THE BIOSYNTHESIS AND PRODUCTION OF HYPOXOSIDE IN *HYPOXIS HEMEROCALLIDEA* FISCH. AND MEY. *IN VIVO* AND *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.

APRIL, 1989

PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg from January 1985 to December 1988, 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.

Bayley.

Arlene Diane Bayley

April 1989

ACKNOWLEDGMENTS

My sincere gratitude is extended to the following people:

Professor J. van Staden, my supervisor, for his guidance and encouragement throughout; Professor R.N. Pienaar, Dr. N.A.C. Brown and Professor C.S. Whitehead, all of whom advised me in my Research Committee meetings; Professor S.E. Drewes for his expert advice pertaining to chemistry and hypoxoside, and for his gifts of chemicals during the past 4 years; Dr. P.M. Drennan for her valuable assistance and suggestions with the preparation and interpretation of material in the anatomical studies;

Mr. V.J. Bandu and Mrs. B.J. White for their assistance with electron microscopy; Mr. M. Hampton and Mr. D. Boddy for their efficient technical assistance with faulty equipment.

Dr. M.T. Smith for his perusal of, and useful comments on the content of my introduction; Mrs. K. Hurly, Miss F.E. Drewes and Miss S. Pridmore for their thorough proof reading; Dr. M. E. Aken, Dr. P. M. Drennan, Mrs P. Donnelly and Mr. V. J. Bandu for their advice and help with the preparation of the anatomical plates; Mr. C.E. Reid of Computer Services for his help with the printing; and the postgraduates and research assistants of the Botany Department who have helped make the last 4 years enjoyable workwise and socially.

I wish to especially thank:

My parents for their unfailing support and belief in me throughout my university education; Mr. C.H. Norris for maintaining my sanity,

being patient and supportive and for his artistic renditions of many of my figures; and Miss C. Ackermann for her assistance with anatomical interpretation and the production of my plates but most importantly for her friendship, moral support and patience with a bad tempered distracted flatmate.

I also gratefully acknowledge financial support from the Council for Scientific and Industrial Research from 1985 to 1986.

ABSTRACT

Hypoxoside, a phenolic diglucoside, with a diarylpentane-type structure, is thought to be the medicinally active constituent of corm extracts of *Hypoxis hemerocallidea* Fisch. & Mey. which are reputed to alleviate the symptoms of prostate hypertrophy and urinary infections. The biosynthesis and production of this unique phytochemical were investigated in *H. hemerocallidea* using both *in vivo* and *in vitro* systems.

It was found, in root-producing callus, that ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acid were efficient precursors for hypoxoside in comparison to ^{14}C-sodium acetate and ^{14}C-acetyl coenzyme-A, which were not incorporated into the phenolic compound. Thus, at least one aryl moiety of hypoxoside was derived, via phenylalanine and *t*-cinnamic acid, from the shikimate pathway. The acetate pathway did not appear to be involved in the biosynthetic process. The data supports the hypothesis that the molecule is formed from two cinnamate units with the loss of a carbon atom, in opposition to the proposal that the molecule is derived from head-to-tail condensation of acetate units onto a propenylic moiety.

Despite the structural similarities between hypoxoside and caffeic and *p*-coumaric acids, these two hydroxycinnamic acids were not efficient precursors for hypoxoside *in vivo* or *in vitro*. A number of reasons are put forward to explain this finding.

It was found that the greatest concentration of hypoxoside was located in the corms of intact plants. The major biosynthetic site of the molecule was also found to be located in this organ. Since the roots

did accumulate the phytochemical to a small extent, the biosynthetic potential of these organs has not been disregarded. That of the leaves has been, however.

The report by PAGE (1984) that the upper region of the corm contained a greater concentration of hypoxoside than the lower portion, is substantiated in this study, where this region was found to be more biosynthetically active than the lower half. Light microscopic and electron microscopic studies revealed that starch storing cells, which accumulated phenolics in their vacuoles, contained seemingly synthetically active tubular endoplasmic reticulum in their cytoplasm. A greater number of these cells were concentrated in the upper region as opposed to the lower half of the corm. It is postulated that these cells are the site for biosynthesis and accumulation of hypoxoside.

The shikimate pathway, from which the precursors for hypoxoside are derived, was found, through the exposure of intact plants to ^{14}C-carbon dioxide, to be located mainly in the leaves. It is postulated from the above study and one in which ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid were applied to intact plants, that phenylalanine and/or cinnamic acid are the transported form of the shikimate derivatives. *p*-Coumaric and caffeic acids, which are metabolically more stable, are envisaged to be the sequestering forms.

The investigation of the seasonal production of hypoxoside revealed that most of the synthesis and accumulation occurred after the corms had broken winter dormancy and after the flush of leaf growth had

slowed down. During dormancy the production of hypoxoside appeared to cease.

The *in vitro* studies, where the effects of light, temperature, nutrients, plant growth regulators and supply of potential precursors, on hypoxoside production by root-producing callus were investigated, indicate that this metabolite is not simply a "shunt" metabolite. A number of factors other than precursor availability enhanced, or reduced the *in vitro* production of this phytochemical. Furthermore, production of the phytochemical and growth were not always antagonistic.

Hypoxoside, the biosynthesis of which requires a more thorough investigation, is, however, according to this investigation, a typical secondary metabolite in many respects.

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Linda Cowan 1978

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

Since ancient times members of the genus *Hypoxis* L. have been used worldwide as herbal remedies for numerous health complaints. In Asia, particularly China and India, the rootstock of *H. aurea* Lour. is used as a reconstructive and rejuvenating tonic, as an aphrodisiac and as a cure for wasting diseases, dyspepsia, lassitude, wounds and for ailments of the eyes and ears (LEWIS and ELVIN-LEWIS, 1977; STUART, 1977; WALFORD, 1979). In the West Indies *H. decumbens* L. is collected as a folk cure for tumours of the testicles (HARTWELL, 1967).

It is in Africa, however, that the most extensive usage of species of *Hypoxis* in folk medicine occurs (WATT and BREYER-BRANDWIJK, 1962). On this continent the medicinal properties attributed to the genus are considerable and varied but only recently, with the discovery of the unusual phenolic diglucoside, hypoxoside, (MARINI-BETTOLO, PATAMIA, NICOLETTI, GALEFFI and MESSANA, 1982) has the merit of these remedies been recognised by the scientific, medicinal and commercial communities.

Presently it is believed that hypoxoside is responsible, at least partially, for the medicinal activity of corm extracts of *Hypoxis* (PAGE, 1984). The presence of hypoxoside in the corms of *H. hemerocallidea* Fisch. & Mey. was reported by DREWES, HALL, LEARMONTH and UPFOLD (1984). Since this species is abundant in southern Africa

and produces a large fleshy corm rich in hypoxoside it has become the focal point for the researching of this phytochemical.

1.2. TAXONOMY, DISTRIBUTION AND MORPHOLOGY OF *HYPOXIS HEMEROCALLIDEA* FISCH. & MEY.

The origin of the name *Hypoxis* is Greek; "hypo" meaning beneath and "oxys" meaning sharp. Both describe the inferior ovary with its acute narrow base. The genus goes under a number of common names in South Africa; kaffertulp (Afrikaans), gifbol (Afrikaans), ilabatheka (Zulu), inkomfe-enkulu (Zulu), lotsane (Sotho) and tshuka (Tswana) (WATT and BREYER-BRANDWIJK, 1962; SMITH, 1966).

Members of *Hypoxis* are perennial herbs and geophytes, possessing some form of rootstock, usually a corm. This strategy enables them to survive harsh winters, drought and fire. In the case of *H. hemerocallidea*, the corm, a globose to oblong structure, can vary in size with diameters from 0.5 to 11.0 centimetres depending on plant age. Leaves that are tristichously arranged, strap-shaped and pubescent, arise from the apical region of the corm. The contractile roots are numerous and fleshy, emerging in parallel rows from the lower half of the corm just below its widest part (Figure 1.1). Characteristically the interior of the corm is yellow, quickly blackening when cut open, and mucilaginous. Yellow flowers are produced at the end of long peduncles from September through to February. During this time small black seeds are proliferated. For approximately one month in winter, depending on the severity of the weather, the plants become dormant with the aerial parts dying back. Re-emergence of the leaves occurs in early spring (WOOD, 1976; HEIDEMAN, 1979).

The distribution of the Hypoxidaceae worldwide is extensive but the family is not represented in Europe or the northern parts of North America. It is represented by the genera *Forbesia* Eckl., *Ianthe* Salisb., *Molineria* Colla., *Curculigo* Gaertn., *Hypoxis* L., *Empodium* Salisb., *Pauridia* Harv., *Rhodohypoxis* Nel and *Spiloxene* Salisb. (DAHLGREN and CLIFFORD, 1982). Of these, the latter five are represented in southern Africa (THOMPSON, 1972) while the last four genera are endemic to South Africa (DYER, 1976).

Taxonomic investigations of the genus *Hypoxis* in South Africa (WOOD, 1976; HEIDEMAN, 1979) have proved to be difficult as the morphological variation of the species overlap to the extent that the distinction between species is often an unresolved task.

The distribution of the genus *Hypoxis* is confined predominantly to the warm temperate and tropical regions of the world with no reports of the genus being found in Europe, North and Central Asia, North Africa, the southern regions of South America and the northern parts of North America (Figure 1.2) (WOOD, 1976). In Africa distribution of the genus begins south of the Sahara, with the number of species increasing southwards through Zimbabwe, Mozambique, Swaziland, Botswana and Lesotho (HEIDEMAN, 1979). In South Africa the genus is well represented by some fifty species, the greatest density occurring in the Eastern Cape (DYER, 1976). The numbers of species decreases westwards as the winter rainfall areas of the Cape are approached (HEIDEMAN, 1979). The "smaller" species found in southern Africa include *H. augustifolia* Lam., *H. argentea* Bak., *H. filiformis* Bak., and *H. membranacea* Bak. The "larger" species found in the region are *H. acuminata* Bak., *H. iridifolia* Bak., *H. latifolia* Hook., *H. obtusa* Burch., *H. rigidula* Bak. and *H. hemerocallidea* Fisch. & Mey.

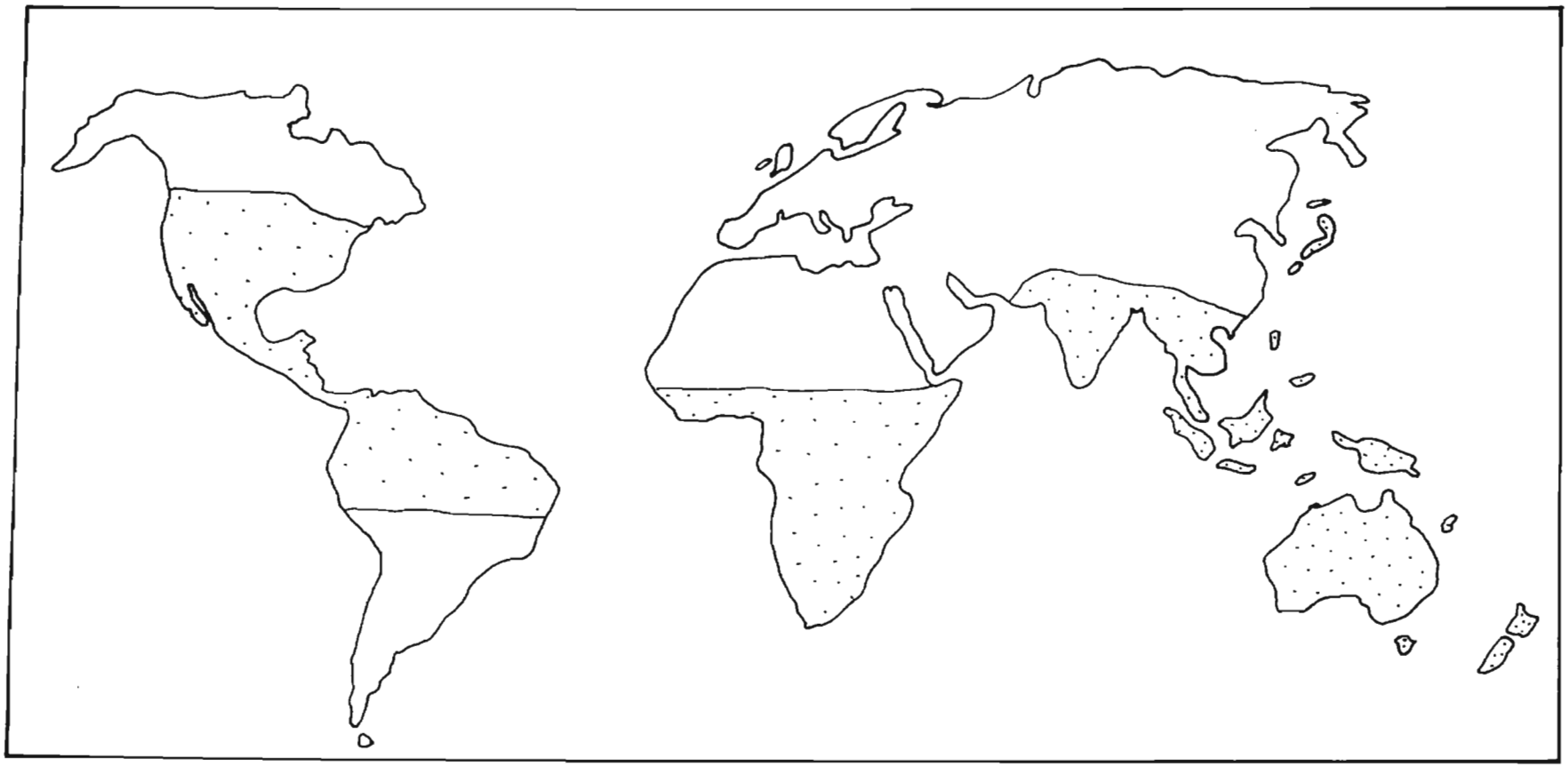


Figure 1.2: General known world distribution of *Hypoxis* L. (PAGE, 1984).

Many of the members of *Hypoxis* occur mainly as perennial grassland species although a diversity of habitats can be occupied by a given species. This is exemplified by *H. hemerocallidea*, the distribution of which varies from mountainous to coastal regions, as well as from dry grassland to damp ravine (DYER, 1976; HEIDEMAN, 1979). Furthermore, WOOD (1976) has reported that the species is most frequently found in grasslands and disturbed areas where almost pure stands of the plants occur.

The family Hypoxidaceae was first described by Robert Brown in 1814 in "General remarks, geographical and systematical, on the botany of Terra Australis" which forms part of Matthew Flinder's "A Voyage to Terra Australis" (STAFLEU and COWAN, 1976). The taxonomic status of this group of plants has been a much disputed issue. BAKER (1878) recognised the Hypoxidaceae as a family while it was regarded as a tribe, Hypoxideae, of the Amaryllidaceae by BENTHAM and HOOKER (1883). BAKER (1896) later followed the latter decision in Flora Capensis. However the group was given the status of family by HUTCHINSON (1959), who placed them in the order Haemodorales, which was derived from the Liliales completely separate from the Amaryllidaceae. On the basis of embryology, biochemical features and leaf anatomy, GEERINCK (1968) proposed the Hypoxidaceae and the Haemodoraceae as distinct families separate from the Amaryllidaceae. The genera he included in the Hypoxidaceae are as follows: *Campynema* Labill., *Campynemanthe* Baill., *Hypoxis* L., (including *Spiloxene* Salisb.), *Pauridia* Harv., *Curculigo* Gaertn. (including *Molineria* Colla and *Empodium* Salisb.) and *Rhodohypoxis* Nel.

The family, Hypoxidaceae, is now a conserved family according to the International Code of Botanical Nomenclature (STAFLEU, 1972).

DAHLGREN, CLIFFORD and YEO (1985) believe it best to place the Hypoxidaceae in the superorder Liliiflorae under the order Asparagales although they concede that the close affinities of the family are uncertain.

HILLIARD and BURTT (1986) have revised *Hypoxis rooperi* T. Moore and designated it *Hypoxis hemerocallidea* Fischer and Meyer. NEL (1914) originally separated *H. hemerocallidea* and *H. rooperi* on the claim that the former had split anther tips while the anther tips in the latter were entire. He did mention, however, that both conditions could be present in *H. rooperi*. HILLIARD and BURTT (1986), on obtaining from Leningrad, the loan of the type specimen for *H. hemerocallidea*, discovered that the anther tips were entire, not split. This fact, together with the observation that the style is no longer than the stigma, means that in the key derived by NEL (1914), *H. hemerocallidea* keys down to *H. rooperi*. Since, FISCHER and MEYER (1845) described *H. hemerocallidea* before MOORE (1852) described *H. rooperi* the name reverts to the former.

1.3. MEDICINAL USAGE OF *HYPOXIS*

Extracts of various *Hypoxis* species are widely used for medicinal and magical purposes by many of the peoples of southern Africa. Most of the usages described in Table 1.1 are centuries old and are steeped in tribal tradition. Amongst the species used only *H. latifolia* is considered to be highly toxic (BRYANT, 1966). It is only in recent times that *Hypoxis* species have found application outside of tribal tradition. White settlers have used hot aqueous decoctions of the corms of *H. hemerocallidea, H. iridifolia, H. obtusa.* and *H. rigidula* as a herbal remedy for enlargement of the prostate gland. This

Table 1.1: Species of *Hypoxis* used by African tribes for food and medicine (PAGE, 1984).

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 cows' udders -African medicine
H. gerrardii Bak. (RILEY, 1963)	Used in Natal	Corm	-To treat stomach trouble
H. hemerocallidea Fisch. and Mey. (WATT and BREYER-BRANDWIJK, 1962; SMITH, 1966)	Lesotho Congo Kwena Lesotho Tswana	Plant Rootstock	-Charm against thunder storms -Tonic for weakly children -Purgative -African medicine -Remedy for burns -Cure for headaches
H. latifolia Hook. (WATT and BREYER-BRANDWIJK, 1962; BRYANT, 1966)	Congo Zulu	Bulb Plant	-poison for small vermin in food -Emetic for disagreeable dreams said to accompany heart weakness -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
H. nyasica Bak. (WATT and BREYER-BRANDWIJK, 1962)	Nyanja Congo	Plant	-Cough remedy -African medicine

Table 1.1 continued

H. obliqua Jacq. (WATT and BREYER BRANDWIJK, 1962; WRIGHT, 1963; SMITH,1966)	Xhosa Congo	Corm/ Rootstock Plant	-Lotion for septic wounds -African medicine
H. rigidula Bak. (WATT and BREYER-BRANDWIJK, 1962; SMITH, 1966)	Sotho Congo	Rootstock Plant	-Remedy for gall-sickness in cattle -African medicine
H. villosa L.f. (WATT and BREYER-BRANDWIJK, 1962)	Lobedu Southern Sotho	Bulb/ Tuber	-Charm against thunder -Repel witches -Emetic 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

remedy, prepared as a tea from the sun dried corms, was sold locally in South Africa under the name Prostamin (PEGEL, 1976a) and is now available as a preparation marketed as Hypoxin.

