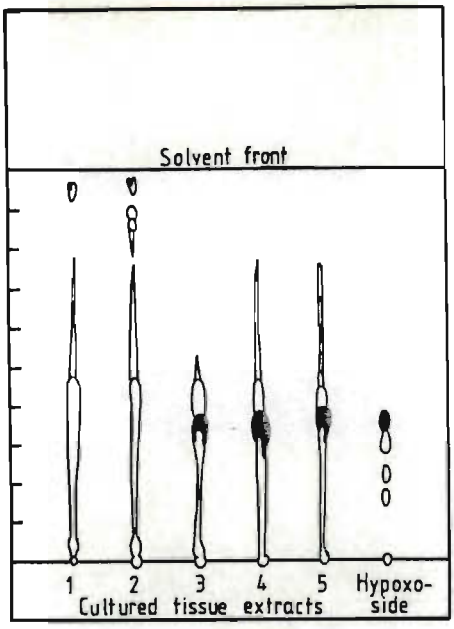


Figure 3.9: Cultured tissue extracts partitioned on Kieselgel 60 F_{254} TLC plates, using butan-2-ol : benzene : distilled water : methanol (4:3:2:1) (solvent I). 1 = undifferentiated *H. rooperi* callus. 2 = shoot forming *H. rooperi* callus. 3 = root forming *H. rooperi* callus. 4 = malformed root tissue established from *H. rooperi* flower buds. 5 = malformed root tissue established from *H. rooperi* corm explants.

Having established that malformed root tissue developed from corm or flower bud explants contained the highest amount of hypoxoside, all further investigations were performed using cultures of this tissue. The first experiment undertaken was to extract and isolate hypoxoside from malformed root tissue, using the entire extraction procedure as outlined in section 3.2.3 (*Qualitative analyses*). Results obtained from this analysis proved that the isolation of hypoxoside from this tissue is not quite as simple as from corm material. The partitioning of extracts of malformed root tissue in solvent I (Figure 3.10) resulted in a large UV fluorescent band extending from Rf 0,30 to 0,44 and a smaller band at Rf 0,21 to 0,26. For authentic hypoxoside, the Rf value was 0,27 to 0,31.

As it was not obvious using the UV fluorescence technique and phenolic spray reagent which compound was hypoxoside, regions 1; 2; 3 and 4 (Figure 3.10) were eluted separately from the TLC plate and subsequently partitioned in solvent II. Results obtained in this analysis (Figure 3.11) indicated quite clearly that only eluants from region 1 and 2 contained hypoxoside. This was further confirmed by analysing the UV fluorescent bands of these eluants once separated in solvent II, using the HPLC. Results obtained (Figure 3.12) indicated that in both eluant 1 and 2, peaks which co-eluted with hypoxoside could be detected. In addition to these peaks of hypoxoside, spurious peaks appeared in the extracts within the first 3 minutes after sample injection. These were clearly related to silica-gel contaminants as they were not present in authentic hypoxoside, but could be detected in the hypoxoside standard which had previously been separated in solvent II.

In addition to hypoxoside, in eluant 1, two other compounds were detected using UV fluorescence (Rf 0,33 to 0,37 and 0,65 to 0,74). Alternatively, in the eluant from region 2, only one other compound was detected (Rf 0,68 to 0,74). Thus the eluant from region 2 apparently contained a purer sample of hypoxoside than the eluant isolated from region 1. Eluants from region 3 and 4 did not contain hypoxoside (Figure 3.11). Instead they were each composed of a single dark UV fluorescent band. In eluant 3 this band extended from Rf 0,66 to 0,72 whereas in eluant 4 it was located between Rf 0,70 and 0,81. The exact nature of these

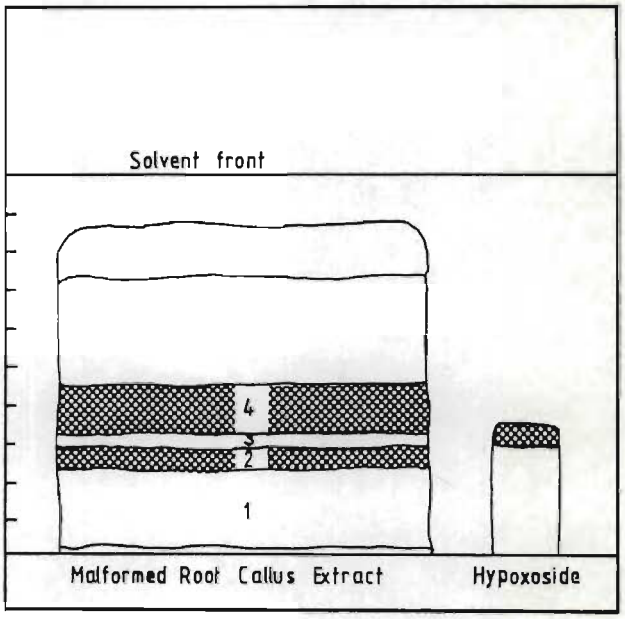


Figure 3.10: Cultured malformed root tissue extract partitioned on Kieselgel 60 F_{254} TLC plates using butan-2-ol : benzene : distilled water : methanol (4 : 3 : 2 : 1) (solvent I).

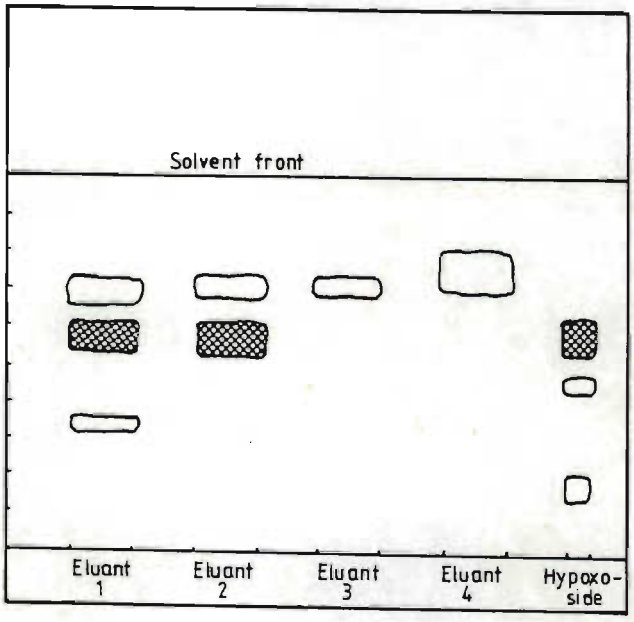


Figure 3.11: Cultured malformed root tissue extracts partitioned on Kieselgel 60 F_{254} TLC plates, using n-butanol : acetic acid : diethylether : water (9 : 6 : 3 : 1) (solvent II).

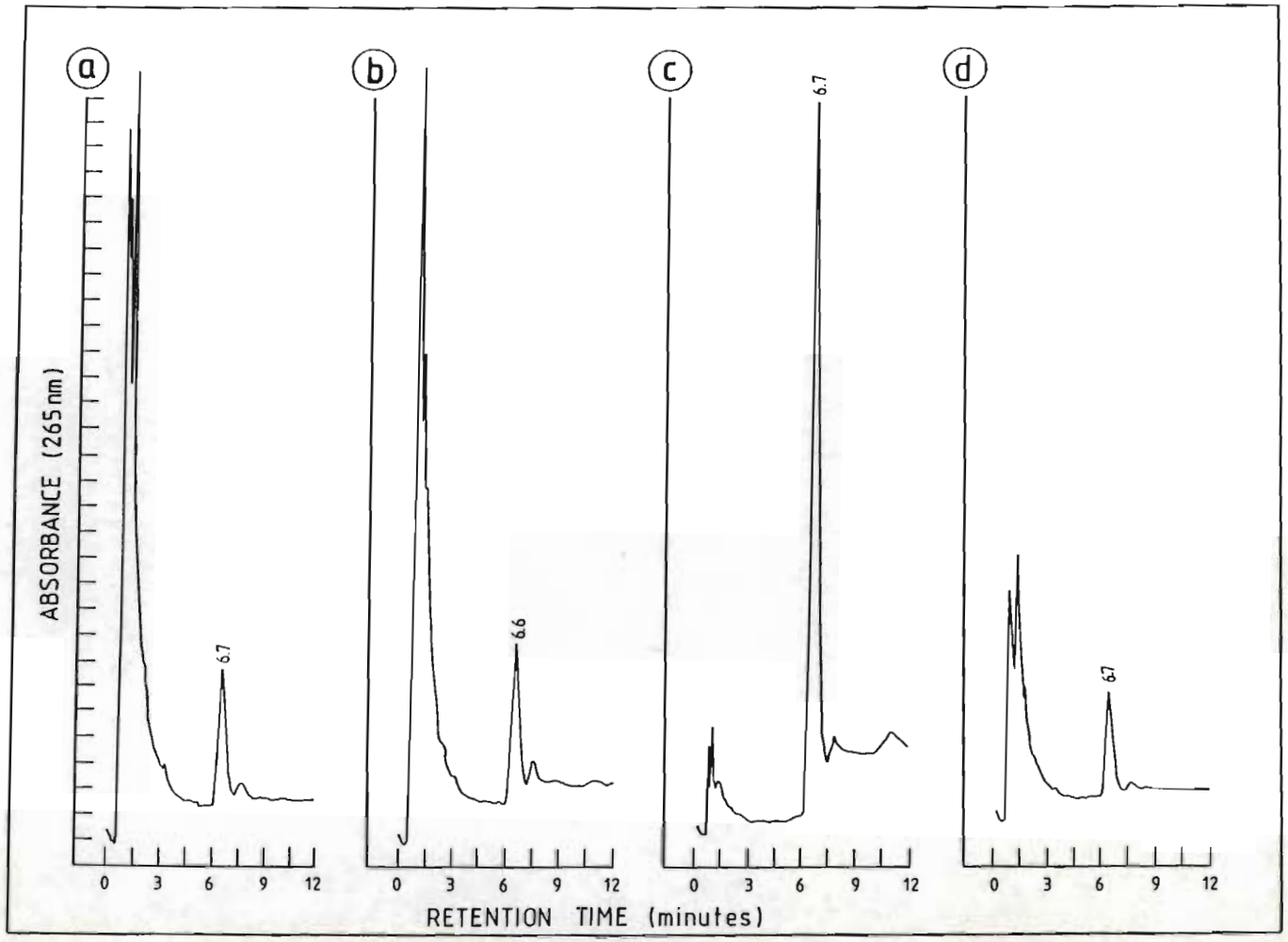


Figure 3.12: High pressure liquid chromatograms of (a) the compound isolated from eluant 1, which co-eluted with authentic hypoxoside, (b) the compound isolated from eluant 2, which co-eluted with authentic hypoxoside, (c) authentic hypoxoside and (d) authentic

compounds is unknown. Further research regarding this aspect could be valuable, as it may lead to the discovery of other pharmacologically useful compounds within *H. rooperi* cultures.

Investigations on the anatomy of cultured malformed roots, indicated that this tissue possessed a structure very similar to the normal contractile roots of *H. rooperi*. In the cultured roots however, the relative proportion of ground parenchyma produced was much higher than in normal roots. Furthermore, in the former tissue's root-tip region, in addition to the normal pattern of cell division occurring, numerous meristematic centres were also present (Plate 3.5). With respect to the ultrastructure of malformed root cells, transmission electron microscope investigations revealed that the majority of the cells (located in the region indicated in Plate 3.5*), contained large nuclei, much cytoplasm and many vacuoles (Plate 3.6a). Occasionally small round or amoeboid-shaped osmiophilic deposits were present in the vacuoles of these cells (Plate 3.6b). In the vacuoles of cells situated nearer the developing stele of malformed roots, larger osmiophilic deposits were more common (Plate 3.6c). Further away from the root-tip region of these roots, the ground parenchyma cells contained larger vacuoles. Osmiophilic deposits were rarely detected in these vacuoles. Generally therefore, the ultrastructural results support the quantitative data, i.e. that relative to corm material, very low levels of hypoxoside are present in malformed root tissue.

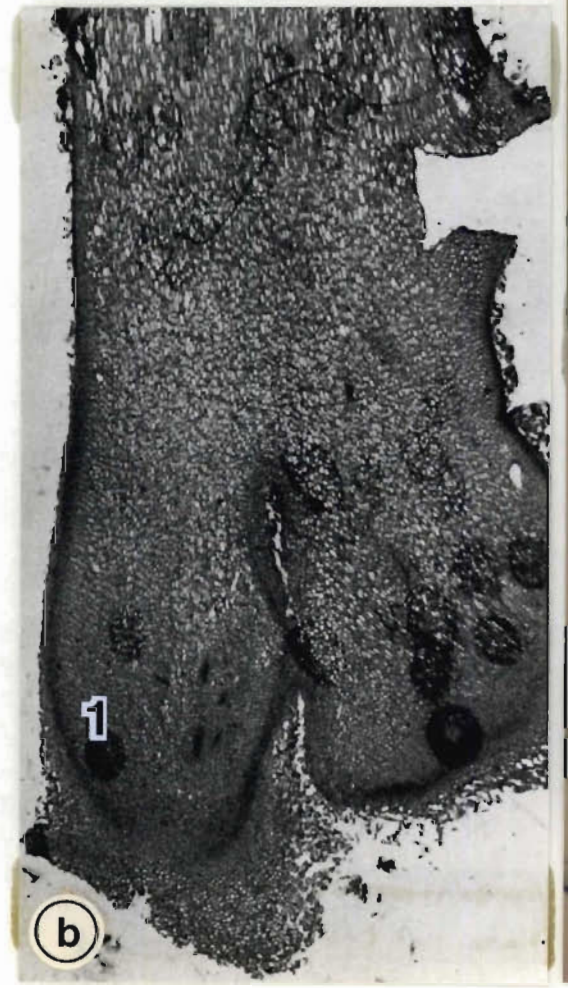
Investigations of the phenolic content of cultures in relation to culture growth, have revealed that many product-growth patterns exist in phenolic producing cultures (CONSTABEL, 1968; FORREST, 1969b; NASH and DAVIES, 1972a; SUGANO, IWATA and NICHI, 1975; IBRAHIM and EDGAR, 1976; WESTCOTT and HENSHAW, 1976; AMORIM, DOUGALL and SHARP, 1977; PHILLIPS and HENSHAW, 1977). For this reason, it is not possible to predict when phenolic levels in cultures will be greatest. As this type of information is valuable in the event of a culture system being commercialized, an investigation of the amount of hypoxoside present in developing malformed root tissue, differentiated from corm explants, was undertaken. The results obtained from this analysis (Figure 3.13a), showed that the levels of hypoxoside were very low in the developing tissue during the first 3 months following culture inoculation. From 3 to 4

Plate 3.5: (a) Malformed root tissue (R) differentiated from a *H. rooperi* corm explant. x 1,6.

(b) Longitudinal section through the root tissue (R) in 3.5a. x 31,5.

(c) Enlargement of region I in 3.5b, indicating clearly the presence of meristemoids in the root tip region. x 90. Insert: Meristemoid composed of actively dividing cells. x 180.