Corm extracts from *H. hemerocallidea*, *H. iridifolia* and *H. rigidula*, when subjected to clinical trials, exhibited remarkable medicinal activity which included antibiotic, anti-inflammatory, hormonal, muscle reactivating and conditional properties (WARREN, 1972). The extracts were also shown to alleviate certain arthritic conditions and conditions due to arteriosclerosis. However, the extracts were most effective in the treatment of urogenital diseases, particularly those affecting the prostate gland, bladder and urinary tract. Patients suffering from benign hypertrophy of the prostate gland, when treated with the corm extracts, showed exceptionally good tolerance in comparison to the medication normally used. The overall result was a reduction in the enlargement of the prostate gland with the disappearance of the accompanying bladder infection (WARREN, 1972). Furthermore, all of the extracts of the members of the Hypoxidaceae that were tested were active against P388, the mouse lympocytic leukemia test (BARCLAY and PERDUE, 1976).

The above evidence has provided the necessary impetus for further research into the nature of the active principal.

1.4. THE MEDICINALLY ACTIVE AGENTS IN CORM EXTRACTS OF *H. HEMEROCALLIDEA*

Since the Hypoxidaceae is closely allied to the Amaryllidaceae and Liliaceae, reports of toxicity in the family would not be unexpected.

The poisonous nature of the Amaryllidaceae and Liliaceae, the former accumulating alkaloids and the latter alkaloids and/or spiroketal glycosides, has been extensively documented (HEGENAUER, 1963). Numerous studies claim that the Hypoxidaceae do not contain alkaloids (HEGENAUER, 1963; WALL, KRIDER, KREWSON, EDDY, WILLAMAN, CORRELL, and GENTRY, 1954; KAPOOR, KAPOOR, SRIVASTAVA, SINGH and SHARMA, 1971), nor is there any mention of the group in any of the alkaloid-containing families (RAFFAUF, 1970; WILLAMAN and HUI-LIN LI, 1970). Nevertheless, there are unsubstantiated reports of the poisonous nature of *Hypoxis* species. BRYANT (1966) considers species belonging to the genus to be very poisonous, particularly *H. latifolia*. In fact, WATT and BREYER-BRANDWIJK (1962) have reported the presence of the alkaloid, haemanthine, in this species and since extracts of this species are used as a vermicide this seems possible.

KAPOOR, KAPOOR, SRIVASTAVA, SINGH and SHARMA (1971) could not detect alkaloids or saponins in two Asian members of the Hypoxidaceae, *H. aurea* and *Curculigo orchiodes* Gaertn. Both species have medicinal properties, while the latter is claimed to have anticancer activity (RAO and BERI, 1950; DHAR, DHAR, DHAWAN, MEHROTRA and RAY, 1968; STUART, 1977; LEWIS and ELVIN-LEWIS, 1977). However, subsequent to these reports, lycorine, a characteristic alkaloid of the Amaryllidaceae, has been detected in *Curculigo orchiodes* (KRISHNA RAO, ALI and REDDY, 1978). Thus the Hypoxidaceae are not completely alkaloid-free. However, the fact that the corms of *Hypoxis* are used for forage in South America and that the root stock of *H. argentea* is eaten by the Xhosa in Southern Africa, suggests that many of the Hypoxidaceae are not toxic (WATT and BREYER-BRANDWIJK, 1962; PEGEL, 1976a).

There are no accounts of the occurrence of steroid saponins (steroid spiroketal glycosides) in this family (PEGEL, 1976a). Furthermore, extensive documentation of the non-toxicity of corm extracts of many *Hypoxis* sp. exists. Studies with rats revealed no toxic, carcinogenic or teratogenic properties (PEGEL, 1976b). WALFORD (1979) reports that no experimentally measurable toxicity could be found in *Hypoxis* species. When patients were given doses of 50 to 1000 milligrams of corm extract per day no acute or chronic toxicity, organ changes or other side effects were found (PEGEL, 1979). WARREN (1972) reports that ingestion of corm extracts resulted in only a very small percentage (less than one percent) of patients complaining of a slight stomach irritation. No heart or circulatory disturbances were observed while toxic and allergic symptoms were absent.

Various aspects of the phytochemistry of the genus *Hypoxis* have been examined. Many of the earlier reports, however, do not mention the species studied. This is is particularly true when considering the phenolics and the organic acids. RAMSTAD (1953) detected chelidonic acid in six species: *H. juncea* Sm., *H. minuta* L., *H. probata* Nel., *H. serrata* L., *H. sellata* L., and *H. villosa* L.. BATE-SMITH (1956) observed ellagic acid in *H. filiformis* but later questioned this finding (HARBORNE and SIMMONDS, 1964). BATE-SMITH (1956) also detected the flavonol, quercetin, as well as *p*-coumaric and caffeic acids in *H. krebsii* Fisch. The Hypoxidaceae are described by him as having: "almost a conventionally primitive phenolic pattern".

In a review of flavonoids in thirty plant families SKRZYPCZAKOWA (1970) reports the presence of flavonols in three unnamed *Hypoxis* species, anthocyanins in one, caffeic acid in three, *p*-coumaric acid in two and ferulic acid in one. However, ILLI, MARTIN and PEGEL

(1976) working with corm extracts of *H. hemerocallidea* did not detect cinnamic acid or any of the following hydroxycinnamic acids: sinapic acid, caffeic acid, ferulic acid, *p*-coumaric acid. Neither were the flavonoids catechin, epicatechin, quercetin, kaempferol nor fisetin detected. Additionally, gallic, ellagic, chelidonic, shikimic, chrysophamic, chlorogenic and trimethylellagic acids were not found. KAPOOR, KAPOOR, SRIVASTAVA, SINGH and SHARMA (1971) did not detect flavonoids in *H. aurea* when surveying a number of Indian plants for this group of compounds.

The presence of the following fatty acids in *H. hemerocallidea* corm extracts has been reported: palmitic, septadecanoic, stearic, sepdecatrienoic, octadecenoic and octadecadienoic acids (ILLI, MARTIN and PEGEL, 1976). In addition the presence and seasonal fluctuation of carbohydrates in *H. hemerocallidea* corms have been investigated. BEWS and VANDERPLANK (1930) studied the changes in the levels of pentosans, hexosans, disaccharides and monosaccharides on a seasonal basis. They concluded that the corm possessed the properties of a typical storage organ.

The sterols and sterol glycosides, detected initially in *H. rigidula* and two unnamed *Hypoxis* species (WALL, KRIDER, KREWSON, EDDY, WILLAMAN, CORRELL and GENTRY, 1954), were first postulated to be the pharmaceutically active agents in corm extracts by REISCH and MÖLLMANN (1974). Sitosterol and sitosteryl *β-D*-glucopyranoside were detected in *H. hemerocallidea* corms by ILLI, MARTIN and PEGEL (1976). The isolation of the sterols, sitosterins and their glycosides for the same species was also reported by WALFORD (1979), although the author does not specify the sitosterins detected. VAN STADEN (1981) gives this detail, listing the following sitosterols as constituents of

H. hemerocallidea corms: sitosterol, campesterol, stigmasterol, and spinasterol (Figure 1.3). This author also states that after extensive clinical testing, corm extracts containing these sitosterols, resulted in a depression of pathologically elevated prostaglandin levels in both humans and animals, even at low doses.

PEGEL (1979) outlined a novel method for the isolation of sterolins from *H. hemerocallidea* corm extracts and suggested that the medicinal activity of the extracts, especially in the treatment of benign prostate hypertrophy and attendant phenomena, was due to the presence of sterolins. The observation that medicinal activity was still present when the extraction techinques were such that reduced yields of sterolins were obtained prompted PEGEL (1979) to note: "It therefore becomes apparent that besides sterolins and sterolin derivatives, other not yet identified compounds are extracted by means of the described procedure and these compounds either as such or in synergistic action with sterolins show a definite activity."

In clinical investigations it was found that doses of 50 to 1000 milligrams of extract per day, administered in doses of 50 to 200 milligrams several times daily produced an effective treatment for a wide range of diseases (Table 1.2).

Subsequent to these fairly extensive clinical tests it was determined by two independent groups that one of the major chemical constituents of *H. hemerocallidea* corms was a phenolic diglucoside. Rhizomes of *H. obtusa* Busch. were found to contain the diglucoside of 1-(3',4'-dihydroxyphenyl)-5-(3",4"-dihydroxyphenyl)-penten-4-yne, trivial name hypoxoside. (MARINI-BETTOLO, PATAMIA, NICOLETTI, GALEFFI and MESSANA, 1982). For a natural product it has a somewhat uncommon structure,

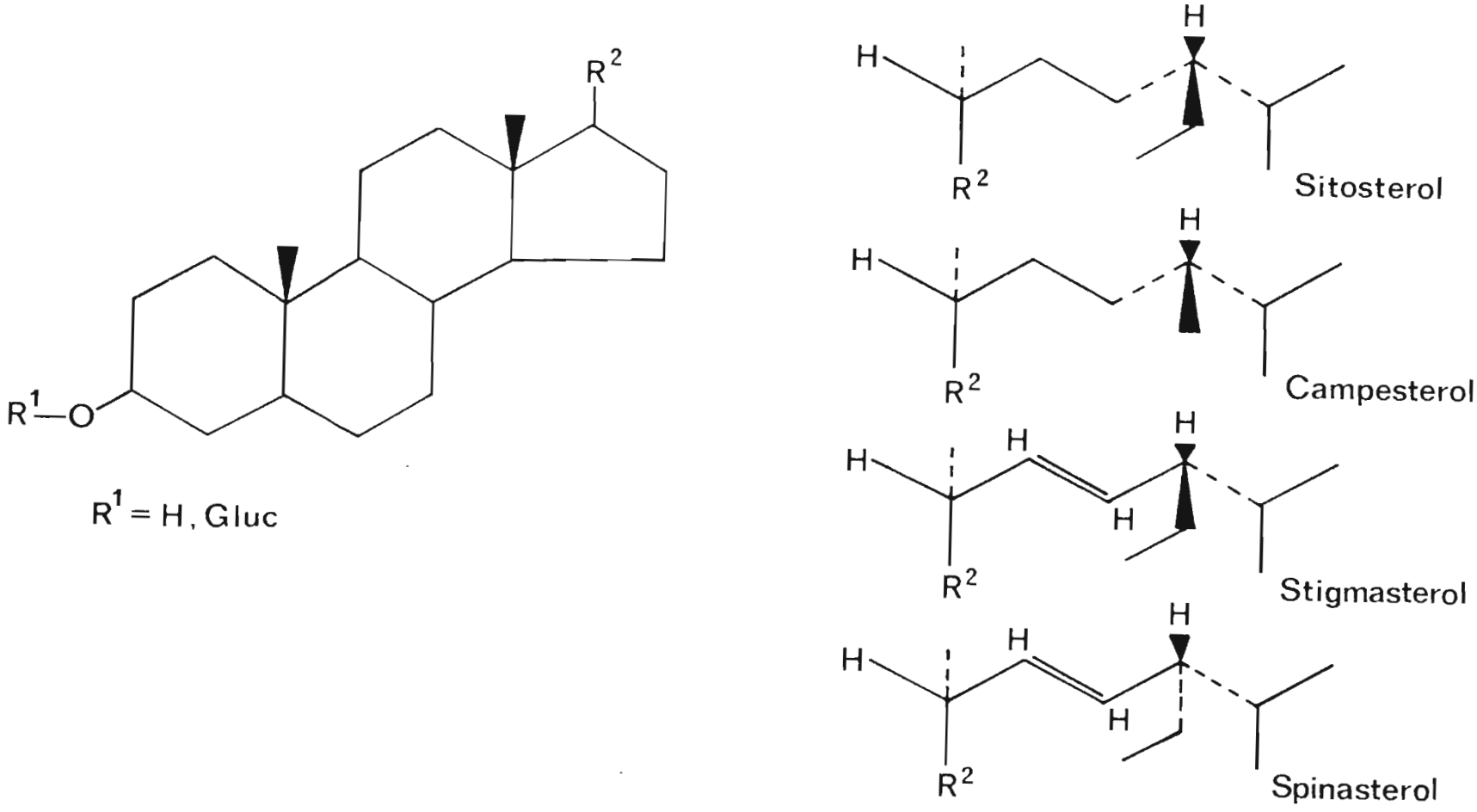


Figure 1.3: Sitosterol constituents of *H. hemerocallidea* corm extracts (VAN STADEN, 1981).

Table 1.2: The beneficial prophylactic and/or curative properties of *H. hemerocallidea* corm extracts (PEGEL, 1979).

SYSTEMS AND/OR ORGANS DISTURBED	DISTURBANCES AND/OR DISEASES RELIEVED BY EXTRACTS
Gastro-intestinal Tract	- ulcers
Urogenital Tract	-prostate hypertrophy and associated complications -diseases of the bladder and urinary tract
Cardiovascular System	-edematous conditions -varicose veins -haemorrhoids
Blood and Blood-forming Organs	-hyperlipidemia and its retrogressive manifestations
Skin	-dermatitis and eczema -acne
Skeletal System and Muscles	-inflammations -arthritis and rheumatism -high uric acid levels

possessing a 1-en-4-yne moiety (Figure 1.4). The hypoxoside was extracted using methanol followed by partitioning of the methanol against water and butan-1-ol; a process that yielded yellow crystals with a melting point of 149 ^{o}C to 151 ^{o}C.

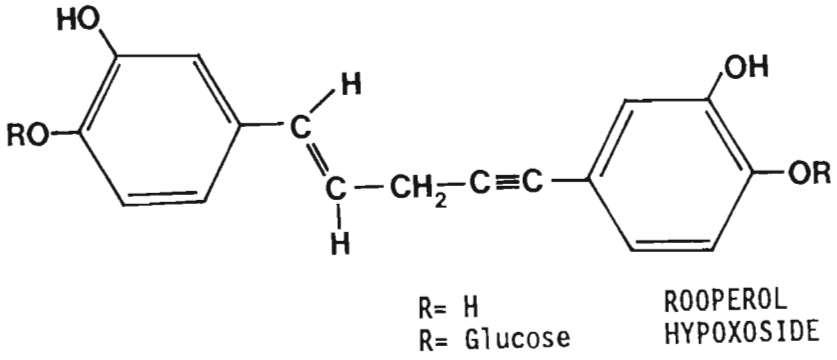


Figure 1.4: Molecular structure of hypoxoside and its aglycone rooperol.

DREWES, HALL, LEARMONTH and UPFOLD (1984) isolated hypoxoside from the corms of *H. hemerocallidea*. The phytochemical was also detected in other members of the Hypoxidaceae including *H. accuminata*, *H. iridifolia*, *H. rigidula*, *H. latifolia* and *Spiloxene schlechteri* (Bolus) Garside. Levels between 3.5 and 4.5 percent of the compound were detected in the rootstock of these species. These researchers also reported that clinical tests revealed that the phenolic showed a low toxicity on oral and intraperitoneal administration to mice and on intravenous administration to rabbits. No foetotoxic or teratogenic effects in mice were observed when hypoxoside was given orally to mice in doses of up to 100 milligrams per kilogram.

It is of interest that VAN STADEN, PAGE and FORSYTH (1986) have discovered that hypoxoside will inhibit kinetin-induced growth of soybean callus. This property was a function of time and its dissipation could be correlated with an accumulation of the phenolic in the cell vacuoles. How cell division is inhibited is not currently known. Of importance, though, was the observation that hypoxoside displayed no toxic effects on the callus.

Hypoxoside is currently considered to be the primary ingredient responsible for the medicinal effectiveness of *Hypoxis* corm extracts (PAGE, 1984). The possible role of the sterols in the potency of these extracts cannot be disregarded, however.

1.5. CURRENT UTILISATION OF *H. HEMEROCALLIDEA* AS A SOURCE OF HYPOXOSIDE

Hypoxoside, to date, has not been listed as a registered pharmaceutical. The phytochemical is however, undergoing thorough clinical testing to fully establish its medicinal properties. Nevertheless, with the advent of its usage, a number of problems are envisaged, one being a cost-efficient source of the phenolic to meet possible demand. Most pharmaceuticals of natural origin are derived from one of two sources; via chemical syntheses or direct extraction from the relevant plant species.

The potential of *in vitro* systems for the production of plant products was recognised over 50 years ago and has gained worldwide acceptance (KURZ and CONSTABEL, 1985). However, the potential of these systems has not been easily realised. To date there has been only one commercially successful venture; Mitsui Petrochemical Industries

Amaryllidaceae are included in this number, only one member of the Hypoxidaceae has been cultured *in vitro*. PAGE and VAN STADEN (1984) established the multiplication of *H. hemerocallidea* initially from corm explants; generating up to 35 plantlets per corm. Although corms provided an effective source of explants for propagation PAGE and VAN STADEN (1986), recognising that this was a time-consuming and destructive method, investigated the inflorescences of *H. hemerocallidea* as an alternative source of explant. Using unopened mature buds a shoot-producing callus was obtained which could be maintained by continuous subculturing. Thus the use of flower buds as explants was more desirable since a continuous system for *in vitro* multiplication, could be obtained. Furthermore, this technique was shown to be very prolific, producing over 80 000 plantlets from 100 *H. hemerocallidea* flower buds within one year (PAGE and VAN STADEN, 1986). The only apparent disadvantage of this method was the fact that flower buds are only available for three months of the year.

Although the *in vitro* methods established are a potential source of *H. hemerocallidea* plants for cultivation and extraction of the medicinal compounds, these plants take approximately three years to produce corms of a suitable size for laboratory extractions. Thus PAGE and VAN STADEN (1987) have investigated the possibility of producing hypoxoside *in vitro*. This, unfortunately, has not yielded very promising results. Different types of *H. hemerocallidea* tissue produced *in vitro* from both flower bud and corm explants were examined for their ability to produce hypoxoside *in vitro*. It was noted that only those tissues displaying some degree of root differentiation contained hypoxoside (Table 1.3). Furthermore *in vitro* levels of hypoxoside were considerably lower than those found *in vivo*

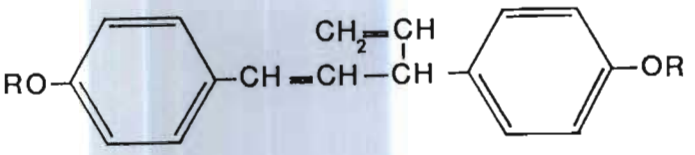
Table 1.3: Hypoxoside content (percentage of fresh mass) of different cultures established from *H. hemerocallidea* corms and flowers (PAGE and VAN STADEN, 1987).

TISSUE TYPE	HYPOXOSIDE CONTENT %
Root forming callus	0.004
Malformed root tissue (flower buds)	0.017
Malformed root tissue (corms)	0.015

Table 1.4: Hypoxoside content (percentage fresh mass) of naturally grown *H. hemerocallidea* corm material (PAGE, 1984).

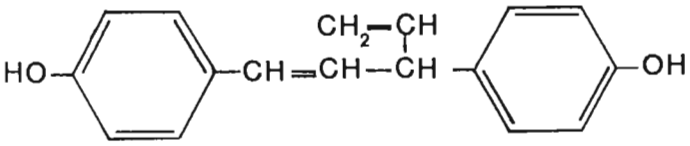
CORM MATERIAL ANALYSED	HYPOXOSIDE CONTENT %
February (upper half of corm)	7.61
May (upper half of corm)	6.14
July (upper half of corm)	4.97
October (upper half of corm)	8.67

linked by a five carbon chain. However, in the former three compounds the carbon chain is branched, the backbone of the chain consisting of

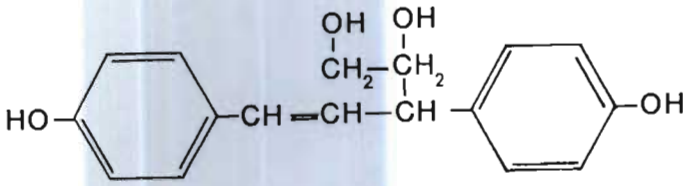


NYASOSIDE

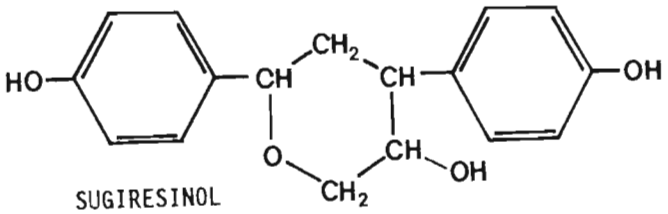
R = GLUCOSE



HINOKIRESINOL



AGATHARESINOL



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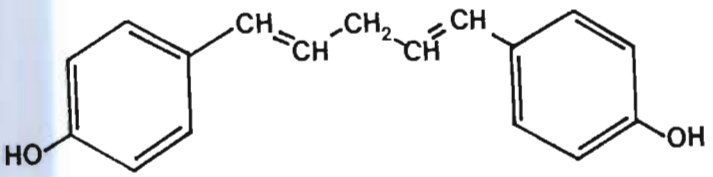


(Z,Z)-4,4'-(1,4-PENTADIENE-1,5-DYL)DIPHENOL

Figure 1.5: The molecular structure of some diarylpentane-type compounds.

three carbon atoms with a two carbon atom side chain. A compound with a structure more closely related to hypoxoside than these three was isolated from the leaves of *Ginkgo biloba* L.. This compound (Z,Z)-4,4'(1,4-pentadiene-1,5-dyl)diphenol (Figure 1.5) was first discovered by PLIENINGER, SCHWARZ, JAGGY, HUBER-PATZ, RODEWALD, IRNGARTINGER and WEINGES (1986) in *Ginkgo biloba* and has subsequently been identified in the rhizomes of *Alpinia galanga* Willd.(BARIK, KUNDU and DEY, 1987).

A group of compounds that can be considered to be structually related to the C_6-C_3.C_2-C_6 phenolics are the diarylheptanoids (Figure 1.6). These phytochemicals are found in a number of families which include the Betulaceae, Zingiberaceae, Myricaceae, Leguminosae, Aceraceae, Dioscoreaceae and the Bruceraceae (INOUE, KENMOCHI, FURUKAWA and FUJITA, 1987).

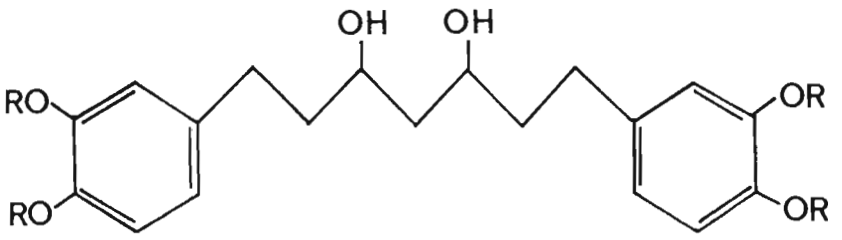


Figure 1.6: The general molecular structure of the diarylheptanoids.

The 9-phenylphenalenones (Figure 1.7), which are found only in the Haemodoraceae, a family closely allied to the Hypoxidaceae (THOMAS, 1961) and the *meta,meta*-bridged biphenyls (Figure 1.8) found in the Myricaceae (CAMPBELL, CROMBIE, TUCK and WHITING, 1970; BEGLEY, CAMPBELL, CROMBIE, TUCK and WHITING, 1971) are considered to be related to the diarylheptanoids.

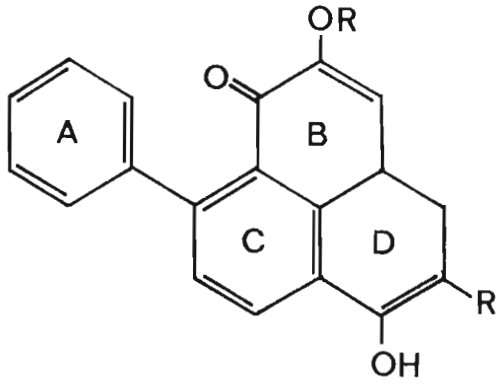


Figure 1.7: The basic molecular structure of the 9-phenylphenalenones.

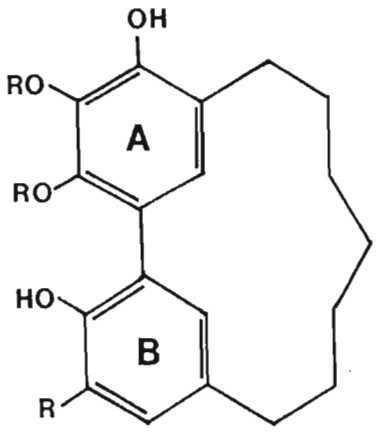


Figure 1.8: The basic molecular structure for the *meta*, *meta*-bridged biphenyls.

The diarylheptanoids consist of two phenolic rings linked by a seven carbon chain. This group of phytochemicals is exemplified by the following natural products: yashabushiketol and dihydroyashabushiketol isolated from *Alnus* L. species in the Betulaceae (ASAKAWA, 1970; SUGA, ASAKAWA and IWATA, 1971), centrolobol, centrolobin and de-O-methylcentrolobin isolated from *Centrolobium* Mart. ex Benth. species in the Leguminosae (ARAGAO CRAVEIRO, DA COSTA PRADO, GOTTLIEB and WELERSON DE ALBUQUEQUE, 1970), the curcuminoids isolated from *Curcuma aromatic* Salib. and *Curcuma longa* L. (JANAKI and BOSE, 1967) and the [7.0]-metacyclophanes isolated from the *Myrica* L. species in the Myricaceae (MALTERUD and ANTHONSEN, 1980).

The Zingiberaceae are a family rich in many phytochemicals, particularly the diarylheptanoids. This family of herbaceous, perennials is widely distributed throughout India, tropical Asia and northern Australia. Zingiberaceous plants are extensively used in traditional medicine in tropical Asia. The importance of this family medicinally, is reflected by some 56 different applications for which its members are used. The main applications include use as anti-

inflammatories, relief for asthmatic symptoms and treatment of skin diseases. The listed biological activity of the Zingiberaceae is as follows: abortifacients, anti-inflammatories, antimalarial, antitumour, antipyretic, antifungal, aphrodisiac, antitussive, cardiotonic and a cure for gastro-intestinal disorders. The medicinal effectiveness of this family has been attributed to the diarylheptanoids, several of which have been shown to be inhibitors of prostaglandin synthesis. As well as the diarylheptanoids this family is rich in terpenoids, flavonoids and arylalkanones (TUNTIWACHWUTTIKUL, PANCHAROEN, KANJANAPOTHI, PANTHONG, TAYLOR and REUTRAKUL, 1986).

The 9-phenylphenalenones or 9-phenylperinaphthenones are pigments. Haemocorin, isolated from Australian members of the Haemodoraceae belonging to the genus *Haemodorum* Sm. (THOMAS, 1971) and lachnanthoside isolated from the roots of *Lachnanthes tinctoria* Ell. (WEISS and EDWARDS, 1969) are two examples of this group of phytochemicals.

Examples of the *meta*, *meta*-bridged biphenyls include myricanol and myricanone extracted from the stem bark of *Myrica nagi* Thunb. ex Murray which is reputed to be a fish poison (BEGLEY and WHITING, 1970; CAMPBELL, CROMBIE, TUCK and WHITING, 1970; BEGLEY, CAMPBELL, CROMBIE, TUCK and WHITING, 1971).

A further group of natural products that bear a structural resemblance to hypoxoside are those phytochemicals consisting of two phenolic rings linked via esteric bonds. These compounds are, in effect, complex glycosides of the hydroxycinnamic acids. Examples of these glycosides include desrhamnosylacteoside (Figure 1.9) found in the

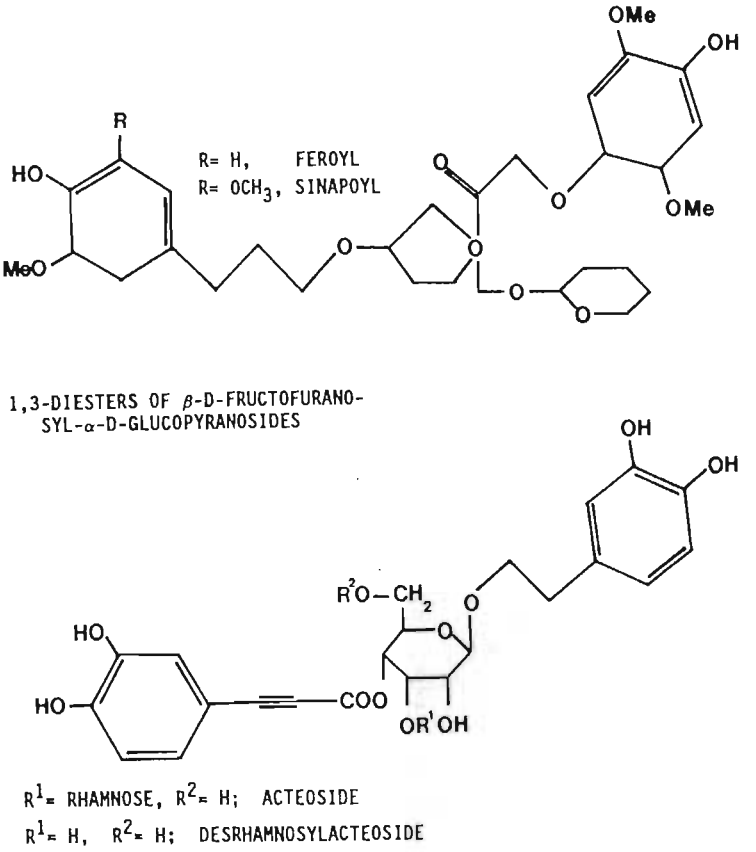


Figure 1.9: Examples of complex hydroxycinnamic acid glycosides.

diseased roots of *Rehmannia glutinosa* Steud. (SHOYAMA, MATSUMOTO and NISHIOKA, 1987) and the compounds isolated from *Polygala chamaebuxus* L. which are 1,3-diesters of *β-D*-fructofuranosyl-*α-D*-glucopyranosides with sinapoyl, feruloyl and acetyl ester moieties on the furanose ring (Figure 1.9)(HAMBURGER and HOSTETTMANN, 1985). Desrhamnosylacteoside and acteoside consist of two phenoxy groups, one with a three carbon and the other with a two carbon side chain which are esterified to glucose. In acteoside a further rhamnose moiety is attached to the glucose. The plant from which these esters are isolated is an important herbal plant in China (SHOYAMA, MATSUMOTO and NISHIOKA, 1987).

1.7. HYPOXOSIDE AND SECONDARY METABOLISM

Hypoxoside and the other diarylpentane-type compounds, and the diarylheptanoids, *meta,meta*-bridged biphenyls and 9-phenylphenalenones are phenolic compounds and may thus be considered to be products of secondary metabolism.

state that secondary metabolites express the individuality of a species in chemical terms.

Unlike the primary intermediates the products of secondary metabolism may accumulate in specialised tissues where they may be sequestered in the vacuoles of cells. In some cases the products may be exuded into canals, as in the case of resins. While primary metabolites are continually produced and utilised, the products of secondary metabolism are often only produced at certain developmental phases or in response to environmental stimuli or stress (MANN, 1987). Another major feature of secondary metabolism is the vast diversity of structure exhibited by its products (HASLAM, 1985,1986; MANN, 1987).

It is possible, using these definitions, to determine what constitutes a secondary or primary metabolite in most cases. However, there are phytochemicals which are less readily categorised. HASLAM (1985) points to the polyamines as such an example. These compounds, putresine, spermine and spermidine appear to be of secondary metabolite origin yet they are ubiquitous in nature. Furthermore they display a high affinity for DNA, stimulate protein synthesis and in some microbial mutants polyamines are an absolute requirement for growth. Despite the apparently essential role the polyamines play there is little evidence to support their function in primary metabolism.

Both cellulose and the phenolic polymer lignin are the major components of cell walls in vascular plants and are therefore vital for cellular development. Once deposited these compounds are metabolically inert. Only lignin and the processes involved in its synthesis and deposition are considered to be part of secondary

metabolism. That this definition is not applied to cellulose and its deposition is indicative of the arbitrary nature of the classification of primary and secondary metabolism (BONNER and GALSTON, 1952; GEISSMAN and CROUT, 1969; MANITTO, 1981).

The inadequacies of the classification of metabolism into two categories is further exemplified by the carotenoids and lipoquinones. The former are pigments present in all green leaves and are of importance in the functioning of photosynthetic organisms. Yet because they are structually diverse and differentially distributed they are defined as secondary metabolites (BONNER and GALSTON, 1952; LIAANEN-JENSEN, 1979). The lipoquinones occur in the mitochondria and chloroplasts of plant cells where they are considered to be part of primary metabolism. Thus the lipoquinones are classified as primary metabolites while the rest of the quinones are considered to be secondary metabolites (LEISTNER, 1981).

The limitations of the definitions of primary and secondary metabolism are broadly acknowledged and alternative classifications have been suggested. That of BONNER and GALSTON (1952) offers a more holistic approach to metabolism seeing it as one cyclically integrated whole, which is fundamentally similar for most organisms, and which is accompanied by side-branches terminating in end products which vary from one organism to the next. The cyclic parts of metabolism are described as the principal "highways", the constituents of which are intermediary in nature. The diversionary pathways into end products are considered to be "byways" and radiate from the tightly- integrated central whole. BONNER and GALSTON (1952) reason that within the confines of this description the Krebs cycle is the central hub of an

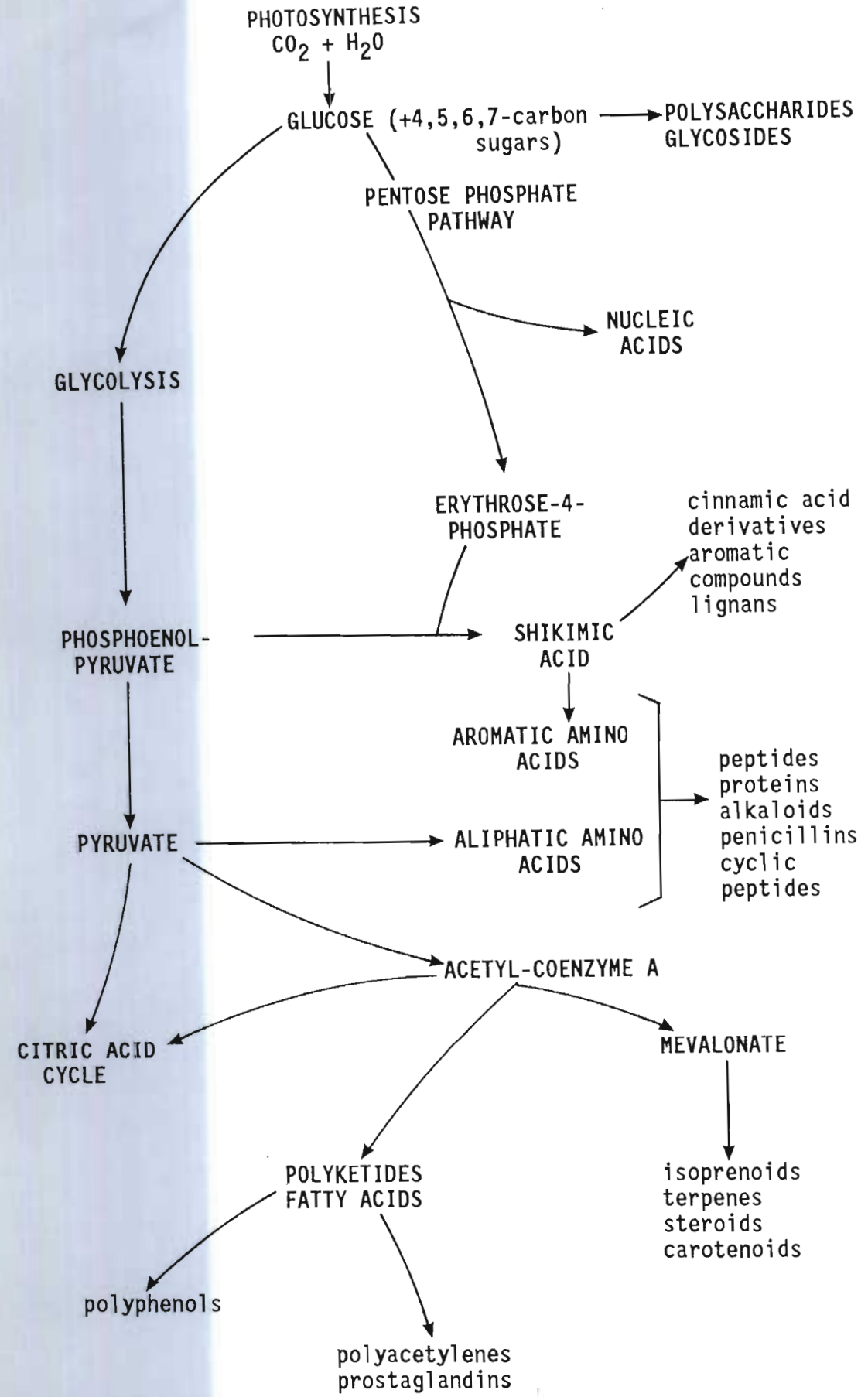


Figure 1.11: An illustration of the points at which secondary metabolism branches from primary metabolism (MANN, 1987).