R
a


1
b

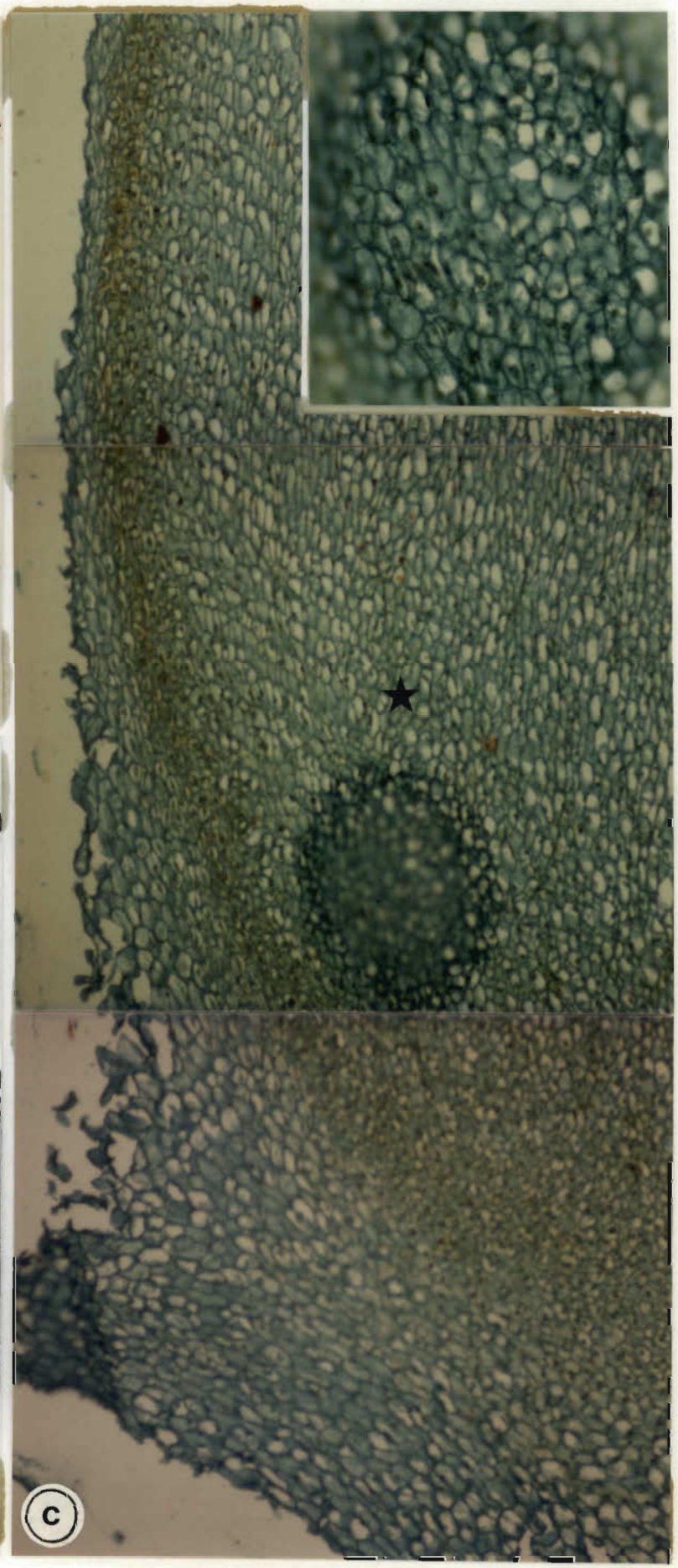
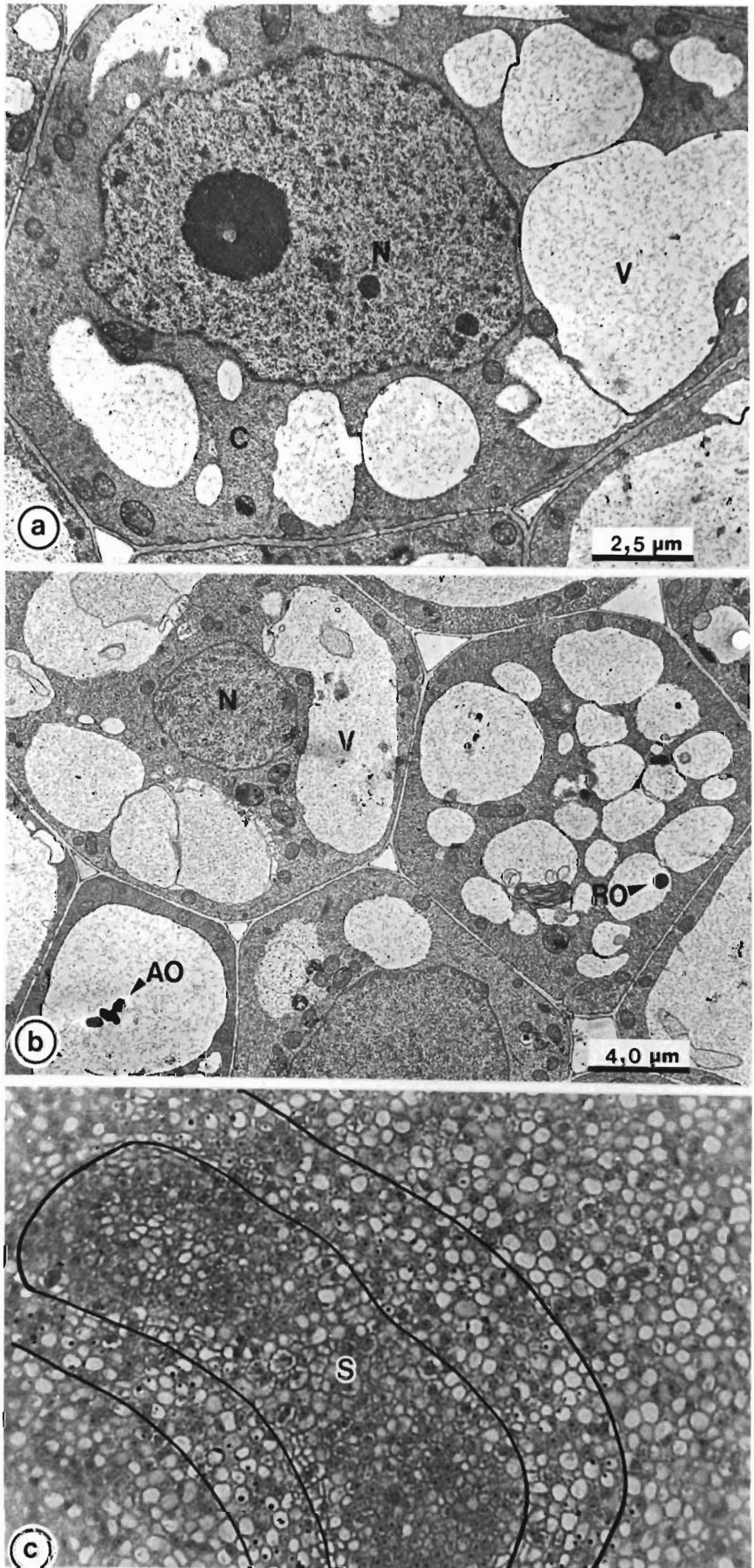

c

Plate 3.6: Ultrastructural detail of malformed root tissue cells established from *H. rooperi* corm explants.

(a) The majority of the cells of this tissue contain a large nucleus (N), much cytoplasm (C) and many small vacuoles (V).

(b) Small round (RO) and/or amoeboid-shaped (AO) osmiophilic deposits were occasionally detected in the vacuoles (V) of these cells.

(c) Cells containing osmiophilic deposits (as indicated by the demarcated area), were most often found surrounding differentiating stelar regions (S).


N
V
C
a
2,5 µm
N
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RO
AO
b
4,0 µm
S
c

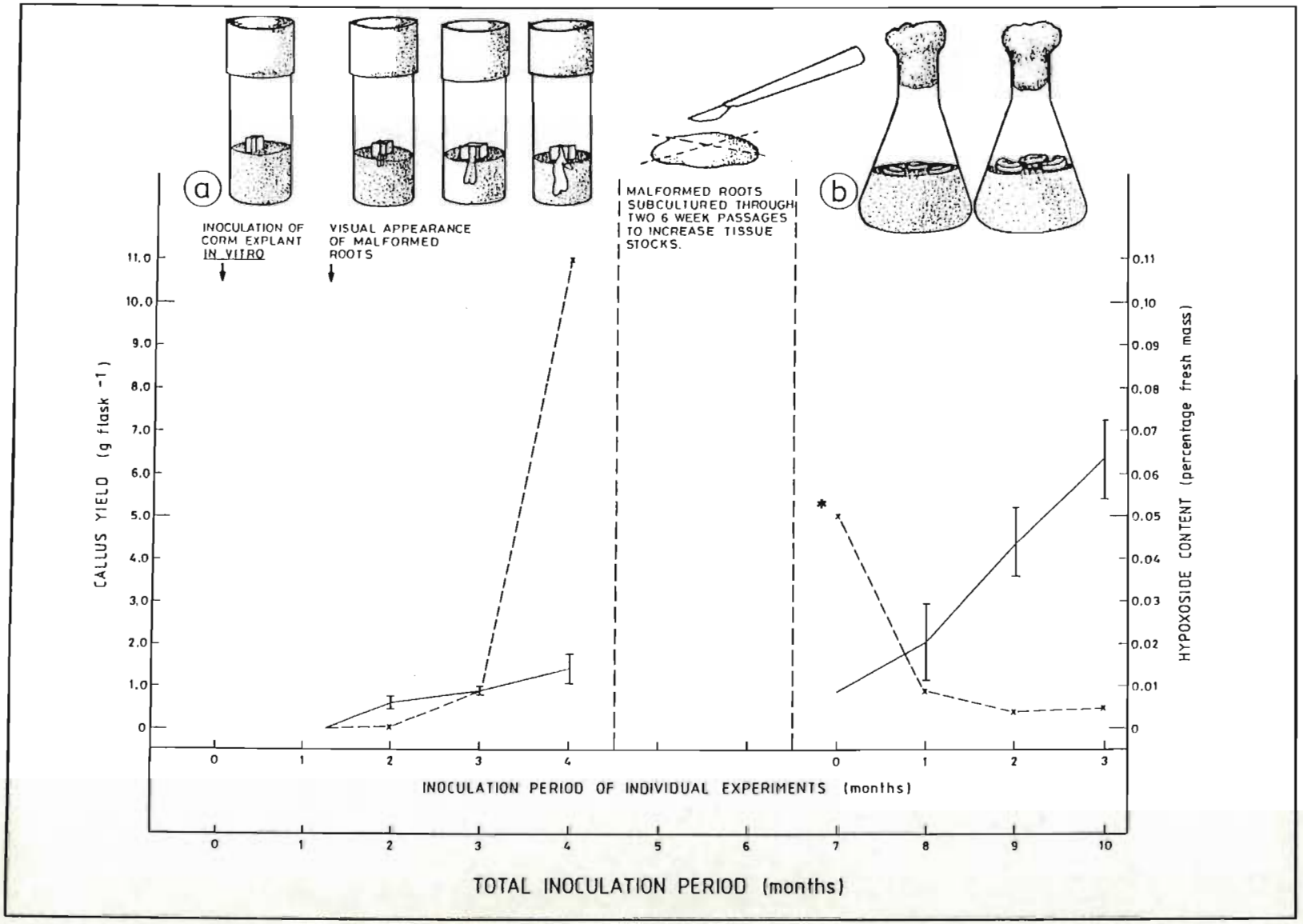

Figure 3.13: Changes in the growth rate (———) and hypoxoside content (x---x) of malformed root tissue established from *H. nogueri* corm explants, as influenced by inoculation period.

months the hypoxoside content increased markedly in this tissue and at 4 months, 0,11 per cent hypoxoside was detected in the malformed roots. Following subculturing of this tissue and subsequent treatment as illustrated in Figure 3.4, the results represented in Figure 3.13b were obtained. These results indicate that during the process of malformed root tissue accumulation via subculturing, the levels of hypoxoside in this tissue decreased to almost 50 per cent (Figure 3.13b*) of the amount detected in 4 month old malformed roots. After a further month in culture, the percentage hypoxoside present in this tissue was almost negligible. During the rest of the culture period, the hypoxoside content remained low. In contrast to this, the amount of tissue increased at an exponential rate.