The isoprenoids have been suggested to be the most widely distributed and abundant of the natural products (HASLAM, 1985). This group, which is divided primarily into the terpenes and the steroids, are of acetate origin via mevalonate. These phytochemicals are all polymers of isoprene and fall into a number of subgroups depending on polymer length and chain end substitution. The terpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes and polyterpenes make up all the essential oils, resins, steroids, carotenoids and *cis*-polyisoprenoids produced by the plant kingdom. The essential oils and resins play an important role in mediating the interaction of plants with their environment, functioning in chemical interactions of a plant-plant, plant-insect and plant-animal nature. Some of the isoprenoids are vital components of primary metabolism. This is exemplified by the prenylquinones and the carotenoids; the former being essential for electron transport and the latter for photosynthesis.

For man the existence of the isoprenoids has been highly beneficial. These compounds were used extensively by the mediaeval herbalist and are still employed today in herbal remedies. The monoterpenes and sesquiterpenes are of great importance in industry as they form the basis of many plant essences used for perfumes, cosmetics, soaps, detergents and flavourings for confectionery and oral preparations. Finally, the use by man of natural rubber, is extensive (BONNER and GALSTON, 1952; HASLAM, 1985; MANN, 1987).

The shikimate pathway, for which the primary intermediates, phosphoenolpyruvate and erythrose-4-phosphate are precursors, is one of two major pathways via which aromatic phytochemicals are formed. The shikimic acid produced in this pathway gives rise to the simpler

aromatic structures from which a vast number of structurally diverse compounds are produced.

The phenolic acids form one group of the simpler shikimate derivatives and are defined as organic compounds with at least one phenolic hydroxyl group and a carboxyl function. These acids exist naturally as glucose esters or esters in combination with quinic acid. Existence of the acids in the free form is a rare occurrence. The phenolic acids are ubiquitous in the plant kingdom, being principally important as precursors for many other secondary metabolites. The cinnamic acids are precursors for the cinnamyl alcohols, coumarins, flavonoids and xanthones. The benzoic acids are thought to originate by side chain degradation of the cinnamic acids and are utilised as precursors for compounds such as the ubiquinones. The phenylacetic acids are also employed as precursors for some of the quinones (GROSS, 1981; HASLAM, 1985; MANN, 1987).

One of the major products formed from the phenolic acids is lignin an integral component of the cell walls of vascular plants. It is believed that lignification was the predominant factor which allowed plants to adopt a terrestrial habit. Lignification contributes to the compressive strength of cell walls and thus provides the building material for woody stems and the water-conducting elements of vascular plants. In woody plants such a large proportion of the phenylpropanoids are incorporated into lignin that most of the aromatic rings in nature are found in wood. Lignin is polymeric, consisting of chains of molecules held in a matrix of cellulose fibrils. The three major types of lignin are composed of polymers of *p*-hydroxycinnamyl alcohol, coniferyl alcohol and sinapyl alcohol (GRISEBACH, 1981; MANN, 1987).

The aromatic amino acids, phenylalanine, tyrosine and tryptophan, products of the shikimate pathway, apart from contributing to protein synthesis are also important precursors for the biosynthesis of the alkaloids and phenolic compounds. The alkaloids are defined as nitrogen containing compounds which are at least partially basic. Most alkaloids are heterocyclic and as a group are the most structurally diverse of the secondary metabolites. Because of their complexity the nomenclature of the alkaloids has not been satisfactorily resolved. Most alkaloids are derived either from the metabolism of the aliphatic amino acids, ornithine and lysine or the aromatic amino acids, phenylalanine, tyrosine or tryptophan. Those derived from ornithine and lysine include the pyrrolidine, piperidine, pyrrolizidine, quinolizidine and pyridine alkaloids. The phenylalanine-, tyrosine- and tryptophan-derived alkaloids are divided into four structural types, the simple monocyclic compounds, the isoquinolines, the benzylisoquinolines and the Amaryllidaceae alkaloids.

The alkaloids are a valuable source of drugs and poisons. Morphine, quinine, codeine, cocaine and ephedrine are examples of alkaloids which are used as drugs, some of which have been used for centuries. Strychnine, ricinine and nicotine are extensively utilised as poisons, a property for which the alkaloids are well renowned. Because they are poisonous, or at best bitter, it is believed that they serve to protect plants against insects and herbivores. Speculation as to why the synthesis of these compounds arose include suggestions that they are waste products of nitrogen metabolism or a reserve of amino acids (WALLER and DERMER, 1981; MANN, 1987).

The polyketides are acetate-derived and were first defined as a group of natural products by BIRCH and DONOVAN (1953). These phytochemicals function primarily as precursors, often in conjunction with products of the shikimate pathway, for a large variety of secondary metabolites which include polyphenols, flavonoids, tannins, quinones, coumarins, xanthones and tannins (HASLAM, 1985; MANN, 1987). The cyclisation of polyketide rings is another pathway via which aromatic amino acids are formed in plants (MANN, 1987).

The flavonoids are one of the most characteristic and conspicuous groups of secondary metabolites, occurring almost universally in higher plants as the pigments responsible for most flower colours and as flavouring. The subgroups of the flavonoids are divided according to the patterns of substitution on the C ring, on the heterocyclic ring oxidation state and of the position of the B ring (Figure 1.12). The major flavonoid groups are the chalcones, isomeric flavanones, flavones, anthocyanins and isoflavonoids. The flavanones, flavones, flavonols, and the anthocyanins have the B ring substituted on the second position of the heterocyclic ring C while in the isoflavonoids ring B occupies position three on the C ring. Apart from these larger groups there are a few smaller groups such as the oligomeric flavonoids, biflavonyls and the proanthocyanidins. Most flavonoids occur naturally as glucosides. The A ring of the flavonoids is derived from the polyketides whilst the B ring and carbon atoms two, three and four of heterocyclic ring C are formed from phenylalanine and are thus of shikimate origin.

The chalcones and dihydrochalcones are by comparison quite rare. The flavanones, which are also relatively rare, are found in fruits, flowers, leaves and wood. Examples include the bitter-tasting

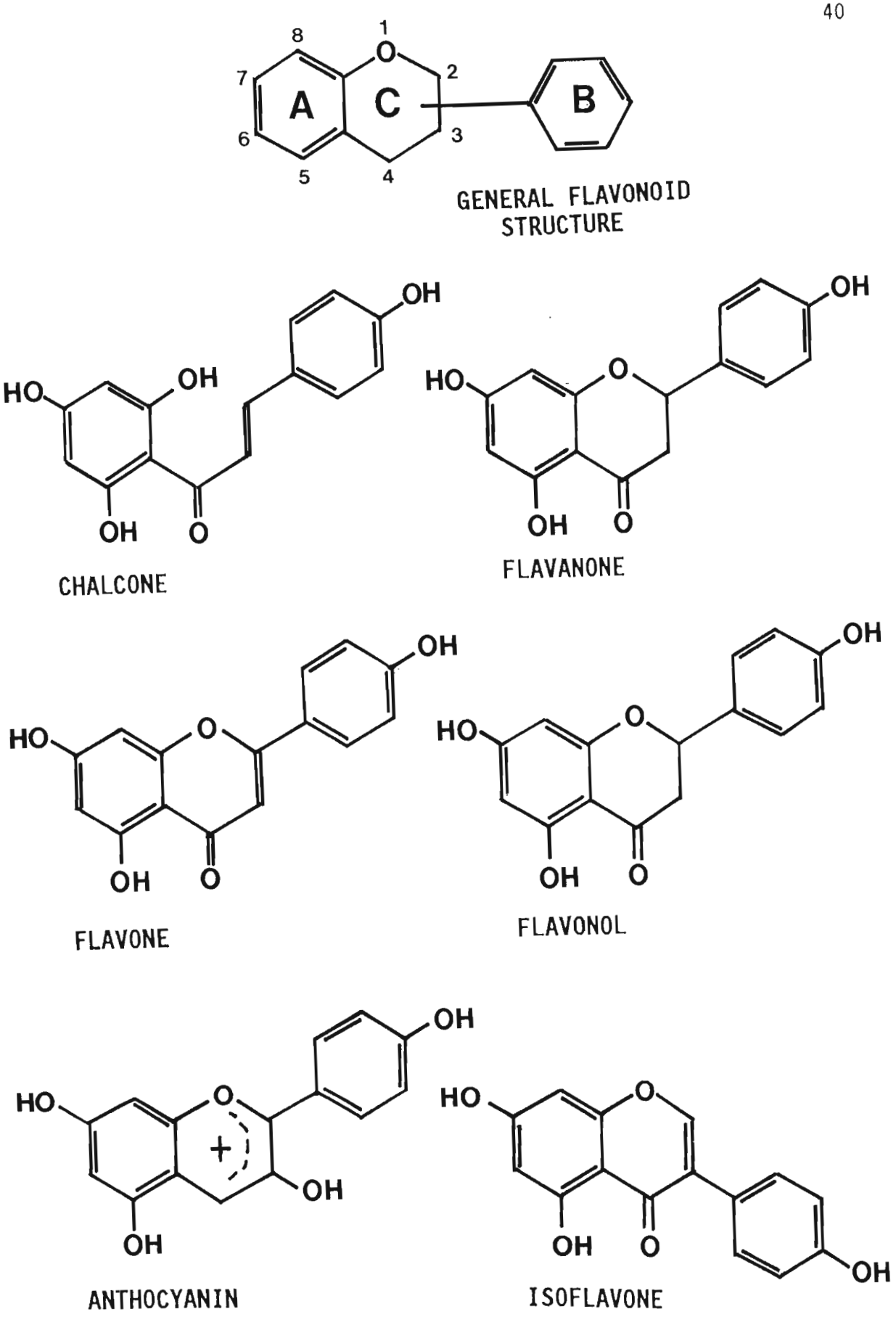


Figure 1.12: General structure of representative members of the different categories of the flavonoids.

compounds found in citrus peel; naringin in grapefruit and hesperidin in orange. The flavones and flavonols are much more common and widely distributed being found in leaves, bark, flowers and fruit. While the flavones and flavonols are not brightly coloured they absorb ultraviolet wavelengths and are thus conspicuous to insects, often being present at the center of flowers where they act as guides to the nectaries. The isoflavonoids are confined mainly to the Leguminosae.

The anthocyanins, the glucosides of the anthocyanidins, are the pigments that cause the red, blue and violet colours of flowers and fruits. These compounds tend to show a pH-dependent colour, often being red at low pH, violet at neutral pH and blue at higher pH. The obvious function of pigments in flowers and fruits being to attract pollinators or dispersers of seed. The large range of colour tones displayed by the anthocyanins is the result of different hydroxyl substitution and methylation.

Apart from providing attracting colours for pollinators it is believed that the flavonoids absorb ultraviolet rays and in so doing provide screens for the sensitive endogenous nucleic acids, NADH and other coenzymes (HAHLBROCK, 1981; HASLAM, 1985; MANN, 1987).

The tannins derive their name from the ability of extracts of the root, stem, fruits, pods, bark, wood and leaves of species, such as *Acacia mearnsii* De Wild., *Quercus* sp. and *Eucalytus* sp., to tan hides and skins. During this process the tannins cause crosslinking of the collagen chains in the skins thus protecting the fibres against microbial attack, as well as producing a greater resistance to heat and water. The tannins are classified into two groups which are structurally different and have different biosynthetic origins. The

"condensed" tannins or proanthocyanidins are polymers of flavandiols. The "hydrolysable" tannin, which can be cleaved by acids and bases, are phenolic acid esters with sugars, normally glucose. The proanthocyanidins originate from acetate and the polyketides via the flavonoid pathway whilst the "hydrolysable" tannins are derived from the shikimate pathway.

The tannins have strong astringent properties which are thought to deter predators. The effectiveness of the astringency is the result of the powerful protein precipitating properties of the tannins which interfere with the salivary proteins making the plant material unpalatable. Thus the tannins are considered to fulfil a predominantly protective function (HASLAM, 1974, 1981; MANN, 1987).

The quinones are a group of natural compounds that are somewhat unevenly distributed in the plant kingdom. Most naturally-occurring quinones are the *p*-quinones. The quinones are biosynthetically derived from many pathways, precursors for which include acetate, the aromatic amino acids, phenylalanine and tyrosine, shikimic acid and mevalonic acid. The quinones are divided into the benzoquinones, naphthoquinones and anthroquinones (LEINSTER, 1981).

Over five hundred naturally-occurring coumarins exist and these phytochemicals are used extensively as anticoagulants, drugs, laser dyes and fluorescent brighteners. The coumarins are formed from one of two distinct pathways. In the 3- and 4-phenylcoumarins the aromatic ring is polyketide in origin while the three aliphatic carbons are derived from the shikimate pathway. In the other coumarins the aromatic nucleus and the three carbon side chain of the lactone ring are produced by the shikimate pathway. The coumarins

are widely distributed throughout the plant kingdom and are attributed with a variety of biological activities. These include enhancement and inhibition of seed germination, chromosomal changes, inhibition of mitosis, stimulation and retardation of flowering and retardation of root and bud growth and leaf elongation. Coumarins are utilised commercially, examples of which include umbelliferone, an ultraviolet wavelength absorber, used in suntan preparations and warfarin a powerful rodenticide (BROWN, 1981; MANN 1987).

So far only the major groups of secondary metabolites have been discussed. Apart from these large and fairly widely distributed classes there are several smaller groups that share a limited distribution such as the xanthones, stilbenes, betaxanthanins, biflavonyls and betacyanins (HERBERT, 1981; HASLAM, 1985; MANN, 1987). Structually, hypoxoside and the other C_6-C_3.C_2-C_6 compounds, the diarylheptanoids and the 9-phenylphenalenones, do not fit into any of the groups discussed above. Natural products are usually classified according to two criteria; structure and biosynthetic origin. On the basis of structure these compounds may all be described as phenolics. The term phenolic is one which can be broadly applied to many secondary products since many possess the necessary phenolic hydroxyl function. By this criterion phenolic compounds include the simple phenols, phenolic acids, hydroxycinnamic acids, coumarins, flavones, flavanones, isoflavones, isoflavonoids, flavonols, anthocyanins, chalcones, quinones and xanthones (HARBORNE and SIMMONDS, 1964; RIBÉREAU-GAYON, 1972).

That the term phenolic should be such a widely-applied and well-recognised one is owed to the unique chemical properties that the phenolic function imparts. The oxygen in the phenolic hydroxyl group

has doublets of unshared electrons that interact with the loosely held π-electrons of the benzene ring producing a mesomeric effect resulting in a reduction of the electron density on the oxygen. Thus the hydroxyl oxygen holds a weak positive charge and therefore displays weak acidic properties. The phenolic hydroxyl groups also cause hydrogen bonding between phenolic molecules which alters physical properties, such as melting points, boiling points, solubility and the ultraviolet and infrared absorption spectra of the compounds. Intermolecular hydrogen bonding is a phenomenon of frequent occurrence for natural phenolics (THOMPSON, 1964). The formation of hydrogen bonds reduces the reactivity of the phenolic groups.

The presence of the phenolic hydroxyl group in some compounds results in the formation of complexes with metal ions (JURD and GEISSMAN,1956) which often affects the colour of the compound, an important consideration in nature. The phenolic function forms ether linkages with sugar moieties to such an extent that phenolic compounds are rarely found in the free form. Because of the phenolic hydroxyl group, many phenolic compounds are susceptible to oxidation, a process that is endogenously controlled by enzymes in plants such as the phenoloxidases (PRIDHAM, 1963). The browning of plant material in response to wounding is caused by the oxidation of phenolic compounds (RIBÉREAU-GAYON, 1972).

1.9. THE ROLE OF SECONDARY METABOLISM

When confronted by the abundancy and diversity of products of secondary metabolism it is not unnatural to seek evolutionary origins and functions for such compounds. Hypoxoside, according to reports (DREWES, HALL, LEARMONTH and UPFOLD, 1984), occurs at concentrations

in excess of four percent of the dry mass in *H. hemerocallidea* corms. Although the biosynthesis of levels as high as these must involve considerable energy expenditure for the plant, there is, at present, no evidence to suggest what benefit the plant derives from such products.

While some secondary products have, through the course of evolution, derived a primary physiological role in plant metabolism, or an importance at the level of plant-environmental interactions, the present role of these products cannot necessarily be linked to their biosynthetic origin.

HASLAM (1986) lists a number of the more accepted hypotheses for the possible role of secondary metabolism but points out that there is not enough evidence to support any one in particular. Four proposals are offered:

(i) Apparently useless secondary metabolites at some point do or did have a role in the plants metabolism.

(ii) The secondary metabolites are products of waste or detoxification.

(iii) The production of secondary metabolites has evolved as a strategy for survival in that by being able to produce a wide range of phytochemicals the plant has a greater chance of attracting or repelling other organisms.

(iv) The actual process of secondary metabolism and not the products thereof, is what is of major importance to the organism. In changed circumstances secondary metabolism allows the enzymes of primary metabolism to continue functioning until the unfavourable conditions change and growth and the associated metabolic activity can resume.

The last hypothesis does not discount the fact that, while it is mainly the process of secondary metabolism that is of importance to the plant, the products of this process may have acquired an important function as well. The latter is seen as a bonus rather than an absolute necessity.