Consideration of the results illustrated in Figure 3.13b separately from those represented in Figure 3.13a, leads to the conclusion that an inverse relationship exists between hypoxoside content and tissue growth. Alternatively, the amalgamation of both sets of data more correctly indicates that this antagonistic relationship occurs between the levels of hypoxoside and the subculturing process. A number of factors may be responsible for this association. It is possible that;

1. successive subculturing of malformed root tissue resulted in the loss of the cells potential to produce hypoxoside,
2. within the cultured tissue the rates of hypoxoside degradation were higher than the rates of synthesis,
3. in culture, the intermediates necessary for hypoxoside synthesis were absent, and
4. the culture conditions required for tissue growth, were selective of low yielding hypoxoside cell lines.

For the above factors to be operative, the synthesis of hypoxoside by the cultured tissue, is a necessary prerequisite. If not, how would the above relationship have developed? It is possible, seeing as the corm explants utilized for *in vitro* cultures contain high levels of hypoxoside (Table 3.1), that this chemical may have been directly translocated from the explant to the developing root tissue. The excision of the malformed root tissue from the initial explant, would thus halt this process. Successive subculturing of the material would subsequently

result in a decrease of the accumulated hypoxoside. If this process was operative, the production of hypoxoside using *H. rooperi* cultures would not be possible. Hence the commercial potential of the technique would be lost. For this reason, an investigation was initiated to determine whether malformed root cultures of *H. rooperi* can synthesise hypoxoside.

It is well established that carbohydrates (sucrose and glucose), nitrogen, and light influence the production of phenolics in both intact plants (McCLURE, 1979) and *in vitro* cultures (BUTCHER, 1977; DOUGALL, 1979; HARBORNE, 1980). Generally in the latter system, high levels of carbohydrate promote the synthesis of phenolics (CONSTABEL, 1968; AMORIM, DOUGALL and SHARP, 1977; ZENK, EL-SHAGI and ULBRICH, 1977). Alternatively, the synthesis and accumulation of phenolics is inhibited by high levels of nitrogen (WESTCOTT and HENSHAW, 1976; AMORIM, DOUGALL and SHARP, 1977; MEHTA and SHAILAJA, 1978). With regard to the influence of light upon the production of phenolics by *in vitro* cultures, it has been shown that the formation of phenolics is stimulated by light conditions (REINERT, CLAUSS and VON ARDENNE 1964; FORREST, 1969a; HAHLBROCK and WELLMANN, 1970; DAVIES, 1972a; HAHLBROCK, 1972; STICKLAND and SUNDERLAND, 1972b; KREUZALER and HAHLBROCK, 1973; BUTCHER, 1977).

Bearing this information in mind, a brief analysis of the influence of sucrose, nitrogen and light upon hypoxoside production, was undertaken. From this analysis, the results obtained (Table 3.4) demonstrated that;

1. tissue cultured under control conditions (treatment 1), contained low levels of hypoxoside throughout the culture period,
2. neither a decrease nor an increase in the levels of sucrose in the culture medium (treatments 2 and 3 respectively), improved hypoxoside synthesis,
3. low levels of nitrogen in the culture medium (treatment 4) resulted in an increase in the amount of hypoxoside produced by the cultured tissue. (This increase being manifest during the latter half of the culture period),
4. the culturing of tissue in continuous darkness (treatment 5) markedly improved hypoxoside production. In contrast to

Table 3.4: Hypoxoside content in 5 g (fresh mass) of malformed root tissue, after being grown under various culture conditions.

TREATMENT				HYPOXOSIDE CONTENT OF MALFORMED ROOT TISSUE (percentage of the fresh mass)	
Number	Sucrose Concentration ($g\ell^{-1}$)	Nitrogen Concentration (NH_4NO_3 and KNO_3, $g\ell^{-1}$)	Light : Dark (Hours)	One month after inoculation on fresh medium	Three months after inoculation on fresh medium
1 CONTROL	30	165+95	16 : 8	0,009	0,005
2	20	165+95	16 : 8	0,000	0,000
3	40	165+95	16 : 8	0,001	0,000
4	30	82,5+47,5	16 : 8	0,006	0,055
5	30	165+95	0 : 24	0,036	0,045

treatment 4 however, in treatment 5 the higher levels of hypoxoside were evident within 1 month of the tissue being cultured under dark conditions.

Although in this investigation high levels of sucrose and light did not improve hypoxoside production as expected, the results obtained clearly showed that cultured malformed root tissue can synthesise hypoxoside *in vitro*.

3.4 CONCLUSION

From a layman's viewpoint, the production of valuable natural plant products in culture, is seen as the growing of plant cells in large fermentation tanks from which vast quantities of the desired chemicals can be tapped. Although this degree of success is seldom attained, many researchers foresee tissue culturing as a means of producing valuable commodity chemicals such as steroids, anti-cancer agents and precursors for contraceptives. From the series of qualitative and quantitative investigations undertaken in this study, it was found that only 3 of the 5 morphogenetic forms of *in vitro* cultured *H. rooperi* tissue contained hypoxoside. Of these 3 forms of material, the malformed root tissue established from corm explants contained the highest amount of this chemical. Unfortunately however, relative to the hypoxoside content of *in vivo* grown corms, these levels were found to be extremely low.

Analyses to try and elucidate why the levels of hypoxoside in cultured malformed root tissue were low, eliminated the possibility that there existed a direct translocation of the chemical from the initial explant to the developing tissue. From investigations undertaken it was established that in fact an antagonistic relationship exists between the hypoxoside content of cultured malformed roots and the process of subculturing. Further it was determined that the effect of subculturing could be overcome either by growing the tissue on a MURASHIGE and SKOOG (1962) medium in which the levels of nitrogen had been reduced, or by eliminating light from the culture environment of the tissue. From these investigations it was shown that cultured malformed root tissue can synthesise hypoxoside *in vitro*. The extent to which this synthetic capacity can be manipulated and enhanced, remains to be determined. Possible methods which could

be investigated with regard to this aspect include: the establishment of malformed root cultures following the four-step strategy presented by DEUS and ZENK (1982) and advice given by YEOMAN, MIEDZYBRODZKA, LINDSEY and Mç LAUCHLAN (1980); precursor feeding as described by TABATA (1977) and KURZ and CONSTABEL (1979); and mycelial homogenate inoculation, a process found to be successful for enhancing phenolic production in potato (ROBERTSON, FRIEND, AVEYARD,UFFEEE and HOMANS, 1968; ÉRSEK and SZIRÁKI, 1980), pea (BAILEY, 1970), bean (DIXON and FULLER, 1976) and tobacco (HELGESON, BUDDE and HABERLACH, 1978) cultures.

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APPENDIX 1A: List of monocotyledonous plants with demonstrated potential for regeneration *in vitro* (excluding the Orchidaceae).

FAMILY	SPECIES	REFERENCE
Agavaceae	*Agave* L. species	GROENEWALD, WESSELS and KOELEMAN, 1977
	Cordyline banksii Hook. f.	HUSSEY, 1978b
	C. terminalis (L.) Kunth	KUNISAKI, 1975; MILLER and MURASHIGE, 1976; KUNISAKI, 1977; MEE, 1978
	Cordyline Adans. species, hybrids and cultivars	DEBERGH, 1976; DE FOSSARD, 1981
	Dracaena godseffiana Hort.	MILLER and MURASHIGE, 1976; DE FOSSARD, 1981
	Dracaena L. species, hybrids and cultivars	MURASHIGE, 1974; DEBERGH, 1975, 1976; OGLESBY, 1978; DE FOSSARD, 1981
	Sanseveria trifasciata Prain	MURASHIGE, 1974; BERRY-LOWE and PERTUIT, 1977
	Yucca gloriosa L.	DURMISHIDZE, GOGOBERIDZE and MAMALADZE, 1983
	Yucca L. species	MURASHIGE, 1974; LITZ and CONOVER, 1977
Amaryllidaceae	*Alstroemeria* L. species	ZIV, KANTEROVITZ and HALEVY, 1973
	Amaryllis L. species	MURASHIGE, 1974; BAPAT and NARAYANASWAMY, 1976; HOLDGATE, 1977; PAJERSKI and ASCHER, 1977
	Clivia Lindl. species	HOLDGATE, 1977
	Furcraea gigantea Venten.	LAKSHMANAN and JANARDHANAN, 1977
	Hippeastrum hybridum Hort.	MII, MORI and IWASE, 1974; HUSSEY, 1975a, 1976b

FAMILY	SPECIES	REFERENCE
	Hippeastrum Herb. species and cultivars	SEABROOK and CUMMING, 1977; HUSSEY, 1978b: GRAVES, REID and MATHES, 1978
	Ipheion Rafin. species, hybrids and cultivars	HUSSEY, 1975a; HOLDGATE, 1977
	Narcissus biflorus Curt.	KOUL and KARIHALOO, 1977
	N. pseudonarcissus L.	HUSSEY, 1975a
	N. tazetta L.	STONE, 1973; HOSOKI and ASAHIRA, 1980a; STEINITZ and YAHEL, 1982
	N. triandrus L.	McCHESNEY, 1971
	Narcissus L. species, hybrids and cultivars	HUSSEY, 1975a, 1976b; SEABROOK, CUMMING and DIONNE, 1976; HOLDGATE, 1977; HUSSEY, 1977a, 1978b; SEABROOK and CUMMING, 1978; HUSSEY, 1982a; SEABROOK and CUMMING, 1982
	Nerine bowdenii W. Watts.	PIERIK and IPPEL, 1977; GROOTAARTS, SCEL and PIERIK, 1981
	N. sarniensis Herb.	PIERIK and IPPEL, 1977
	Nerine Herb. species, hybrids and cultivars	HAKKAART, MAAT and QUAK, 1975; PIERIK and IPPEL, 1977; HUSSEY, 1978b
Araceae	*Alocasia cucullata* Schott	KUNISAKI, 1977
	Amorphophallus rivieri Dur.	MOREL and WETMORE, 1951
	Anthurium andraeanum	PIERIK, STEEGMANS and VAN DER MEYS, 1974; PIERIK, 1976; KUNISAKE, 1980
	A. crystallinum Linden and Andŕe	MURASHIGE, 1979; DE FOSSARD, 1981
	A. scherzerianum Schott	PIERIK and STEEGMANS, 1976a; GEIER, 1982