Amongst physiologists this latter hypothesis has gained the most support as it also provides a feasible argument as to how secondary metabolism arose. It was FOSTER (1947) who, while working on antibiotics in bacteria, introduced the term shunt metabolites to describe how the pool of primary intermediates could be altered to yield antibiotics which are considered to be secondary products. BU'LOCK (1961) working in the same field proposed that secondary metabolites, particularly antibiotics, are metabolic products whose induction is the result of the abnormal stress presented by growth limitation on cellular metabolism. Thus the array of antibiotics produced is dependent on the enzymes possessed by a particular species of bacteria acting upon a few primary metabolites which have accumulated during growth limitation. The ultimate thrust of this argument is that an evolutionary advantage is conferred on an organism in the shunting of excess primary metabolites into secondary metabolism (BU'LOCK, 1961,1980). Shunting, he proposes, maintains the mechanism of primary metabolism and thus the potential for growth and multiplication during unfavourable conditions. This situation allows the rapid resumption of growth and multiplication once favourable conditions return. BU'LOCK (1961) therefore views secondary metabolism as an important maintenance mechanism for primary metabolism. How the production of these secondary metabolites occurs is presented in a second more refined theory (BU'LOCK and POWELL, 1964). The theory presents the sequence for the induction of

secondary metabolism as follows:

(i) termination of balanced growth, leading to

(ii) sudden accumulation of metabolic intermediates, leading to

(iii) induced synthesis of secondary metabolite, leading to

(iv) further induction of metabolism and hence diversification of the secondary metabolites.

Thus the termination of balanced growth results in an accumulation of certain primary intermediates which causes the induction of new enzymes in order to relieve the situation. The enzymes that act upon the primary intermediates are involved in the first step of secondary metabolism (Figure 1.13) and produce the first secondary metabolites, the presence of which induces further enzymes that have a low substrate specificity. This leads to the production of an array of biogenetically-related secondary metabolites, the pattern of which can be used as a fingerprint for a particular species.

Generally three theories have been proposed to describe the evolution of biosythetic pathways and the accompanying enzymes (CHAPMAN and RAGAN, 1980). The first is the subtractive theory, whereby the initially inefficient but potentially broad metabolism of an organism evolves to more defined pathways of increased specificity. The accumulative theory proposes the derivation of the more complex and diverse extant pathways from simpler ancestral pathways (HOROWITZ, 1945). The last type of theory advocates enzyme recruitment from other well-established pathways, implying that enzymes of greater antiquity displayed less substrate specificity than more recently-evolved ones (JENSEN and PIERSON, 1975).

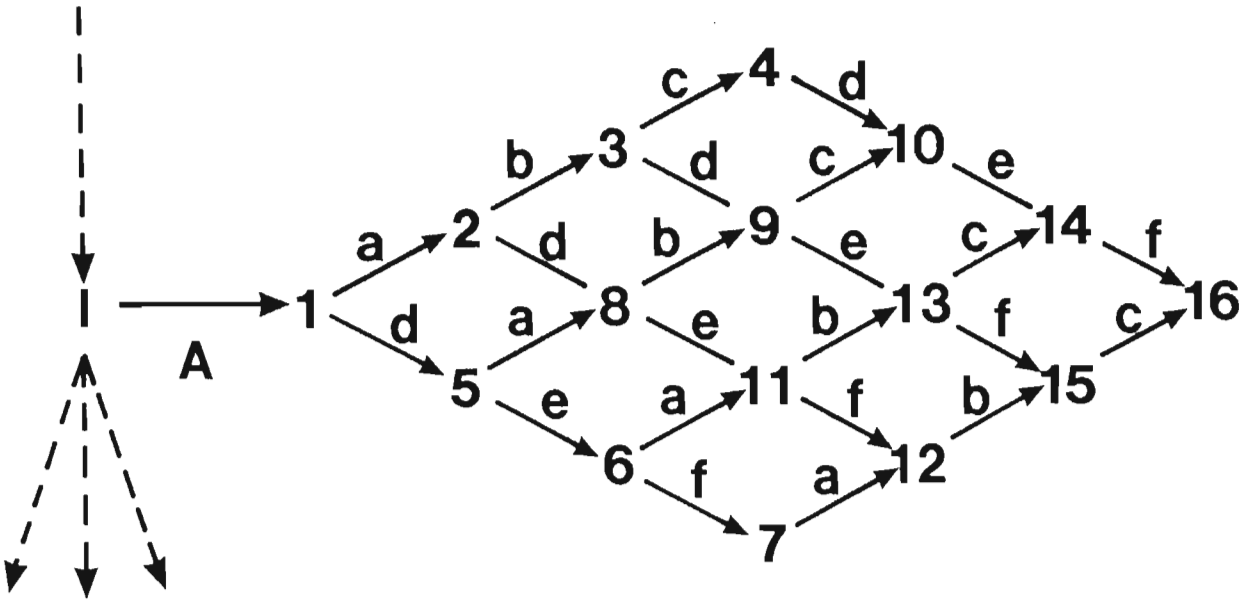


Figure 1.13: The scheme presented by BU'LOCK and POWELL (1964) for the biogenesis of a class of secondary metabolites derived from the hypothetical primary intermediate, I. A build up of I in primary metabolism (broken line) leads to reaction A, the first step into secondary metabolism (solid line), which initially yields product 1. Further reactions, a-f, with low substrate specificity lead to the formation of products 2-16. Higher substrate specificity would result in a smaller range of products. All products resultant from reaction A belong to one biogenetic class.

In terms of enzyme evolution the first theory suggests an initial array of enzymes with low substrate specificity being fined down to fewer with higher specificity and thus the development of a more efficiently controlled metabolism. The second theory implicates the induction of new enzymes resulting in increased complexity of pathways while the third proposes the importation and modification of enzymes from established pathways to those that are developing.

BU'LOCK (1961) and BU'LOCK and POWELL's (1964) theories for the induction and evolution of secondary metabolism favour the accumulative and recruitment theories for enzyme evolution. While BU'LOCK (1980) views secondary metabolism as a survival strategy of evolutionary significance for an organism, other views differ. WOODRUFF (1966) agrees with the proposal of BU'LOCK (1961) on how secondary metabolism originated in micro-organisms but he maintains that it is not selectively advantageous. His argument being that while an organism is growing it creates a demand for primary intermediates. When growth stops, because the feedback mechanisms are not efficient, an accumulation of primary intermediates occurs. This has led to the evolution of secondary metabolism in order to alleviate an abnormal situation. Thus WOODRUFF (1966) does not support the proposal that secondary metabolism is a means of maintaining primary metabolism during unfavourable conditions. He views it rather as the result of the unfortunate inefficiency of control in primary metabolism which forces the organism to produce secondary metabolites which are often toxic and disposed of by excretion to the surrounding environment. WOODRUFF (1966) believes that certain micro-organisms have solved this problem by the evolution of cells that have a precise control over their primary metabolic functions, such that antibiotic production is no longer necessary.

While WOODRUFF's (1966) theory may be applicable to micro-organisms the situation is not as simply defined in plants. Both BU'LOCK (1961) and WOODRUFF's (1966) proposals for secondary metabolism link to the theories that secondary metabolites serve as a means of detoxification or are waste products for primary metabolism when the levels of the primary intermediates become too high. In plants many secondary metabolites are toxic and inhibitory to the enzymes of primary metabolism, requiring that these compounds be stored in the vacuoles of specialised cells or that they be secreted into the environment or into canals within the plant. In plants, however, while the initiation of secondary metabolism may have been the result of inefficient control of primary metabolism, the evolutionary gain has been substantial. The derivation of secondary metabolism, and in particular such processes as lignification, it is thought, has allowed the successful colonisation of the terrestrial habitat by plant life. Therefore, in plant cells secondary metabolism has been evolutionary advantageous, not so much through its maintenance of primary metabolism but, rather, through the fortuitous role that the subsequent products have derived. In bacteria the evolutionary advanced situation may be to develop more efficient control of primary metabolism and thus make secondary metabolism redundant; in other words to move away from complexity towards a simpler but more tightly controlled metabolism. In plants, however, the increase in complexity and flexibility of metabolism has been significantly advantageous not only in the colonisation of the land from a structural point of view but in helping plants compensate for their sedentary habit.

The extent to which some secondary metabolites have become essential to the survival of plants has prompted some researchers to propose

that it is the selective pressures of the external environment that have brought fourth the *de novo* synthesis of secondary metabolites. The fact that the sequestering in the plant tissue of the often toxic and conjugated secondary metabolites also fulfils the function of repellent or attractant in plant-animal interactions, phytoalexins in the case of microbial attack or allelopathogens in the case of plants, is undoubtebly true.

STAHL (1888) was the first to suggest that the secondary metabolites stored in plant tissues had evolved in response to predation. He proposed that the possible effect that predation could have on the evolution of plant chemical composition could be equated with the effect that this predation has had on plant morphology. This argument is extended by FRAENKEL (1959), an entomologist, in studies of the eating habits of insects. He points out that the eating preferences of insects are not dependent on what is nutritionally available in a plant, most plants offering similar primary metabolites but rather whether the plant is palatable or not, a feature that is governed by the secondary metabolites that are present. These secondary metabolites, which developed early on in the evolution of plants, he suggests, are of little nutritional value, serving mainly to repel insect foragers, some of which have evolved a tolerance for these compounds and actively seek out plants containing them. FRAENKEL (1959) contends that it is reasonable to assume that secondary metabolites play a protective role with regard to fungi and bacteria as well. He postulates further that the existence of secondary metabolites in plants is the result of, and is solely dependent on, their purpose as an attracting or defence system for the plant.

JANZEN (1969) supports FRAENKEL (1959) in that he upholds the hypothesis that it is the pressures of natural selection that may call up the *de novo* evolution of a biosynthetic pathway capable of producing compounds that are toxic to foraging herbivores.

MULLER (1969) points out however, that these theories are essentially confusing the primary and principal functions of secondary metabolites. He concedes that presently these compounds produced by the plant may have the chief function of protection but he asserts that primarily they are the toxic wastes of primary metabolism, capable of totally disrupting it if they are not sequestered or secreted into the environment. MULLER (1969) argues that viewing these compounds purely in the context of animal toxins provides no explanation as to how they came to be. If, however, these metabolic pathways had arisen as waste from primary metabolism, as detoxification products or as shunt metabolites, then several simultaneous but alternative pathways would be functioning in the plant producing a wide variety of secondary products some of which may be evolutionary advantageous to the plant. Selective pressure applied by predation on these metabolites could result in one or more of the pathways being of more importance to the plant and thus these pathways would predominate over the others. MULLER (1969) sums up his argument as follows: "the toxicity to animals of these metabolic wastes, no matter how important eventually, is subsequent and secondary to their elimination from the protoplasm".

HASLAM (1986) maintains that while MULLER (1969) sees the protective functions of secondary metabolism as acquired rather than original, many biologists who support the concepts of chemical ecology find it difficult to accede to the energy utilised by secondary metabolism

unless these compounds confer some selective advantage. HASLAM (1986) is in favour of the concepts of MULLER (1969). He quotes examples where the precursors of end products are more effective deterrents than the end products themselves. The leaves of *Quercus* L. species produce two tannins, vescalagin and castalagin, which impart an astringency and ability to complex proteins. However, the precursor, penta-O-galloyl-β-*D*-glucose, for these tannins possesses these properties to a much greater extent. Clearly, the production of the two tannins is not as advantageous to the plant as their precursor. Thus it is probable that these compounds owe their continued existence to the fact that their presence is neither advantageous nor disadvantageous for the plant.

Despite the controversy over the origin of secondary metabolites HASLAM (1986) maintains that the role played by these compounds is auxilliary to the metabolic event that caused them. In order to understand the origin of secondary metabolism it would be of more value to examine the metabolic events and their causes. The precursors for secondary metabolites are just a few primary intermediates, namely phosphoenolpyruvate, pyruvate, acetyl co-enzyme A, mevalonic acid and a few amino acids. The latter two are derived from the former compounds. HASLAM (1986) has drawn attention to the fact that all these substrates have a common feature; they all occur in the final sequence of the glycolytic pathway just before entry into the Krebs cycle. Obviously this is where accumulation of primary intermediates would occur if a change arose in the energy requirements and building processes of primary metabolism. Of all the hypotheses the shunt or overflow theories provide the best explanation as to the origin of secondary metabolism (HASLAM, 1986).

1.10. ACCUMULATION OF SECONDARY METABOLITES

YEOMAN, MIEDZYBRODZKA, LINDSEY and McLAUCHLAN (1980) report that as a general rule the actively-growing parts of a plant, such as meristems, expanding leaves and the early stages of fruit and seed development, do not accumulate secondary metabolites. It is usually in the older fully-differentiated parts, such as the mature leaves, roots, fruits and seeds that accumulation occurs. YEOMAN, MIEDZYBRODZKA, LINDSEY and McLAUCHLAN (1980) suggest that an antagonistic relationship exists between primary metabolism, involving the processes of growth, cell division and protein synthesis and secondary metabolism. These authors conclude that with the slowing down of growth by plant cells there is a concomitant increase in cellular differentiation and organisation along with secondary product synthesis and accumulation.

The inverse relationship between growth and secondary metabolism may exist because these processes represent different developmental phases in the life of a plant cell. Obviously once growth has slowed, precursors from primary metabolism are available for channelling into secondary metabolism. Thus secondary metabolism is a process that occurs subsequent to primary metabolism after growth slows down and this interpretation would favour the shunt metabolite hypothesis for the origin of secondary metabolism. Investigation of secondary metabolism and the developmental stages at which it occurs may elucidate, to a certain extent, how the processes arose. However the complexity of developmental processes in plants makes such a study difficult. The situation is further complicated by the short duration of the activity of these pathways and the confinement of these processes to specialised tissues. Recovery of enzymes from these pathways is made difficult by the accumulation of the secondary

products themselves, as they are often very inhibitory to enzyme action (LOOMIS and BATTAILE, 1966; HEIDE and LEISTNER, 1983).

Due to the above situation *in vitro* methods have gained in popularity as a means of studying secondary metabolism. The reasons for this include the stability and homogeneity of these systems coupled with the ability to exert control over the culture environment and supply of nutrients and hormones in a microbial-free environment (ELLIS, 1984). There are some serious drawbacks in using these techniques, however. Firstly, genome instability is often markedly prevalent in cell cultures (MEINS, 1983). This factor coupled with the reduced cellular differentiation in cell cultures can inhibit the phenotypic display of secondary metabolism and often leads to a reduced accumulation or total lack of the desired metabolites when compared to the parent tissue (ELLIS, 1984). Nevertheless, interesting and conflicting trends between growth and phenolic production have been reported for cell cultures.

Generally it is accepted that secondary metabolites accumulate in cell suspension cultures when these systems enter the stationary phase of growth (KURZ and CONSTABEL, 1985). Therefore the inverse relationship between growth and secondary metabolite production that occurs in the whole plant is clearly displayed, in most cases, in the production of these compounds *in vitro*. There have been many reports that phenolic production in cell cultures takes place chiefly in the stationary phase (CONSTABEL, 1968; FORREST, 1969; NASH and DAVIES, 1972; WESTCOTT and HENSHAW, 1976; PHILLIPS and HENSHAW, 1977; MUHITCH and FLETCHER, 1985). Observations of continued cell division taking place during secondary metabolite production (NASH and DAVIES, 1972; WESTCOTT and HENSHAW, 1976) or conversely secondary metabolite production by

actively dividing cells have been made. It has been argued, however, that the growth cycle of the cell cultures in these examples were not completely synchronous and that some of the cells had already entered the stationary phase (PHILLIPS and HENSHAW, 1977).

A number of theories prevail as to why production of secondary metabolites occurs in the stationary phase. The situation is seen as an ordered expression of the genome with the synthesis or activation of the relevant enzymes occurring at a specific developmental stage (LUCKNER, 1980). Some view it as a competition between primary and secondary metabolism. PHILLIPS and HENSHAW (1977) postulate that the production of phenolics in *Acer pseudoplatanus* L. is rate-limited by the diversion of precursors for this process into protein synthesis. FORREST (1969) concludes that a decrease in the growth rate in cell cultures due to environmental conditions causing a nutrient imbalance, as in higher plants, leads to channeling of the accumulated carbohydrates into polyphenol synthesis. MIZUKAMI, KONOSHIMA and TABATA (1977) working with *Lithospermum* cell cultures found that the addition of inhibitors of RNA and protein synthesis stimulated the production of shikonin derivatives. The conclusion derived from these results was that the enzymes of the biosynthetic pathways responsible for the production of the secondary metabolite were present at all times. Thus it was the availability of nutrients and the efficiency of primary intermediate balance in primary metabolism that determined whether shunting into secondary metabolism occurred or not.

The way in which this switch to secondary biosynthetic pathways occurs has been postulated by YEOMAN, MIEDZYBRODZKA, LINDSEY and McLAUCHLAN (1980) in two theories. The first suggests that the entire enzyme complement for the biosynthetic pathways are present at all times and

that all that is required is the switching on of a key enzyme. The second theory postulates that the synthesis of the enzymes for a particular pathway occurs when the conditions for the production of the secondary metabolite are favourable. The results obtained by MIZUKAMI, KONOSHIMA and TABATA (1977) favour the first theory because in their experiment the *de novo* synthesis of enzymes was blocked. If the shunt metabolite approach is applied to this situation then as KURZ and CONSTABEL (1985) argue the *de novo* synthesis of enzymes postulated by the second theory would not be as evolutionary advantageous to the plant as the first theory.

These examples illustrate the value of *in vitro* methods for studying secondary metabolism.

1.11. *H. HEMEROCALLIDEA* AND SECONDARY METABOLISM

Investigating the production of hypoxoside *in vivo* and *in vitro* by *H. hemerocallidea* would aid in elucidating the possible role this phytochemical has, if any,in the plant. It would also reveal whether hypoxoside is a typical secondary metabolite. A study of this sort requires that a number of areas be investigated. Thus a knowledge of precursors and where precisely the production of this compound "fits" into the plants metabolism is required. Secondly, information regarding the seasonal fluctuations in the production of the phytochemical linked to the growth rates of the plant during this time and thus the availability of precursors are necessary. Thirdly, the organ and cellular site where synthesis occurs are important since the plant has a dormancy period. Finally a study of the effects of nutrient availability, physical factors and hormonal control of the process, using an *in vitro* approach is needed.

With these considerations in mind an investigation of hypoxoside production in *H. hemerocallidea* was initiated.

CHAPTER 2

BIOSYNTHETIC INVESTIGATIONS

2.1. INTRODUCTION

The investigation of biosynthetic pathways in plants can be approached through a number of methods. Initially, however, the molecular structure of the phytochemical in question must be determined. While the processes by which the primary intermediates are formed in plants are complex, obscuring their origin, in the case of secondary metabolites structure often allows fairly accurate speculation about precursor origin (HERBERT, 1981). This is made possible by the fact that secondary metabolites are derived from only a few of the primary intermediates.

From structural elucidation, hypotheses for precursors and biosynthetic mechanisms for a phytochemical can be devised. The biosynthesis of many secondary products has been found in actuality to closely resemble the original hypothetical proposals. The proposed precursors, intermediates and biosynthetic pathways for a secondary product can be examined with the use of a number of methods which include:

(i) the *in vitro* use of extracted and purified enzymes,

(ii) the *in vivo* use of mutants with blocked biosynthetic pathways and

(iii) the use of isotopically labelled molecules (RIBÉREAU-GAYON, 1972; HASLAM, 1985).

Until recently the enzymatic approach for biosynthetic studies of secondary metabolism has been hindered by a number of problems. Extraction of enzymes from tissues rich in secondary products such as organic acids, tannins and phenolics is often very unsatisfactory as these phytochemicals frequently inhibit and/or denature the enzymes. The situation is further exacerbated firstly, by the low concentrations in which the enzymes of secondary metabolism are usually present and secondly, their localised distribution in specialised tissues. Furthermore, the enzymes of a particular secondary pathway may only be present in the tissue during specific developmental phases (RIBÉREAU-GAYON, 1972; HASLAM, 1985). These obstacles have made enzymology an unfavourable method for studying biosynthesis. However, with the advent of more refined methods for enzyme extraction and purification it is becoming a more useful technique (HUTCHINSON, 1986).

Biosynthetic techniques involving the use of mutants have been employed extensively in studies with micro-organisms. Mutant strains, that have lost the ability to synthesise certain constituents of a biosynthetic pathway due to a lack of the appropriate enzyme, are selected for. Compounds that are thought to be intermediates, which occur after the blocked step, can then be fed to the culture medium to determine if the desired end product will be formed. Mutation is induced artificially in micro-organisms by exposure to X-rays, ultraviolet radiation or mutagenic chemicals, all of which have a damaging effect on the organisms' genome. In this way microbial populations can be exposed and then screened for useful mutations. Mutation is, however, often lethal. In plants the production and selection of mutant lines is not an easy task. Nevertheless, cell suspensions have been used successfully for this purpose. The

criteria, however, require that a cell suspension of the plant under study be initiated and that this produces the required secondary metabolite at levels sufficient for its study. Thus this method is not frequently used (RIBÉREAU-GAYON, 1972; HERBERT, 1981; VICKERY and VICKERY, 1981; HASLAM, 1985; MANN 1987).

The most effective and commonly used method for studying biosynthetic pathways utilises isotopic incorporation either on its own or in combination with subsequent molecular degradation to determine positions labelled in the end product. Isotopic studies can also be used in combination with enzymatic and mutant methods. It is ideal to utilise as many methods as possible in biosynthetic studies in order to reduce the diversity of interpretation. However, it is often impossible to achieve this.

Isotopic labels can be divided into two categories:

(i) *radioactive isotopes*, for example carbon-14 and tritium, which can be assayed very sensitively by scintillation counting and

(ii) *stable isotopes*, for example carbon-13, nitrogen-15, oxygen-18 and deuterium, which are assayed for by much less sensitive methods, such as mass spectrometry and nuclear magnetic resonance spectroscopy (NMR). The advantage of NMR being that it is non-destructive. However, the use of stable isotopes requires expensive instrumentation (HERBERT, 1981).

The feeding of the isotopically labelled precursor to the experimental system presents problems. Where cell suspensions or cell free extracts are used these problems are not as great as when intact plants or organs are used. Methods generally used for administering

isotopically labelled compounds to intact plants or organs include:

(i) The wick method where a wick is threaded through the plant stem and the end dipped into an aqueous solution containing the isotopically labelled compound.

(ii) Assimilation via the roots which are placed into an aqueous solution containing the isotopically labelled compound.

(iii) Injection of an aqueous solution of the isotopically labelled compound into a hollow stem or seed capsule.

(iv) Incubation of excised organs in an aqueous solution or nutrient medium containing the isotopically labelled compound (HERBERT, 1981).

Obviously, the method chosen for the application of the isotopically labelled compounds, is dependent on the morphology of the plant in question.

Despite the fact that isotopic labelling methods have been successfully employed to elucidate many biosynthetic pathways in plants and micro-organisms, there are a number of problems which can lead to the erroneous interpretation of results.

Firstly a negative result may be due to a number of factors other than that the applied compound is not a precursor. These include a lack of penetration to the biosynthetic site caused by poor translocation; a lack of permeability of the tissues to the suspected precursor; or the enzymatic degradation of the applied molecule *en route*. Generally incorporation of radioactivity by plants is low usually between 0.0001 to 0.01 percent of the total applied (MANN, 1987). Time of application is very important as the precursor must be applied at a

time when the plant is naturally producing the secondary metabolite under scrutiny.

Low incorporation of the labelled compound into the secondary metabolite being studied is a result that must be treated with caution. CORNFORTH (1973) states that in relation to incorporation being indicative that one compound is a precursor for another, "It is surprising how many workers have tended to regard this appearance in yields, however miserable as sufficient demonstration".

The low incorporation can be due to a dilution of radioactivity brought about by the applied compound serving as a precursor for a number of other metabolic pathways. Alternatively, the exogenous application may disrupt the endogenous balance of the tissue. Thus the incorporation of the suspected precursor may not mean that it is an obligatory intermediate but rather that this just represents a convenient way for the plant to deal with the exogenous excess. The enzymes of secondary metabolism often have a broad substrate specificity so that applied molecules that resemble the natural precursor may be incorporated into the end product in an analogous pathway, which is distinct from the normal major pathway (RIBÉREAU-GAYON, 1972; HASLAM, 1985; MANN, 1987).

Hence the comment that it is "much easier to show that a compound can serve as a precursor for a cell constituent than to determine whether it is a normal obligatory intermediate in the biosynthesis of that constituent" (DAVIS, 1955).

Therefore, the results obtained from the application of isotopically labelled compounds should be consistent and degradation of the end

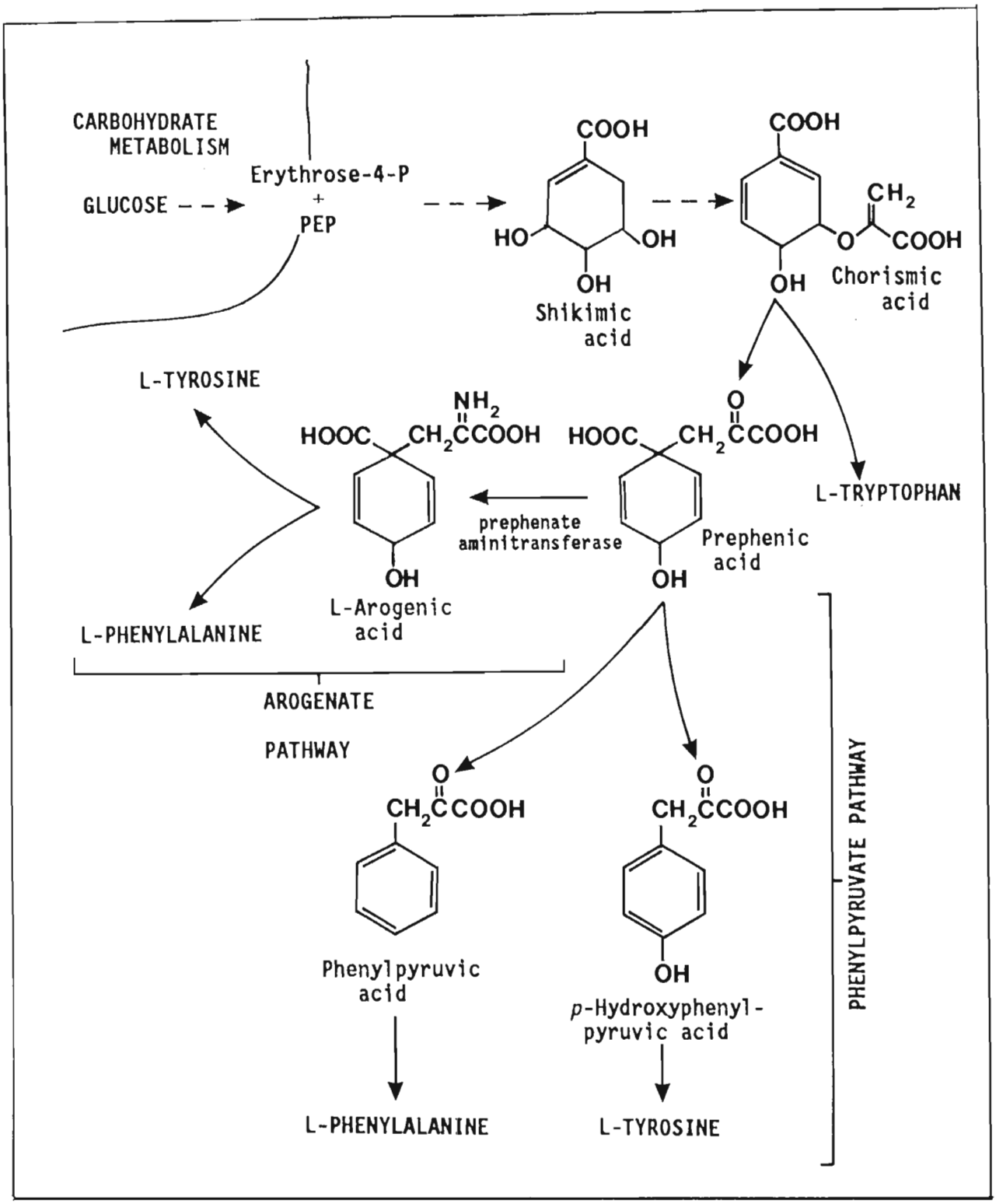


Figure 2.1.1: The shikimate pathway, illustrating the two alternative branches via which the aromatic amino acids can be formed from prephenate, namely the arogenate and the phenylpyruvate pathways.

only the aromatic amino acids but shikimate itself can form a connecting link between this pathway and secondary metabolism (JENSEN, 1986). Apart from the great diversity of secondary metabolites derived from the shikimate pathway, the output of the pathway is estimated to be up to 60 percent or more of the final plant mass (JENSEN, 1986).

The shikimate pathway was first elucidated by DAVIS (1955) who was studying the biosynthesis of aromatic amino acids in the micro-organism *Escherichia coli*. The scheme derived by DAVIS (1955) (Figure 2.1.1) for the pathway in *E. coli* involved the formation of phenylpyruvic and *p*-hydroxyphenylpyruvic acids from prephenic acid, which served as direct precursors for phenylalanine and tyrosine, respectively. It was suggested that a similar pathway existed in higher plants (BROWN and NEISH, 1955). However, it has subsequently been found that other prokaryotes use L-arogenate as an intermediate between prephenate and the aromatic amino acids, phenylalanine and tyrosine (BYNG, KANE and JENSEN, 1982). Since this discovery it has become apparent that this is the major pathway present in plants although exceptions exist (CONNELLY and CONN, 1986; JENSEN, 1986; SCHMAUDER and GRÖGER, 1986).

The enzyme, phenylalanine ammonia lyase has been described as " the first committed enzyme of phenylpropanoid metabolism" (HANSON and HAVIR, 1979) serving as the "bridge" between the shikimate pathway and the vast array of phenylpropanoid biosyntheses (CAMM and TOWERS, 1973; STAFFORD, 1974)(Figure 2.1.2). Phenylalanine ammonia lyase, first shown by KOUKOL and CONN (1961) to convert L-phenylalanine to *t*-cinnamic acid and L-tyrosine to *p*-coumarate (NEISH, 1961), has now been characterised for numerous plants (HASLAM, 1985). It is widely

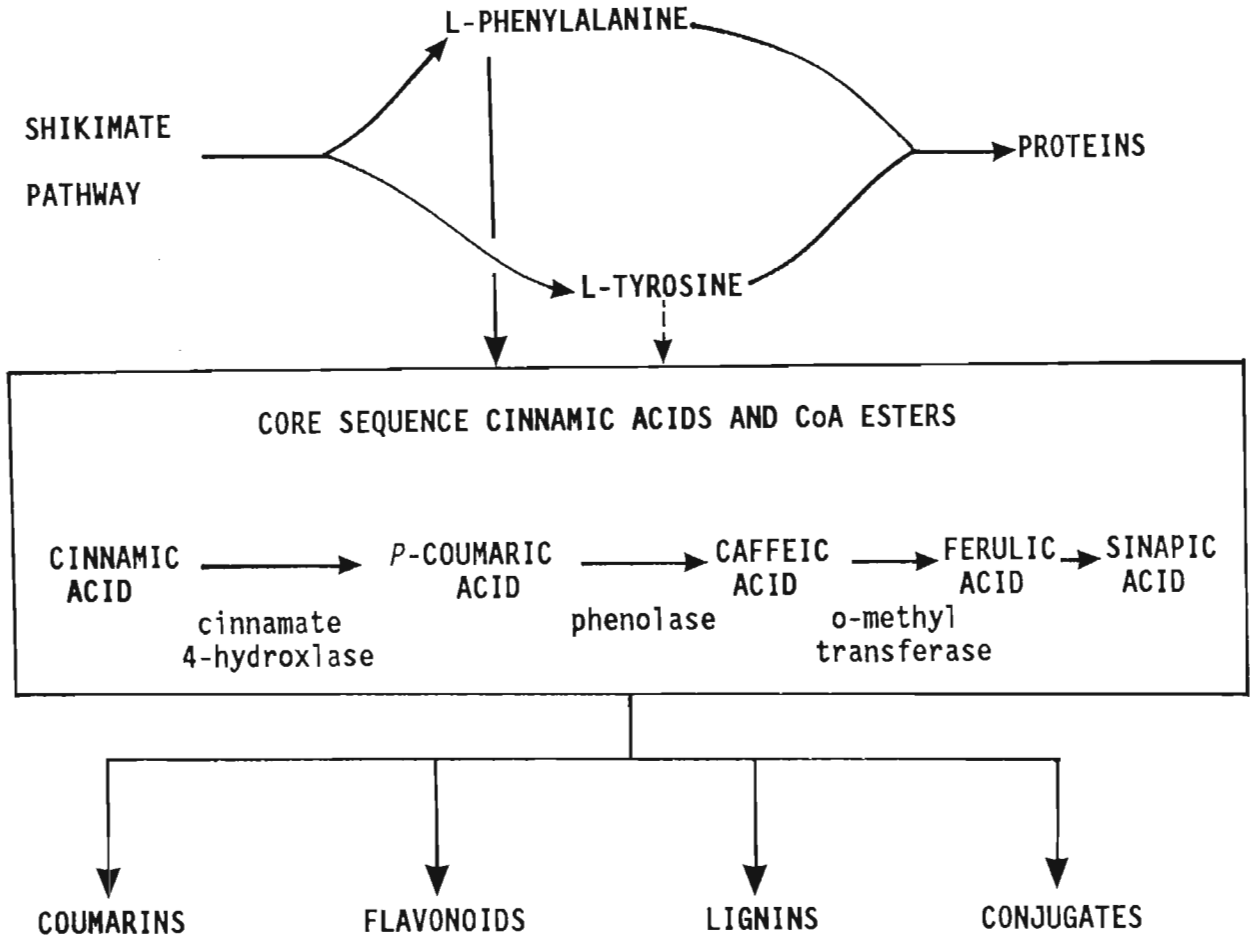


Figure 2.1.2: A schematic diagram of phenylpropanoid biosynthesis.

held that subsequent to the action of this enzyme, the various hydroxycinnamic acids (Figure 2.1.2) are derived sequentially by successive hydroxylations and O-methylations (NEISH, 1964; HANSON and HAVIR, 1979). Since the hydroxycinnamic acids are ubiquitously present in plant tissues as glycosides, esters or amides, it has been suggested that this conjugation is a detoxification mechanism or a means of enhancing solubility and/or decreasing sensitivity to enzymatic oxidation (HASLAM, 1985). The hydroxycinnamic acids and the corresponding conjugates thereof, play a major part in phenolic biosynthesis, their being the first phenylpropanoid compounds formed, from which the other more complex phenylpropanoid derivatives are believed to be derived (HANSON and HAVIR, 1979; HASLAM, 1985).

The acetate hypothesis, derived and confirmed by BIRCH and DONOVAN (1953), extrapolates the involvement of acetic acid as a building block for fatty acids and steroids to phenolic compounds providing an additional route to the formation of aromatic nuclei in plants (HASLAM, 1985). The "head-to -tail" linkage of acetate units proposed by BIRCH and DONOVAN (1953) has also been conclusively proved by GRISEBACH (1957).

The basic mechanism of the acetate hypothesis is summerised by HASLAM (1985) as follows:

(i) Acetate units are joined by the formal elimination of water in head-to-tail linkage with each other or with other naturally occurring carboxylic acids to form β-polyketomethylene chains.

(ii) The β-polyketomethylene chains may undergo secondary changes, notably they may cyclise to form aromatic rings.

(iii) The carbon skeleton thus formed may be modified by the introduction of alkyl groups.

(iv) Secondary processes of reduction and of oxidation may occur either before or after cyclisation.

The pattern of hydroxyl substitution on the aromatic nuclei of plant phenols can aid in indicating their origin. For example, the aryl nuclei of hypoxoside are disubstituted with hydroxyl groups that hold an *ortho* relationship relative to each other (Figure 2.1.3). Generally it is considered that this pattern of hydroxyl substitution

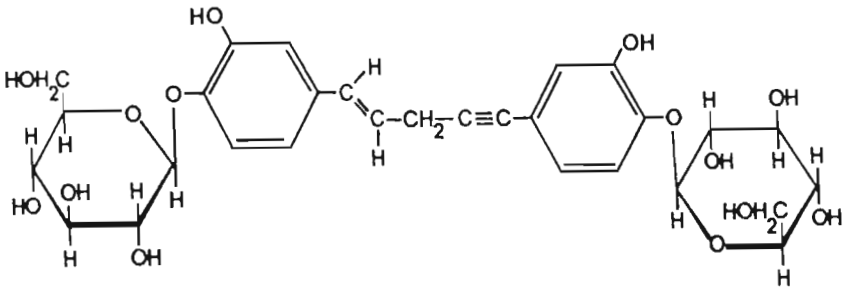


Figure 2.1.3: The molecular structure of hypoxoside.

is of shikimate origin (HASLAM, 1985). The elucidation of the biosynthesis of the flavonoid, quercetin, illustrates this feature of shikimate derivates. UNDERHILL, WATKIN and NEISH (1957) showed that shikimic acid, phenylalanine, *p*-coumaric acid and cinnamic acid are the most efficient precursors for the B ring of quercetin (Figure 2.1.4).