FAMILY	SPECIES	REFERENCE
	Caladium bicolor Vent.	SAHAVACHARIN, 1982
	Caladium Vent. species	HARTMAN, 1974; HOLDGATE, 1977
	Colocasia esculenta (L.) Schott	NYMAN, GONZALES and ARDITTI, 1983
	Diffenbachia picta Schott	KNAUSS, 1976
	Diffenbachia Schott species	HOLDGATE, 1977; LITZ AND CONOVER, 1977; DE FOSSARD, 1981
	Monstera deliciosa Liebm.	MURASHIGE, 1979; FONNESBECH and FONNESBECH, 1980
	Monstera Adans. species	DE FOSSARD, 1981
	Philodendron cordatum Kunth	DE FOSSARD, 1981
	P. lacernum (Jacq.) Schott	KUNISAKI, 1977
	P. oxycardium C. Koch and Sello	KUNISAKI, 1977
	P. setloum C. Koch	MURASHIGE, 1979
	Philodendron Schott species	MURASHIGE, 1974; HOLDGATE, 1977
	Pinellia ternata Druce	SHOYAMA, HATANO and NISHIOKA, 1983
	Sauromatum guttatum Schott	MOREL and WETMORE, 1951
	Scindapsis aureus Engler	MILLER and MURASHIGE, 1976; KUNISAKI, 1977
	Spathiphyllum Schott species, hybrids and cultivars	KUNISAKI, 1977; STRODE and OGLESBY, 1978; OGLESBY, 1978; FONNESBECH and FONNESBECH, 1979
	Syngonium podophyllum Schott	MILLER and MURASHIGE, 1976; MAKINO and MAKINO, 1978; DE FOSSARD, 1981

FAMILY	SPECIES	REFERENCE
Bromeliaceae	*Aechmea distichantha* Lem.	ZIMMER and PIEPER, 1976
	A. fasciata (Lindl.) Baker	JONES and MURASHIGE, 1974; MURASHIGE, 1974; ZIMMER and PIEPER, 1976; HOLDGATE, 1977; HOSOKI and ASAHIRA, 1980b
	Aechmea Ruiz and Pav. hybrids	ZIMMER and PIEPER, 1976
	Ananas comosus (L.) Merr.	JONES and MURASHIGE, 1974; LAKSHMI-SITA, SINGH and IYER, 1974; MURASHIGE, 1974; PANNETIER and LANAUD, 1976; HOLDGATE, 1977; MATHEWS and RANGAN, 1979, 1981; ZEPEDA and SAGAWA, 1981
	A. sativus Schult.	MATHEWS, RANGAN and NARAYANASWAMY, 1976
	Cryptanthus bivittatus Regel	JONES and MURASHIGE, 1974; MURASHIGE, 1974; DAVIDSON and DONNAN, 1977; HOLDGATE, 1977
	Cryptanthus Otto and Dietr. species	JONES and MURASHIGE, 1974; ZIMMER and PIEPER, 1976
	Cryptbergia meadii Hort.	JONES and MURASHIGE, 1974
	Cryptbergia Hort. hybrids and cultivars	MURASHIGE, 1974; HOLDGATE, 1977
	Dyckia sulphurea C. Koch	JONES and MURASHIGE, 1974; MURASHIGE, 1974
	Guzmania lingulata Mez	MEKERS, 1977
	Guzmania Ruiz and Pav. species	JONES and MURASHIGE, 1974; HOSOKI and ASAHIRA, 1980b

FAMILY	SPECIES	REFERENCE
	Neoregelia L.B. Smith species	HOLDGATE, 1977
	Nidularium Lem. species	HOLDGATE, 1977
	Quesnelia quesneliana (Brongn.) L.B. Smith	HOSOKI and ASAHIRA, 1980b
	Tillandsia polystachya Jacq.	MEKERS, 1977
	Vriesea heliconioides Lindl.	MEKERS, 1977
	V. poelmanii Lindl.	HOSOKI and ASAHIRA, 1980b
	V. splendens Lem.	MEKERS, 1977
	Vriesea Beer species	MEKERS, 1977; MEKERS and VAN ONSEM, 1983
Commelinaceae	*Tradescantia paludosa* Anderson and Woods	SAWHNEY and NAYLOR, 1982
Cyperaceae	*Cyprus rotundus* L.	FISHER, 1977
	Pterotheca falconeria Hook.	WILKINS and HOLOWINSKY, 1965
Dioscoreaceae	*Dioscorea alata* L.	MANTELL, HAGUE and WHITEHALL, 1978
	D. bulbifera L.	FORSYTH, 1982; FORSYTH and VAN STADEN, 1982
	D. deltoidea Wall.	GREWAL and ATAL, 1976; MASCARENHAS, HENDRE, NADGIR, GHUGALE, GODBOLE, PRABHU and JAGANNATHAN, 1976
	D. discolor Kunth	DE FOSSARD, 1981
	D. floribunda Mart and Gal.	LAKSHMI-SITA, BAMMI and RANDHAWA, 1976; AMMIRATO, 1978
	D. rotundata Poir.	MANTELL, HAGUE and WHITEHALL, 1978
	D. sansiberensis Pax	RAO, 1969
	D. sylvatica (Kunth) Eckl.	FORSYTH, 1982

FAMILY	SPECIES	REFERENCE
Gramineae	*Agropyron cristatum* (L.) Gaertn.	LO, CHEN and ROSS, 1980
	A. smithii Rydb.	LO, CHEN and ROSS, 1980
	Alopecurus arundinaceus Poir	LO, CHEN and ROSS, 1980
	Andropogon gerardii Vitman	CHEN, STENBERG and ROSS, 1977
	Avena fatua L.	RINES and McCOY, 1981
	A. sativa L.	CARTER, YAMADA and TAKAHASI, 1967; CUMMING, GREEN and STUTHMAN, 1976; LÖRZ, HARMS and POTRYKUS, 1976; RINES and McCOY, 1981; HEYSER and NABORS, 1982; NABORS, HEYSER, DYKES and DEMOTT, 1983
	A. sterilis L.	RINES and McCOY, 1981
	Bromus inermis Leyss.	GAMBORG, CONSTABEL and MILLER, 1970; LO, CHEN and ROSS, 1980
	Dactylis glomerata L.	DALE, 1977; CONGER and CARABIA, 1978; CHEN, CHEN, LO and ROSS, 1982; McDANIEL, CONGER and GRAHAM, 1982; DALE and DALTON, 1983
	Eleusine coracana Gaertn.	RANGAN, 1976
	Festuca arundinacea Schreb	DALE, 1977; LOWE and CONGER, 1979; DALE and DALTON, 1983
	F. pratensis Hudson	DALE and DALTON, 1983
	Hordeum vulgare L.	NORSTOG, 1970; CLAPHAM, 1973; CHENG and SMITH, 1975; DALE and DEAMBROGIO, 1979; DALE and DALTON, 1983
	Hordeum L. hybrids	PERSHINA and SHUMNY, 1981
	Lactuca sativa L.	KOEVARY, RAPPAPORT and MORRIS, 1978; BERRY, LU, PENTAL and COCKING, 1982

FAMILY	SPECIES	REFERENCE
	Lolium multiflorum Lam.	AHLOOWALIA, 1975; DALE, 1975, 1980; DALE and DALTON, 1983
	L. perenne L.	AHLOOWALIA, 1975; DALE and DALTON, 1983
	L. rigidum Gaudin	SKENE and BARLASS, 1983
	Lolium L. hybrids	KASPERBAUER, BUCKNER and BUSH, 1979; DALE and DALTON 1983
	Oryza sativa L.	NISHI, YAMADA and TAKAHASHI, 1968; NISHI and MITSUOKA, 1969; GUHA, IYER, GUPTA and SWAMINATHAN, 1970; IYER and RAINA, 1972; NAKANO, TASHIRO and MAEDA, 1975; HENKE, MANSUR and CONSTANTIN, 1978; WERNICKE, WAKIZUKA and POTRYKUS, 1981; YAN and ZHAO, 1982; NABORS, HEYSER, DYKES and DEMOTT, 1983; WONG, KO AND WOO, 1983
	Panicum maximum Jacq.	BAJAJ, SIDHU and DUBEY, 1981; LU and VASIL, 1981; LU, VASIL and VASIL, 1981; LU and VASIL, 1982
	P. miliaceum L.	RANGAN, 1974; BAJAJ, SIDHU and DUBEY, 1981; NABORS, HEYSER, DYKES and DEMOTT, 1983; RANGAN and VASIL, 1983
	P. miliare Lam.	RANGAN and VASIL, 1983
	Paspalum scrobiculatum L.	RANGAN, 1976
	Pennisetum americanum (L.) K. Schum.	VASIL and VASIL, 1980, 1981a, 1981b, 1982a, 1982b
	P. purpureum Schum.	BAJAJ and DHANJU, 1981; WANG and VASIL, 1982
	P. typhiodeum Pers.	RANGAN, 1976; NABORS, HEYSER DYKES and DEMOTT, 1983
	Pennisetum Rich. hybrids	HAYDU and VASIL, 1981; VASIL and VASIL, 1981a
	Phleum pratensis L.	DALE and DALTON, 1983