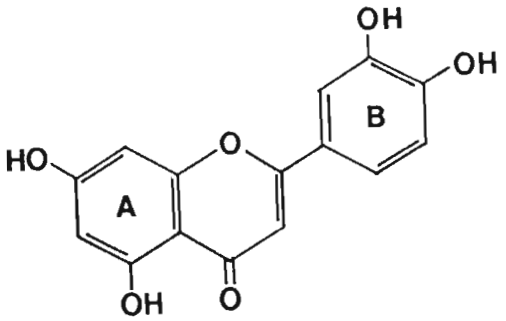
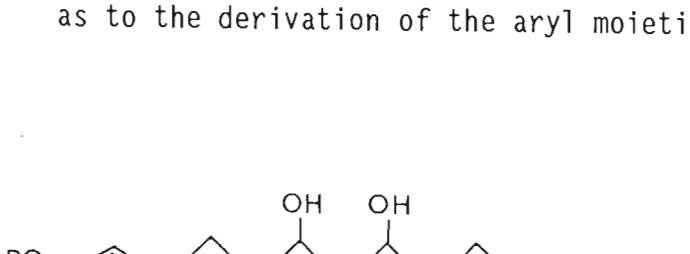


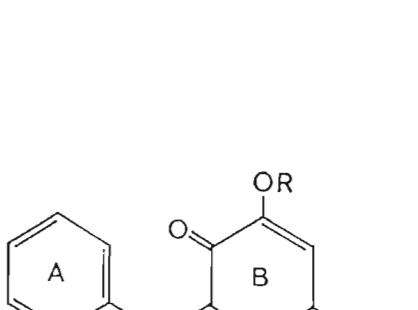
Figure 2.1.4: The structure of the flavonoid, quercetin.

The A ring of this flavonoid was shown to be of acetate origin (WATKIN, UNDERHILL and NEISH, 1957) with the dihydroxyl substituents sharing a *meta* relationship with respect to one another. The separate biosynthetic origins of the aryl moieties of the flavonoids has been substantiated by other researchers (GRISEBACH, 1957; HUTCHINSON, TAPER and TOWERS, 1959; CO and MARKAKIS, 1966). In the case of the flavonoids, the pattern of hydroxyl substitution on the acetate derived A ring is generally *meta* (RIBÉREAU-GAYON,1972; HASLAM,1985). Whether, the type of hydroxyl substitution on an aryl moiety can always be used as an indication of its origin, is debatable.

The biosynthetic origin of the aromatic nuclei of the phenolic compounds that can be regarded as structurally related to hypoxoside, namely the diarylheptanoids and 9-phenylphenalenones, is controversial. Like hypoxoside, these compounds contain aryl groups with dihydroxylation substitution that is *ortho*, suggesting a similar origin for all the aromatic nuclei present in the molecule (Figure 2.1.5). The results that deal with the biosynthesis of the diarylheptanoids and 9-phenylphenalenones published thus far, conflict as to the derivation of the aryl moieties.



DIARYLHEPTANOID



9-PHENYLPHENALENONE

Figure 2.1.5: Generalised molecular structures for the diarylheptanoids and 9-phenylphenalenones.

GEISSMAN and CROUT (1969) suggested that the biosynthesis of the diarylheptanoid, curcumin, involved the linkage of two cinnamate units by a central carbon supplied from malonate. Subsequent testing of this hypothesis by ROUGHLEY and WHITING (1971; 1973) yielded surprising results. In studies with *Curcuma longa* L. both [1-^{14}C]- and [3-^{14}C]phenylalanine served as precursors for at least one cinnamate unit with no unexpected rearrangement of the propanoid side chain occurring. Application of [1-^{14}C]- and [2-^{14}C]acetate and [1-^{14}C]- and [2-^{14}C]malonate resulted in radioactivity being incorporated into curcumin; a result that was not unexpected until molecular degradation of curcumin was carried out. Radioactivity was not found to be confined to the central carbon of the heptane chain, as expected, but was also present in the degradation products containing the aromatic portions of the molecule. The possibility that acetate participation was occurring through pyruvate eventually into shikimate metabolism or through degradation to carbon dioxide was ruled out by the fact that [2-^{14}C]acetate incorporation was considerable in comparison to that of [1-^{14}C]acetate. Thus ROUGHLEY and WHITING (1973) have suggested an alternative route for the biosynthesis of curcumin. This route involves one cinnamate unit as a starter which is extended by five acetate or malonate units with eventual cyclisation of this side chain to give the second aryl nucleus. Hydroxylation at C-7 of the heptane chain would complete the molecule.

The extension of a cinnamate starter by acetate/malonate units has been observed for the derivation of the [n]-gingerols, particularly 6-gingerol (Figure 2.1.6) in *Zingiber officinalis* Roscoe. , a plant rich in diarylheptanoids (DENNIFF, MACLEOD and WHITING, 1980).

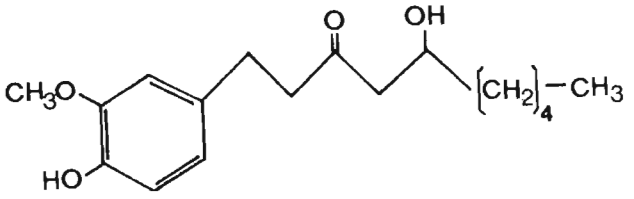


Figure 2.1.6: The structure of 6-gingerol.

The origin for the aryl groups of the cyclic diarylheptanoid, acerogenin A, as determined by INOUE, KENMOCHI, FURUKAWA and FUJITA (1987), disagrees with the findings of ROUGHLEY and WHITING (1971; 1973), in that the aryl units of acerogenin A are of like origin in contrast to curcumin where their origin is separate. The former group of researchers studied the biosynthesis of acerogenin A in *Acer nikoense* Maxim.. They proposed that acerogenin A was derived from centrolobol. The hypothetical derivation of the centrolobines, constituents of *Curcuma longa*, involves the coupling of two cinnamate units with the carbon atom of a precursor of unknown origin or alternatively it involves the chain extension of a cinnamate unit by five acetate units (ARAGAO CRAVEIRO, DA COSTA PRADO, GOTTLIEB and WELERSON DE ALBUQUEQUE, 1970). INOUE, KENMOCHI, FURUKAWA and FUJITA (1987) with the application of [1-^{14}C]phenylalanine, [3-^{14}C]cinnamic acid, [1-^{14}C]acetate, [2-^{14}C]acetate and [2-^{14}C]malonate followed by degradation of the radioactive acerogenin A recovered from the plant material, concluded that acerogenin A was formed, via centrolobol, from two *p*-coumarate residues and one malonate unit. Since centrolobol is found in both *Curcuma longa* (Zingiberaceae) and in *Acer nikoense* (Aceraceae) these results suggest that either the biosynthetic routes to curcumin and centrolobol in *Curcuma longa*

differ or that the biogenetic origins of the diarylheptanoids are different in the Zingiberaceae and Aceraceae.

THOMAS (1961) determined that the fungal 9-phenylphenalenones were of polyketide origin. He separates plant 9-phenylphenalenones biogenetically from the fungal 9-phenylphenalenones on the results obtained for the biosynthesis of haemocorin in *Haemodorum corymbosum* Vahl (THOMAS, 1971). He claims that the more effective incorporation into haemocorin of [2-^{14}C]tyrosine, [1-^{14}C]- and [2-^{14}C]phenylalanine than [1-^{14}C]- and [2-^{14}C]acetate supports the hypothesis that the plant 9-phenylphenalenones are derived from phenylalanine and tyrosine linked with the methyl carbon of acetate. This hypothesis was apparently further validated by the work of EDWARDS, SCHMITT and WEISS (1972) and that of HARMON, EDWARDS and HIGHET (1977) who studied the incorporation of [U-^{14}C]-, [1-^{14}C]- and [3-^{14}C]phenylalanine and [U-^{14}C]-, [1-^{14}C]- and [3-^{14}C]tyrosine into the aglycone of lachnanthoside (Figure 2.1.7) in *Lachnanthes tinctoria* Ell.. They conclude from the data, that the fact that the applied tyrosine labelled the C-5 position specifically confirms the hypothesis of THOMAS (1971) that the molecule is formed from the linkage of phenylalanine and tyrosine by a methyl carbon.

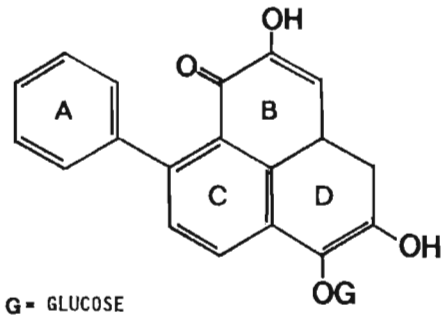


Figure 2.1.7: The structure of lachnanthoside.

However, ROUGHLEY and WHITING (1973) have drawn attention to this deduction by THOMAS (1971) and EDWARDS, SCHMITT and WEISS (1972) that tyrosine and phenylalanine were being incorporated into different aromatic rings of lachnanthoside, namely the A and B rings respectively , claiming that this supposition is not substantiated as no degradations were performed in order to ascertain whether phenylalanine was labelling ring A as well as ring B. Furthermore, no significance was attached to the incorporation of [1-^{14}C]-acetate into the molecule as the labelling sites were not determined. Thus, ROUGHLEY and WHITING (1973) have argued, it was not determined that ring B was of cinnamate origin and they contend that its origin could well be via acetate. NMR data that shows an incorporation of [1-^{13}C]phenylalanine into the C-7 position of lachnanthoside is sufficient evidence according to HARMON, EDWARDS and HIGHET (1977) to defend the belief that the B ring is derived purely from phenylalanine.

Thus at present the biosynthesis of the diarylheptanoids and 9-phenylphenalenones remains unresolved, while no literature is available on the biosynthetic derivation of the diarylpentane-type compounds.

MARINI-BETTOLO, PATAMIA, NICOLETTI, GALEFFI and MESSANA (1982) have suggested a biosynthetic origin from two phenylalanine units for hypoxoside. However, with the discovery of nyasoside their postulation for the derivation of hypoxoside has become less specific, suggesting that the molecule is formed from the junction of an acetylenic unit on the γ-position of a propenylic moiety (Figure 2.1.8) with no proposed origin for these aryl units being given

(MARINI-BETTOLO, NICOLETTI, MESSANA, GALEFFI, MSONTHI and CHAPYA,1985).

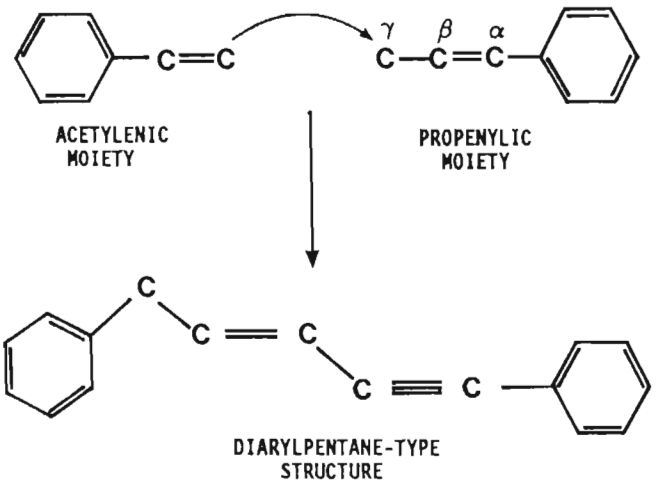


Figure 2.1.8: The possible mechanism for the formation of hypoxoside proposed by MARINI-BETTOLO, NICOLETTI, MESSANA, GALEFFI, MSONTHI and CHAPYA (1985).

On the basis of information available two hypotheses have been developed for the possible origin of the aryl moieties and linking pent-1-en-4-yne chain, namely:

(i) both aryl moieties are derived from cinnamate possibly via a mechanism similar to that postulated by MARINI-BETTOLO, NICOLETTI, MESSANA, GALEFFI, MSONTHI and CHAPYA (1985) which would involve the loss of the terminal carbon of the propenyl chain of one of these cinnamate units or that,

(ii) one aryl group is of cinnamate origin with the propenyl side chain of the moiety being extended by the addition of four acetate units followed by ring closure which would give the second aryl group and the five carbon linkage chain.

It is envisaged that if the first hypothesis is correct then acetate units would not be included in the hypoxoside molecule while, if the

second hypothesis is true then precursors of both acetate and shikimate origin would be utilised for hypoxoside biosynthesis.

In order to investigate the above hypotheses various experiments were devised utilising the following carbon-14 isotopically labelled compounds: L[U-^{14}C]phenylalanine, [3-^{14}C]*t*-cinnamic acid, [U-^{14}C]acetic acid, [1-^{14}C]acetyl coenzyme A, [2-^{14}C]*p*-coumaric acid and [2-^{14}C]caffeic acid.

Two experimental systems were investigated,

(i) an *in vitro* system consisting of root producing callus derived from *H. hemerocallidea* and

(ii) an *in vivo* system where clonal plants of *H. hemerocallidea* were used.

Because of the report by PAGE and VAN STADEN (1987) that the various types of cultured tissue of *H. hemerocallidea* displayed a different proficiency for hypoxoside production, the *in vitro* system was examined before any precursor studies were performed. There are a number of advantages in the employment of an *in vitro* system for biosynthetic studies; these being that

(i) sterile conditions are easy to maintain,

(ii) precursors can be applied more specifically, and

(iii) external physical factors are easy to control.

It was hoped that the results obtained from the tissue culture system could then be extrapolated to the whole plant.

2.2. MATERIALS AND METHODS

2.2.1. *IN VITRO* INVESTIGATIONS

2.2.1.1. Hypoxoside concentrations present in *in vitro* tissues

Tissues of *H. hemerocallidea* initiated and cultured *in vitro* were evaluated for their hypoxoside content in order to obtain a hypoxoside producing line that could be used for biosynthetic studies.

Material

Shoot-producing, root-producing and malformed root-producing calli (Figure 2.2.1) were initiated in January 1985 from the corm of a plant of *H. hemerocallidea* collected from the field, in the environs of Pietermaritzburg. The methods described by PAGE and VAN STADEN (1984, 1987) were used to initiate the cultures.

Corms were washed and the upper halves were cut into three slices (Figure 2.2.2) which were sterilised as follows: 0.1 percent benzimidazole (benomyl) (5 minutes), 95 percent ethanol (5 minutes) and 0.1 percent mercuric chloride (30 minutes). Sterilisation was followed by one 30 minute and two 20 minute washes with sterile distilled water.

Dead and damaged tissue was removed from the corm slices. Explants were cut from the primary thickening meristematic region of the corm slices and innoculated onto 15 ml basal medium (BM) in 25 mm x 80 mm glass tubes capped with CAP-O-TEST tops. The BM consisted of MURASHIGE and SKOOG (1962) micro- and macro-nutrients (Table 2.2.1) supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol,

Figure 2.2.1: Shoot-producing callus (**A**), root-producing callus (**B**), and malformed root-producing callus (**C**), produced *in vitro* from corm explants of *H. hemerocallidea* 10 months after initiation and 8 weeks after subculture

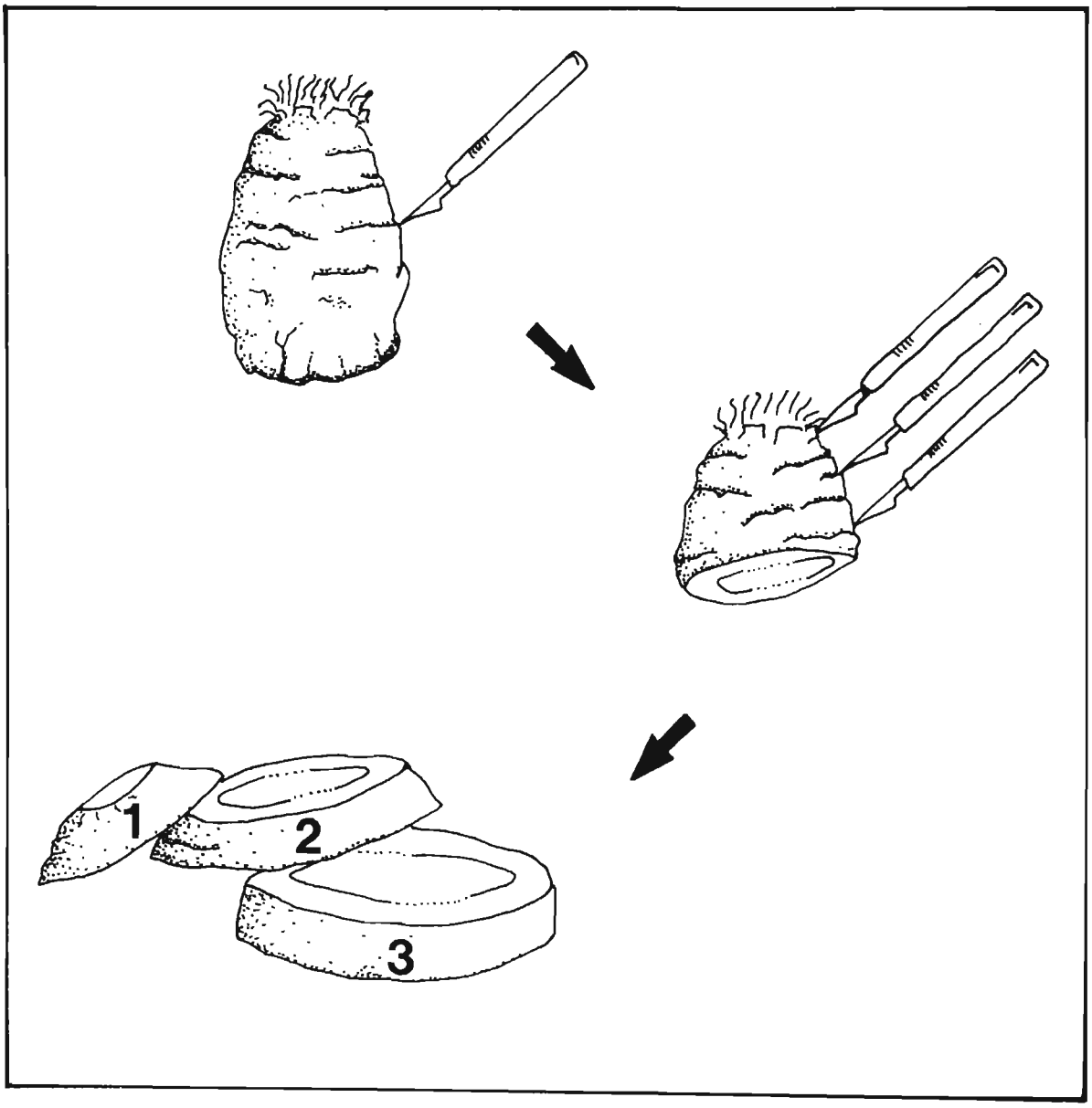


Figure 2.2.2: Diagrammatic representation of the slicing of the upper halves of the corm of *H. hemerocallidea* prior to sterilisation.