FAMILY	SPECIES	REFERENCE
	Phragmites communis L.	SANGWAN and GORENFLOT, 1975
	Saccharum officinarium L.	HEINZ and MEE, 1969, 1971; MORI, 1971; DE FOSSARD, 1976; NADAR AND HEINZ, 1977; MARETZKI and HIRAKI, 1980; LARKIN, 1982
	Secale L. species	EAPÉN and RAO, 1982b
	Sorghastum nutans L.	CHEN, LO and ROSS, 1979
	Sorghum bicolor (L.) Moench	MASTELLER and HOLDEN, 1970; GAMBORG, SHYLUK, BRAR and CONSTABEL, 1977; THOMAS, KING and POTRYKUS, 1977; DUNSTAN, SHORT, DHALIWAL and THOMAS, 1979; WERNICKE and BRETTELL, 1980
	Stipa viridules Trir	LO, CHEN and ROSS, 1980
	Triticum aestivum L.	DUDITS, NEMFT and HAYDU, 1975; CHIN and SCOTT, 1977; SHIMADA, 1978; GOSCH-WACKERLE, AVIV and GALUN, 1979; OZIAS-AKINS and VASIL, 1982; NABORS, HEYSER, DYKES and DEMOTT, 1983
	T. dicoccum Schrank	SHIMADA, SASAKUMA and TSUNEWAKI, 1969
	T. durum Desf.	BENNICI and D'AMATO, 1978
	T. longissimum Bowden	GOSCH-WACKERLE, AVIV and GALUN, 1979
	T. monococcum L.	GOSCH-WACKERLE, AVIV and GALUN, 1979
	T. speltoides (Tausch) Godron	GOSCH-WACKERLE, AVIV and GALUN, 1979
	T. tauschii (Cass.) Schmalh.	GOSCH-WACKERLE, AVIV and GALUN, 1979
	T. timopheevii Zhuk	PROKOROV, CHERNOVA and FILIN-KOLDAKOV, 1974; GOSCH-WACKERLE, AVIV and GALUN, 1979
	T. turgidum (L.) Thell	GOSCH-WACKERLE, AVIV and GALUN, 1979

FAMILY	SPECIES	REFERENCE
	Triticum L. species, hybrids and cultivars	SHIMADA, SASAKUMA and TSUNEWAKI, 1969; SHARMA, BELLO and SAPRA, 1978; GOSCH-WACKERLE, AVIV and GALUN, 1979; EAPÉN and RAO, 1982a, 1982b; NAKAMURA and KELLER, 1982a, 1982b
	Zea mays L.	GRESSHOFF and DOY, 1973; GREEN and PHILLIPS, 1975; HARMS, LÖRZ and POTRYKUS, 1976; RAMAN, WALDEN and GREYSON, 1980; LU, VASIL and OZIAS-AKINS, 1982; VASIL, LU and VASIL, 1983
Hypoxidaceae	*Hypoxis rooperi* S. Moore	PAGE and VAN STADEN, in press
Iridaceae	*Dierama latifolium* C. Koch	PAGE and VAN STADEN, in press
	Freesia K latt species and hybrids	BRANTS and VERMEULEN, 1965; BRANTS, 1968; BAJAJ and PIERIK, 1974; HUSSEY, 1975a; PIERIK and STEEGMANS, 1975b; HUSSEY 1976b; PIERIK and STEEGMANS, 1976b; HUSSEY, 1977a; STIMART and ASCHER, 1978b
	Gladiolus grandiflorus Andr.	WILFRET, 1971; BAJAJ, SIDHU and GILL, 1983
	G. hortulans Bailey	HILDEBRANDT, 1971; SIMONSEN and HILDEBRANDT, 1971
	Gladiolus L. species, hybrids and cultivars	ZIV, HALEVY and SHILO, 1970; HUSSEY, 1975a, 1976b, 1977a, 1977b; ZIV, 1979
	Iris L. species, hybrids and cultivars	BARUCH and QUAK, 1966; MORI, 1971; FUJINO, FUJIMURA and HAMADA, 1972; MEYER, FUCHIGAMI and ROBERTS, 1975; HUSSEY, 1976a, 1976b, 1977a; REUTHER , 1977; WEILER and EMERSHAD, 1977

FAMILY	SPECIES	REFERENCE
	Neomarica coerulea (Ker-Gawl.) Sprague	HOSOKI, 1975
	Schizostylis coccinea Backh. and Harvey	HUSSEY, 1975a, 1976b
	Schizostylis Backh. and Harvey species and hybrids	HOLDGATE, 1977
	Sparaxis bicolor Ker-Gawl.	HUSSEY, 1976b
	Sparaxis Ker-Gawl. species and hybrids	HUSSEY, 1975a; HOLDGATE, 1977
Liliaceae	*Allium cepa* L.	FRIDBORG, 1971; HUSSEY, 1978b; DUNSTON and SHORT, 1979a
	A. porrum L.	DEBERGH and STANDAERT-DE-METSENAERE, 1976
	A. sativum L.	MORI, 1971; KEHR and SCHAEFFER, 1976; ABO EL-NIL, 1977; BHOJWANI, 1980
	A. tuberosum Rotler	ZEE, FUNG and YUE, 1977
	Aloe pretoriensis Pole-Evans	GROENEWALD, KOELEMAN and WESSELS, 1975; GROENEWALD, WESSELS and KOELEMAN, 1977
	Asparagus myriocladus Thunb.	KUNISAKI, 1977
	A. officinalis L.	GORTER, 1965; TAKATORI, MURASHIGE and STILLMAN, 1968; WILMAR and HELLENDOORN, 1968; BUI-DANG-HA and MACKENZIE, 1973; HASEGAWA, MURASHIGE and TAKATORI, 1973; YANG and CLORE, 1973, 1974; BUI-DANG-HA, NORREEL and MASSET, 1975; YANG and CLORE, 1976; REUTHER, 1977; YANG, 1977

FAMILY	SPECIES	REFERENCE
	A. orientalis Bess.	MURASHIGE, SHABDE, HASEGAWA, TAKATORI and JONES, 1972
	A. plumosus Bak.	FONNESBECH, FONNESBECH and BREDMOSE, 1977a, 1977b
	A. sprengeri Regel.	HUNAULT, 1976
	Asparagus L. species	MALNASSAY and ELLISON, 1970; STEWARD and MAPES, 1971; DORÉ, 1977; YAKUWA, HARADA and TSUJI, 1982
	Convallaria L. species	HOLDGATE, 1977
	Fritillaria meleagris L.	HUSSEY, 1976b
	Gasteria verrucosa Haw.	BEYL and SHARMA, 1983
	Haworthia angustifolia Haw.	KAUL and SABHARWAL, 1972
	H. atrofusca G.G.Smith	KAUL and SABHARWAL, 1972
	H. chloracantha Haw.	KAUL and SABHARWAL, 1972
	H. fasciata Haw.	BEYL and SHARMA, 1983
	H. maughanii Poelln	KAUL and SABHARWAL, 1972
	H. planifolia var. *setulifera* Poelln	WESSELS, GROENEWALD and KOELEMAN, 1976
	H. retusa (L.) Haw.	KAUL and SABHARWAL, 1972
	H. turgida Haw.	MAJUMDAR, 1970; KAUL and SABHARWAL, 1972
	H. variegata (L.) Bolus	KAUL and SABHARWAL, 1972
	Haworthia Duval species	MAJUMDAR and SABHARWAL, 1968; KAUL and SABHARWAL, 1972; MAJUMDAR and SCHLOSSER, 1972
	Heloniopsus orientalis (Thunb.) T. Tanaka	KATO, 1975
	Hemerocallis L. species, hybrids and cultivars	HEUSER, 1976; HEUSER and APPS,1976; MEYER, 1976b; GRAVES, REID and MATHES, 1978; OGLESBY, 1978: KRIKORIAN and KANN, 1981

FAMILY	SPECIES	REFERENCE
	Hosta decorata Bailey	HAMMER, 1976; PAPACHATZI, HAMMER and HASEGAWA, 1981
	Hosta Jacq. species	MEYER, 1976a
	Hyacinthus orientalis L.	PIERIK and WOETS, 1971; SANIEWSKI, NOVAK and RUDNICKI, 1974; PIERIK and POST, 1975; PIERIK and STEEGMANS,1975a; KIM, HASEGAWA and BRESSAN, 1981; PAEK, 1982
	Hyacinthus L. species hybrids and cultivars	PIERIK and RUIBING, 1973; HUSSEY, 1975a, 1975b, 1976b; HOLDGATE, 1977; HUSSEY, 1977a
	Lachenalia Jacq. hybrids	NEL, 1982
	Lilium auratum Lindl.	NIIMI and ONOZAWA, 1979; TAKAYAMA and MISAWA, 1979, 1982, 1983a, 1983b
	L. candidum L.	COQUEN and ASTIE, 1977
	L. japonicum D. Don	NIIMI and ONOZAWA, 1979
	L. leichtlinii Hook.	KATO and YASUTAKE, 1977
	L. longiflorum Thunb.	SHERIDAN, 1968; HACKETT, 1969; HOLDGATE, 1977; HUSSEY 1977a; GUPTA, SHARMA and CHATURVEDI, 1978; STIMART and ASCHER, 1978a; BENNICI, 1979; LESHEM, LILIEN-KIPNIS and STEINITZ, 1982; TAKAYAMA and MISAWA, 1982
	L. pyrenacium Gouan	HUSSEY, 1976b, 1977a
	L. regal Wils.	MONTEZUMA-DE-CARVALHÒ, LUDOVINA and GUIMARAES, 1974; GRAVES, REID and MATHES, 1978
	L. rubellum Bak.	NIIMI and ONOZAWA, 1979; NIIMI and WATANABE, 1982
	L. speciosum Thunb.	ROBB, 1957; HOLDGATE, 1977; KATO and YASUTAKE, 1977; TAKAYAMA and MISAWA, 1979, 1982, 1983a, 1983b

FAMILY	SPECIES	REFERENCE
	Lilium L. species and hybrids	MORI, 1971; ALLEN, 1974; ASJES, BUNT and VAN SLOGTEREN, 1974; HUSSEY, 1976b; SIMMONDS and CUMMING, 1976; ANDERSON, 1977; HUSSEY, 1977a; ASANO, 1978; NIIMI and ONOZAWA, 1979; STIMART, ASCHER and ZAGORSKI, 1980; NOVÁK and PETRÜ, 1981; TAKAYAMA and MISAWA, 1982; ZIMMERMAN and ASCHER, 1982
	Muscari botryoides Mill.	HUSSEY, 1975a
	Ornithogalum thrysoides Jacq.	HUSSEY, 1975a, 1976c
	Ornithogalum L. hybrids	NEL, 1981
	Scilla sibirica Haw. cultivars	HUSSEY, 1975a
	Scilla L. species	HOLDGATE, 1977
	Tulipa L. species	HOLDGATE, 1977; RIVIÈRE and MULLER, 1979
Maranta-ceae	*Maranta leuconeura* E. Morr.	HENNY, 1980; DE FOSSARD, 1981
Musaceae	*Musa acuminata* L.	SWAMY, RAO and CHACKO, 1983
	M. cavendishii Paxton	MA and SHII, 1972
	M. textilis Neé	MANTE and TEPPER, 1983
	Musa L. species	BERG and BUSTAMANTE, 1974
Palmae	*Cocos nucifera* L.	EEUWENS and BLAKE, 1977; PANNETIER and BUFFARD-MOREL, 1982; THANH-TUYEN and DE GUZMAN, 1983
	Elaeis guineensis Jacq.	RABECHAULT, AHEE and GUENIN, 1970; STARITSKY, 1970; JONES 1974; HANOWER and PANNETIER, 1982; PARANJOTHY and OTHMAN, 1982

FAMILY	SPECIES	REFERENCE
	Phoenix dactylifera L.	RABECHAULT, AHEE and GUENIN, 1970; AMMAR and BENBADIS, 1977; REYNOLDS and MURASHIGE, 1979; TISSERAT, 1979; TISSERAT and DE MASON, 1980
Strelitziaceae	*Strelitzia reginae* Ait.	ZIV and HALEVY, 1983
Zingiberaceae	*Zingiber officinale* Roscoe	HOSOKI and SAGAWA, 1977

APPENDIX 1B: Monocotyledonous plants which have been propagated *in vitro* via callus cultures (*represents callus cultures which give rise to somatic embryos).

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
Agavaceae	*Agave* L. species	seed	GROENEWALD, WESSELS and KOELEMAN, 1977
	Cordyline terminalis (L.) Kunth	stem, young leaf	KUNISAKI, 1975, 1977; MEE, 1978
	Yucca gloriosa L.	bud	DURMISHIDZE, GOGOBERIDZE and MAMALADZE, 1983
Amaryllidaceae	*Amaryllis* L. species	bulb, inflorescence	BAPAT and NARAYANASWAMY, 1976
	Furcraea gigantea Venten.	leaf	LAKSHMANAN and JANARDHANAN, 1977
	Hippeastrum Herb. species	ovary, inflorescence	SEABROOK and CUMMING, 1977
	Ipheion Rafin. species	bulb, inflorescence, ovary, stem	HUSSEY, 1975a
	Narcissus L. hybrids and cultivars	ovary	HUSSEY, 1975a
Araceae	*Anthurium andraeanum* Lind.	embryo, leaf, pedicle, petiole, spathe	PIERIK, STEEGMANS and VAN DER MEYS, 1974; PIERIK, 1976
	A. scherzerianum Schott	spathe	GEIER, 1982
	Caladium bicolor Vent.	young leaf	SAHAVACHARIN, 1982
	Pinellia ternata Druce	tuber	SHOYAMA, HATANO and NISHIOKA, 1983

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
Bromeliaceae	*Aechmea distichantha* Lem.	axillary bud	ZIMMER and PIEPER, 1976
	A. fasciata (Lindl.) Baker	axillary bud	ZIMMER and PIEPER, 1976
	Aechmea Ruiz and Pav. hybrids	axillary bud	ZIMMER and PIEPER, 1976
	Ananas comosus (L.) Merr.	leaf	MATHEWS and RANGAN, 1979, 1981
	A. sativus Schult.	axillary bud	MATHEWS, RANGAN and NARAYANASWAMY, 1976
	Cryptanthus bivittatus Regel	stem	DAVIDSON and DONNAN, 1977
	Cryptanthus Otto and Dietr. species	axillary bud	ZIMMER and PIEPER, 1976
Cyperaceae	*Cyprus rotundus* L.	young rhizome and tuber	FISHER, 1977
	Pterotheca falconeria Hook.	seedling tissue	WILKINS and HOLOWINSKY, 1965*
Dioscoreaceae	*Dioscorea deltoidea* Wall.	hypocotyl	GREWAL and ATAL, 1976
	D. sansiberensis Pax.	bulbil	RAO, 1969
Gramineae	*Agropyron cristatum* (L.) Gaertn	immature inflorescence	LO, CHEN and ROSS, 1980
	A. smithii Rydb	immature inflorescence	LO, CHEN and ROSS, 1980
	Alopecurus arundinaceus Poir	immature inflorescence	LO, CHEN and ROSS, 1980
	Andropogon gerardii Vitman	immature inflorescence	CHEN, STENBERG and ROSS, 1977
	Avena fatua L.	immature embryo	RINES and McCOY, 1981

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
	A. sativa L.	hypocotyl, immature embryo, mature seed, mesocotyl	CARTER, YAMADA and TAKAHASI, 1967; CUMMING, GREEN and STUTHMAN, 1976; LÖRZ, HARMS and POTRYKUS, 1976; RINES and McCOY, 1981; HEYSER and NABORS, 1982; NABORS, HEYSER, DYKES and DEMOTT, 1983
	A. sterilis L.	immature embryo	RINES and McCOY, 1981
	Bromus inermis Leyss.	immature inflorescence	GAMBORG, CONSTABEL and MILLER, 1970*; LO, CHEN and ROSS, 1980
	Dactylis glomerata L.	whole caryopsis, immature inflorescence	CONGER and CARABIA, 1978; CHEN, CHEN, LO and ROSS, 1982; DALE and DALTON, 1983
	Eleusine coracana Gaertn.	mesocotyl	RANGAN, 1976
	Festuca arundinacea Schreb	immature inflorescence, mature embryo	LOWE and CONGER, 1979; DALE and DALTON, 1983
	F. pratensis Hudson	immature inflorescence	DALE and DALTON, 1983
	Hordeum vulgare L.	apical meristem, immature embryo	CHENG and SMITH, 1975; DALE and DEAMBROGIO, 1979
	Hordeum L. hybrids	apical meristem	PERSHINA and SHUMNY, 1981
	Lactuca sativa L.	*in vitro* grown root and shoot, seedling cotyledon protoplast	BERRY, LU, PENTAL and COCKING, 1982

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
	Lolium multiflorum Lam.	embryo, immature inflorescence	AHLOOWALIA, 1975; DALE, 1975; DALE and DALTON, 1983
	L. perenne L.	embryo, immature inflorescence	AHLOOWALIA, 1975; DALE and DALTON, 1983
	L. rigidum Gaudin	germinating seed, immature embryo	SKENE and BARLASS, 1983
	Lolium L. hybrids	immature inflorescence, peduncle, stem	KASPERBAUER, BUCKNER and BUSH, 1979; DALE and DALTON, 1983
	Oryza sativa L.	leaf, seed, seedling	NISHI, YAMADA and TAKAHASHI, 1968; NISHI and MITSUOKA, 1969; GUHA, IYER, GUPTA and SWAMINATHAN, 1970; IYER and RAINA, 1972; NAKANO, TASHIRO and MAEDA, 1975; HENKE, MANSUR and CONSTANTIN, 1978; YAN and ZHAO, 1982; NABORS, HEYSER, DYKES and DEMOTT, 1983
	Panicum maximum Jacq.	immature embryo, immature inflorescence, protoplast from callus or suspension cultures from the scutellum	LU and VASIL, 1981 *; LU, VASIL and VASIL, 1981*; LU and VASIL, 1982*
	P. miliaceum L.	immature inflorescence, mesocotyl, seed	RANGAN, 1974; NABORS, HEYSER, DYKES and DEMOTT, 1983; RANGAN and VASIL, 1983*

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
	P. miliare Lam.	immature inflorescence	RANGAN and VASIL, 1983*
	Paspalum scrobiculatum L.	mesocotyl	RANGAN, 1976
	Pennisetum americanum (L.) K. Schum.	immature embryo, immature inflorescence	VASIL and VASIL, 1981a*, 1981b*, 1982a*, 1982b*
	P. purpureum Schum.	immature inflorescence, immature seed	BAJAJ and DHANJU, 1981; WANG and VASIL, 1982*
	P. typhiodeum Pers.	mesocotyl, seed	RANGAN, 1976; NABORS, HEYSER, DYKES and DEMOTT, 1983
	Pennisetum Rich. hybrids	immature embryo, immature inflorescence	VASIL and VASIL, 1981a*
	Phleum pratensis L.	immature inflorescence	DALE and DALTON, 1983
	Phragmites communis L.	stem	SANGWAN and GORENFLOT, 1975
	Saccharum officinarium L.	apical meristem, inflorescence, leaf	HEINZ and MEE, 1969, 1971; DE FOSSARD, 1976; NADAR and HEINZ, 1977; MARETZKI and HIRAKI, 1980; LARKIN, 1982*
	Secale L. species	immature embryo	EAPÉN and RAO, 1982b
	Sorghastum nutans L.	immature inflorescence	CHEN, LO and ROSS, 1979
	Sorghum bicolor (L.) Moench	immature embryo, seedling shoot	MASTELLER and HOLDEN, 1970; GAMBORG, SHYLUK, BRAR and CONSTABEL, 1977; DUNSTAN, SHORT, DHALIWAL and THOMAS, 1979*
	Stipa viridules Trir	immature inflorescence	LO, CHEN and ROSS, 1980

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
	Triticum aestivum L.	embryo, rachis, seed, stem	DUDITS, NEMFT and HAYDU, 1975; CHIN and SCOTT, 1977; SHIMADA, 1978; GOSCH-WACKERLE,AVIV and GALUN, 1979; NABORS HEYSER, DYKES and DEMOTT, 1983
	T. longissimum Bowden	embryo, rachis, seed	GOSCH-WACKERLE, AVIV and GALUN, 1979
	T.monococcum L.	embryo, rachis, seed	GOSCH-WACKERLE, AVIV and GALUN, 1979
	T. speltoides (Tausch) Godron	embryo, rachis, seed	GOSCH-WACKERLE, AVIV and GALUN, 1979
	T. tauschii (Cass.) Schmalh.	embryo, rachis, seed	GOSCH-WACKERLE, AVIV and GALUN, 1979
	T.timopheevii Zhuk	embryo, rachis, seed	GOSCH-WACKERLE, AVIV and GALUN, 1979
	T. turgidum (L.) Thell	embryo, rachis, seed	GOSCH-WACKERLE, AVIV and GALUN, 1979
	Triticum L. species	immature and mature embryo, inflorescence	SHIMADA, SASAKUMA and TSUNEWAKI, 1969; SHARMA, BELLO and SAPRA, 1978; GOSCH-WACKERLE, AVIV and GALUN, 1979; EAPÉN and RAO, 1982a, 1982b; NAKAMURA and KELLER, 1982a, 1982b
	Zea mays L.	immature embryo, mesocotyl	GRESSHOFF and DOY, 1973; GREEN and PHILLIPS, 1975; HARMS, LÖRZ and POTRYKUS, 1976; VASIL, LU and VASIL, 1983*

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
Iridaceae	*Freesia* Klatt species	anther, corm, leaf, pedical, inflorescence, young flower bud	BAJAJ and PIERIK, 1974; HUSSEY, 1975a, 1977a
	Gladiolus grandiflorus Andr.	bract, denuded flower, flower stalk, inflorescence, leaf, young anther	BAJAJ, SIDHU and GILL, 1983
	G. hortulans Bailey	corm stem tip	SIMONSEN and HILDEBRANDT, 1971
	Gladiolus L. species	immature inflorescence	ZIV, HALEVY and SHILO, 1970
	Iris L. species	inflorescence, shoot apex	MEYER, FUCHIGAMI and ROBERTS, 1975; REUTHER , 1977*
Liliaceae	*Allium cepa* L.	bulb	FRIDBORG, 1971
	A. porrum L.	stem	DEBERGH and STANDAERT DE-METSENAERE, 1976
	A. sativum L.	bulb leaf disc, growing point, stem	KEHR and SCHAEFFER, 1976; ABO EL-NIL, 1977*
	A. tuberosum Rotler	young leaf	ZEE, FUNG and YUE, 1977
	Aloe pretoriensis Pole-Evans	seed	GROENEWALD, KOELEMAN and WESSELS, 1975
	Asparagus officinalis L.	cladode, etiolated bud, hypocotyl, shoot tip, spear	GORTER, 1965; TAKATORI MURASHIGE and STILLMAN 1968*; WILMAR and HELLENDOORN, 1968*; BUI-DANG-HA and MACKENZIE, 1973; BUI-DANG-HA, NORREEL and MASSET, 1975*; REUTHER, 1977

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
	Asparagus L. species	embryo, seedling, stem, stem protoplast	MALNASSAY and ELLISON, 1970; STEWARD and MAPES, 1971*; YAKUWA, HARADA and TSUJI, 1982
	Gasteria verrucosa Haw.	leaf	BEYL and SHARMA, 1983*
	Haworthia augustifolia Haw.	gynoecium, inflorescence	KAUL and SABHARWAL, 1972
	H. atrofusca G.G. Smith	gynoecium, inflorescence	KAUL and SABHARWAL, 1972
	H. chloracantha Haw.	gynoecium, inflorescence	KAUL and SABHARWAL, 1972
	H. fasciata Haw.	leaf	BEYL and SHARMA, 1983*
	H. maughanii Poelln	gynoecium, inflorescence	KAUL and SABHARWAL, 1972
	H. planifolia var. *setulifera* Poelln	leaf	WESSELS, GROENEWALD and KOELEMAN, 1976
	H. retusa (L.) Haw.	gynoecium, inflorescence	KAUL and SABHARWAL, 1972
	H. turgida Haw.	gynoecium, inflorescence, ovary wall	MAJUMDAR, 1970; KAUL and SABHARWAL, 1972
	H. variegata (L.) Bolus	gynoecium, inflorescence	KAUL and SABHARWAL, 1972
	Haworthia Duval species	gynoecium, inflorescence	MAJUMDAR and SABHARWAL, 1968; KAUL and SABHARWAL, 1972; MAJUMDAR and SCHLOSSER, 1972
	Heloniopsus orientalis (Thunb.) T. Tanaka	leaf, stem	KATO, 1975
	Hemerocallis L. species and cultivars	inflorescence, petal	HEUSER, 1976; HEUSER and APPS, 1976; MEYER, 1976b

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
	Hosta Jacq. species	flower	MEYER, 1976a
	Hyacinthus L. cultivars	bulb, inflorescence, leaf, ovary	HUSSEY, 1975a, 1975b
	Lilium auratum Lindl.	bulbscale, petal, peduncle	TAKAYAMA and MISAWA, 1979
	L. leichtlinii Hook.	leaf	KATO and YASUTAKE, 1977
	L. longiflorum Thunb.	stem apex, seed	SHERIDAN, 1968; BENNICI, 1979
	L. regale Wils.	stamen filament	MONTEZUMA-DE-CARVALHÒ, LUDOVINA and GUIMARAES, 1974
	L. speciosum Thunb.	leaf	KATO and YASUTAKE, 1977
	Lilium L. species and hybrids	bulbscale, inflorescence, stem, stem apex	SIMMONDS and CUMMING, 1976; HUSSEY, 1977a; STIMART, ASCHER and ZAGORSKI, 1980; NOVÁK and PETRÜ, 1981
	Muscari botryoides Mill.	bulb, inflorescence, leaf, ovary	HUSSEY, 1975a
	Ornithogalum thrysoides Jacq.	bulb, inflorescence, leaf, ovary, sepal	HUSSEY, 1975a, 1976c
	Scilla sibirica Haw. cultivars	bulb, inflorescence, leaf, ovary, sepal	HUSSEY, 1975a

FAMILY	SPECIES	EXPLANT SOURCE	REFERENCE
Palmae	*Cocos nucifera* L.	young leaf	PANNETIER and BUFFARD-MOREL, 1982*
	Elaeis guineensis Jacq.	embryo, young leaf	RABECHAULT, AHEE and GUENIN, 1970*; HANOWER and PANNETIER, 1982
	Phoenix dactylifera L.	embryo, lateral bud, ovule, shoot tip	RABECHAULT, AHEE and GUENIN, 1970*; TISSERAT, 1979*
Zingiberaceae	*Zingiber officinale* Roscoe	rhizome	HOSOKI and SAGAWA, 1977*