Figure 2.2.3: Four month old malformed roots produced from corm explants of *H. hemerocallidea*. This tissue was subcultured every 8 weeks and gave rise to malformed root-producing callus.

1. Homogeneously ground freeze dried material (0.1 g) (shoot-, root- or malformed root-producing callus) was extracted with 50 ml 80 percent ethanol for 4 hours in the dark at 23 ^{o}C. Extracts were then filtered, the residues given two 25 ml washes with 80 percent ethanol and the combined washes and extracts were reduced to dryness *in vacuo* at 30 ^{o}C.
2. Extracts were redissolved in 0.5 ml 80 percent ethanol and loaded onto Merck silica gel UV fluorescent $60F_{254}$ thin layer chromatography (TLC) plates.
3. TLC plates were double developed with the following solvent system, butan-2-ol: benzene: distilled water: methanol (4:3:2:1, volume for volume, upper phase) for one hour each time. After the TLC plates had been air dried they were viewed at 254 nm and ultraviolet (UV) absorbent bands co-chromatographing with authentic hypoxoside were marked (Figure 2.2.4). All TLC plates were stored at -20 ^{o}C until used for analysis.

High performance liquid chromatographic analysis of hypoxoside-containing extracts

High performance liquid chromatography (HPLC) was used, tentatively, to detect the presence of hypoxoside in the TLC purified extracts. UV absorbent bands co-chromatographing with authentic hypoxoside were removed from the plates and the silica gel was eluted with 50 ml 80 percent ethanol, followed by two 25 ml washes with 80 percent ethanol. Eluants were reduced to dryness *in vacuo* at 30 ^{o}C and redissolved in 100 μl 80 percent redistilled ethanol.

Extracts were subjected to HPLC as outlined by PAGE (1984). A Varian 5000 HPLC fitted with a Hypersil 5 μm 5 ODS (4.6 x 250 mm, C_{18}) reverse phase column. The column was eluted with 20 percent

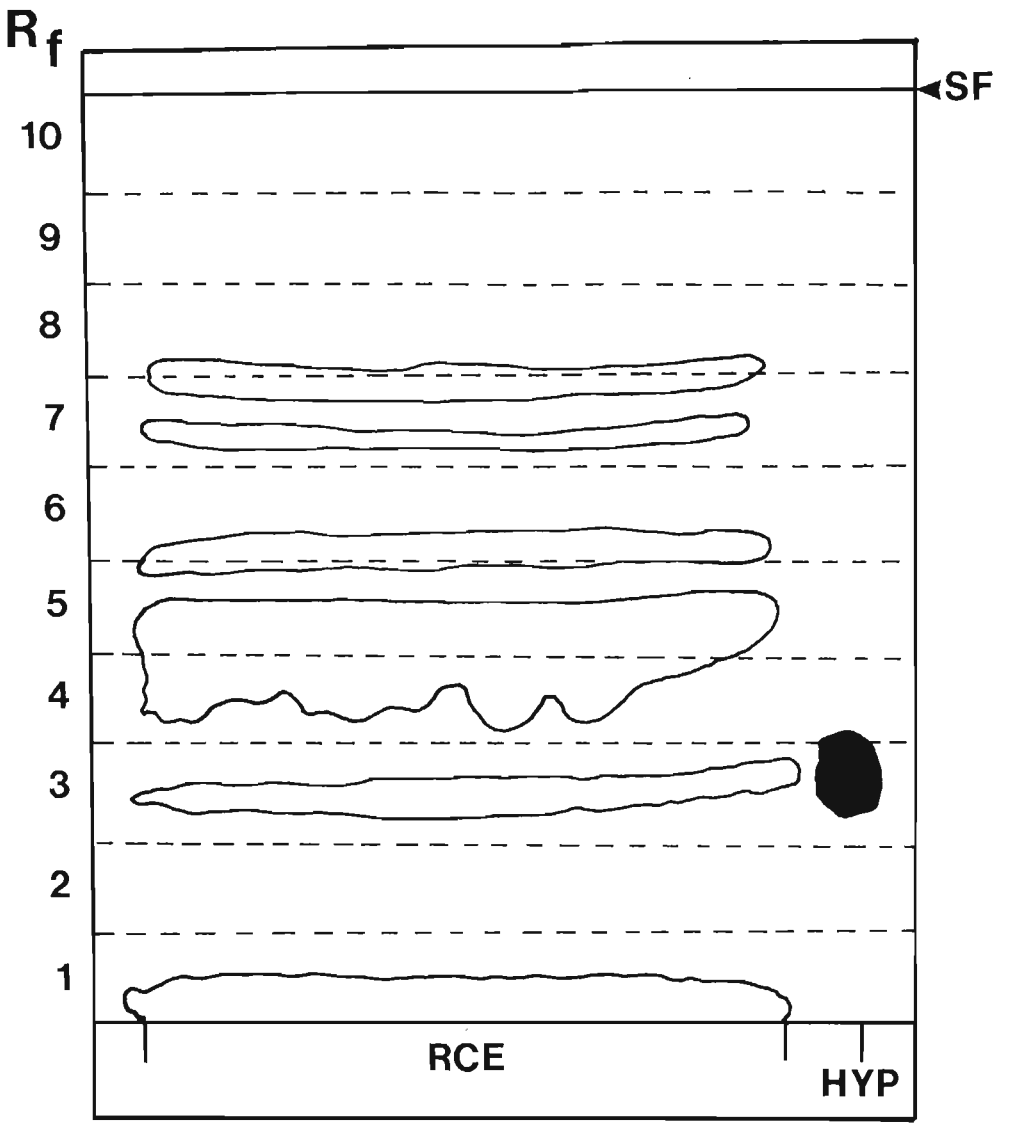


Figure 2.2.4: The bands of UV absorbance detected when an extract of root producing callus was separated by silica gel thin layer chromatography using butan-2-ol: benzene: distilled water: methanol (4:3:2:1:, volume for volume, upper phase) as the solvent. The chromatogram was developed twice, dried and viewed at 254 nm. RCE= root callus extract, HYP= hypoxoside, SF= solvent front.

acetonitrile and 80 percent water over 30 minutes at a flow rate of 1.5 ml min^{-1}. Compounds eluting from the column were detected using a Varian variable wavelength monitor fitted with a 8 μl flow through cell set at 257 nm.

Authentic hypoxoside (4 μl aliquots containing 0.002 mg were used throughout) eluted from the column with a retention time of 12.8 minutes (Figure 2.2.5). The area under peaks in the HPLC separated extracts obtained from cultured tissue were integrated for each sample so that the percentage of hypoxoside in the extract could be used as an indication of the purity of the eluant obtained from TLC separation.

Quantification of hypoxoside

HPLC separation of the extracts from the *in vitro* tissue showed that the eluants obtained from TLC were approximately 77 percent pure with respect to hypoxoside. This level of purity was considered acceptable enough to use TLC as the method for purification prior to quantitative analysis. Extracts from 0.05 g freeze dried tissue (shoot-, root- and malformed root-producing callus) were obtained and separated with TLC as already described. Three replicates from each tissue type were assayed for hypoxoside content as follows:

1. The UV absorbing bands that co-chromatographed with authentic hypoxoside on the TLC plates were removed and the silica gel was eluted with 80 percent ethanol. Eluants were centrifuged in a Hettich Universal 2S centrifuge for 5 minutes at 1000 revolutions per minute.
2. Five milliliters was withdrawn from each sample and 1 ml *p*-nitroaniline reagent was added to each. The *p*-nitroaniline reagent was prepared by adding 200 μl 5 percent sodium nitrite to 2 ml 0.5 percent *p*-nitroaniline in 2M hydrochloric acid at 6 ^{o}C

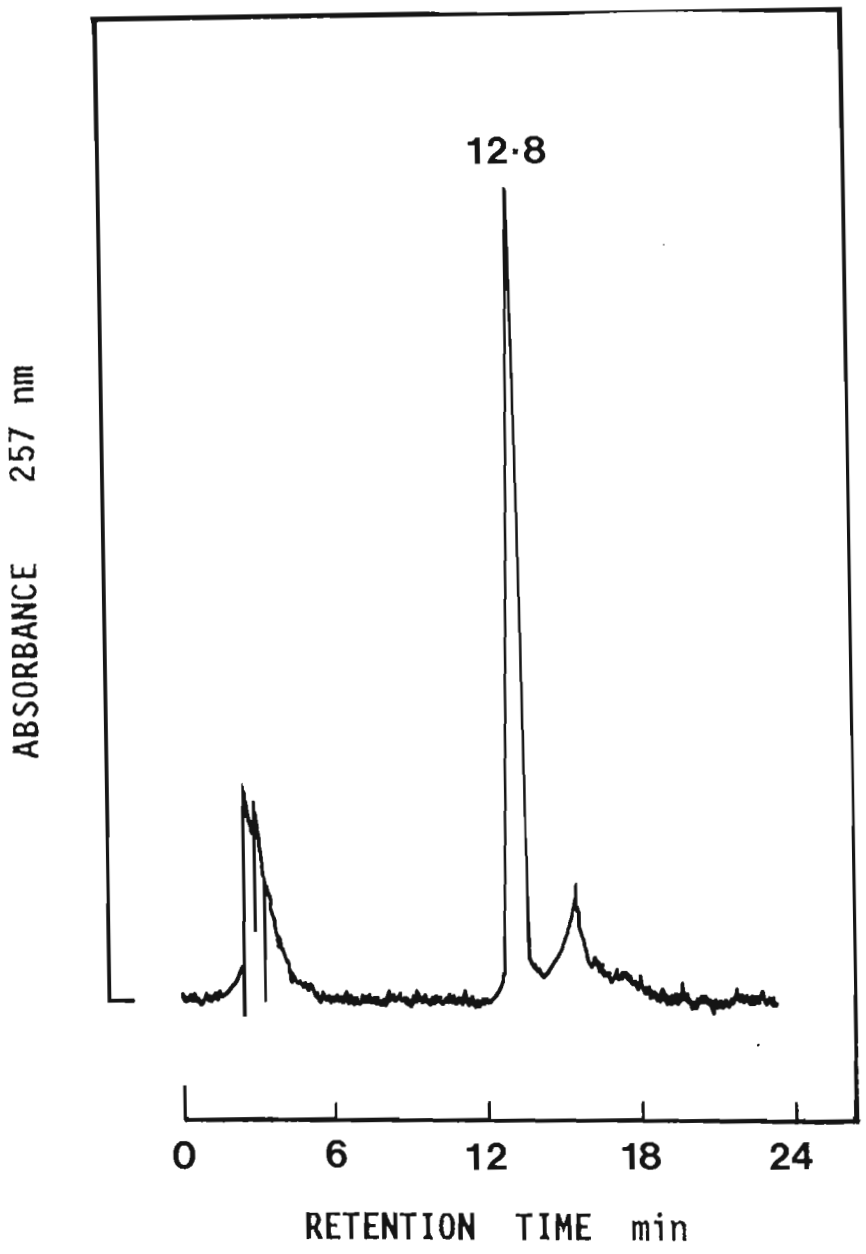


Figure 2.2.5: The trace of UV absorbance at 257 nm obtained upon the HPLC separation of authentic hypoxoside using a Varian 5000 high performance liquid chromatograph fitted with a Hypersil 5ODS C_{18} column and a Varian variable wavelength monitor. The eluant used was a constant mix of 20 percent acetonitrile and 80 percent water over a 30 minute period.

followed by the addition of 8 ml 20 percent sodium acetate. Samples were then vortexed and a further 1 ml 15 percent sodium carbonate was added to each. After vortexing the samples were left to stand in the dark for 10 minutes.

3. The absorbance of the samples was measured on a Varian DMS 90 UV visible spectrophotometer in quartz cuvettes at 522 nm against a blank sample containing 5 ml 80 percent ethanol, 1 ml *p*-nitroaniline reagent and 1 ml 15 percent sodium carbonate. The absorbance was measured at 522 nm as this was where the maximum absorbance occurred when the absorbance of an authentic hypoxoside sample (3ml of 50 mg l^{-1}) was measured between the range of 400 nm and 700 nm (Figure 2.2.6).

4. The following standards of hypoxoside were used to obtain a calibration curve: 1, 5, 10, 20, 40, and 50 mg l^{-1}. In this range an exponential curve which fitted the formula $y = ae^{bx}$ with $r^2 = 0.98$, $a = 4.88$ and $b = 0.41$ (Figure 2.2.7). Three replicates for each concentration were used to obtain separate values for the absorbance recordings used to derive the calibration curve.

5. The calibration curve and the absorbance values for each sample were then used to calculate the hypoxoside concentration present in each sample. A mean percentage hypoxoside per dry mass was obtained for each tissue type from three replicates to which a 95 percent confidence level was applied. A blank sample of eluant from the silica gel was also measured to ascertain if the fluorescent agent on the TLC plates would interfere with the hypoxoside assays. This sample had negligible absorbance. A standard hypoxoside sample was included to determine the recovery of the phytochemical from TLC; these results being used to adjust the sample values accordingly.

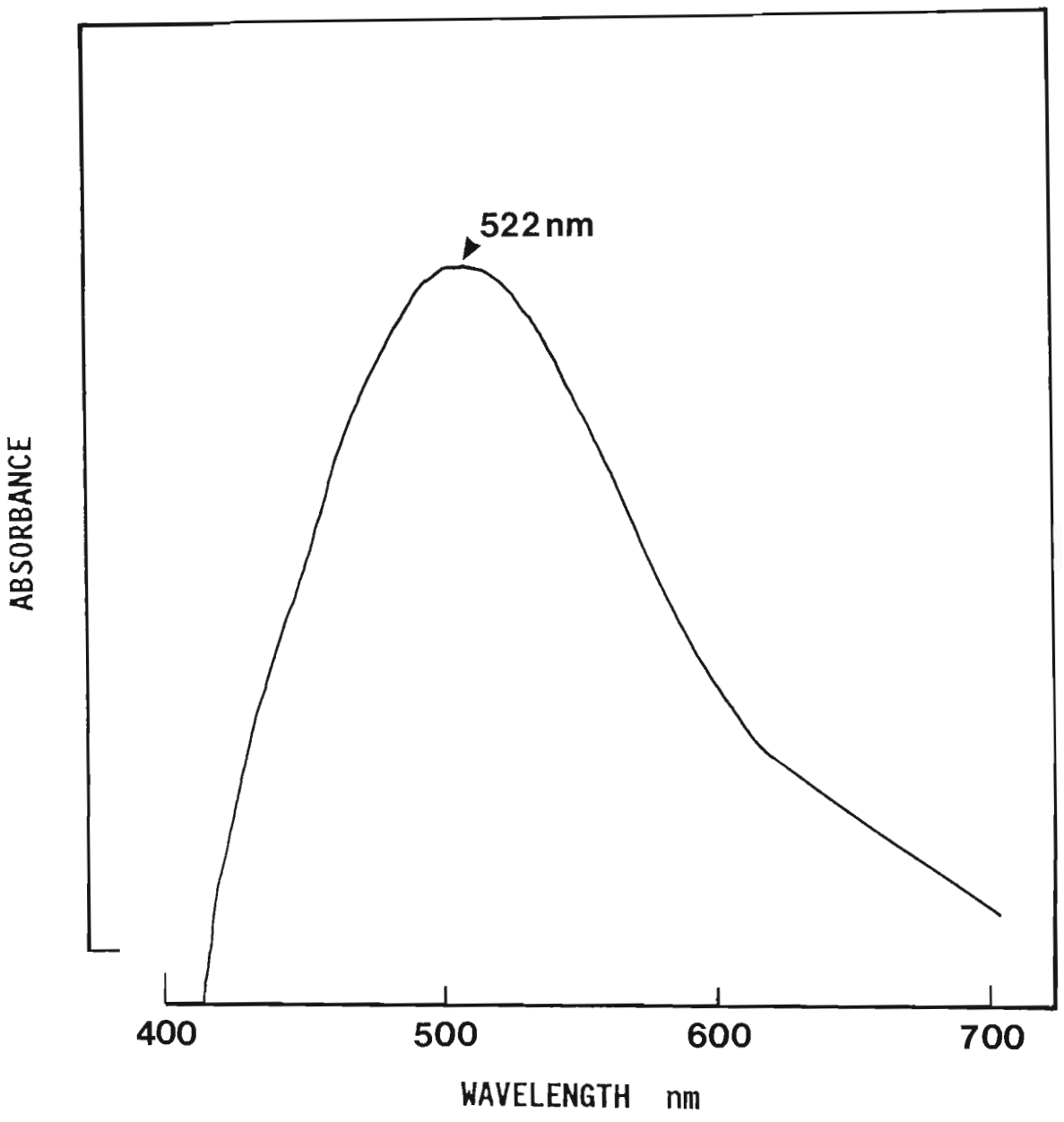


Figure 2.2.6: The absorbance of the hypoxoside / *p*-nitroaniline reagent reaction measured between 400 nm and 700 nm in quartz cuvettes in a Varian DMS 90 UV visible spectrophotometer. Maximum absorbance was achieved at 522 nm.

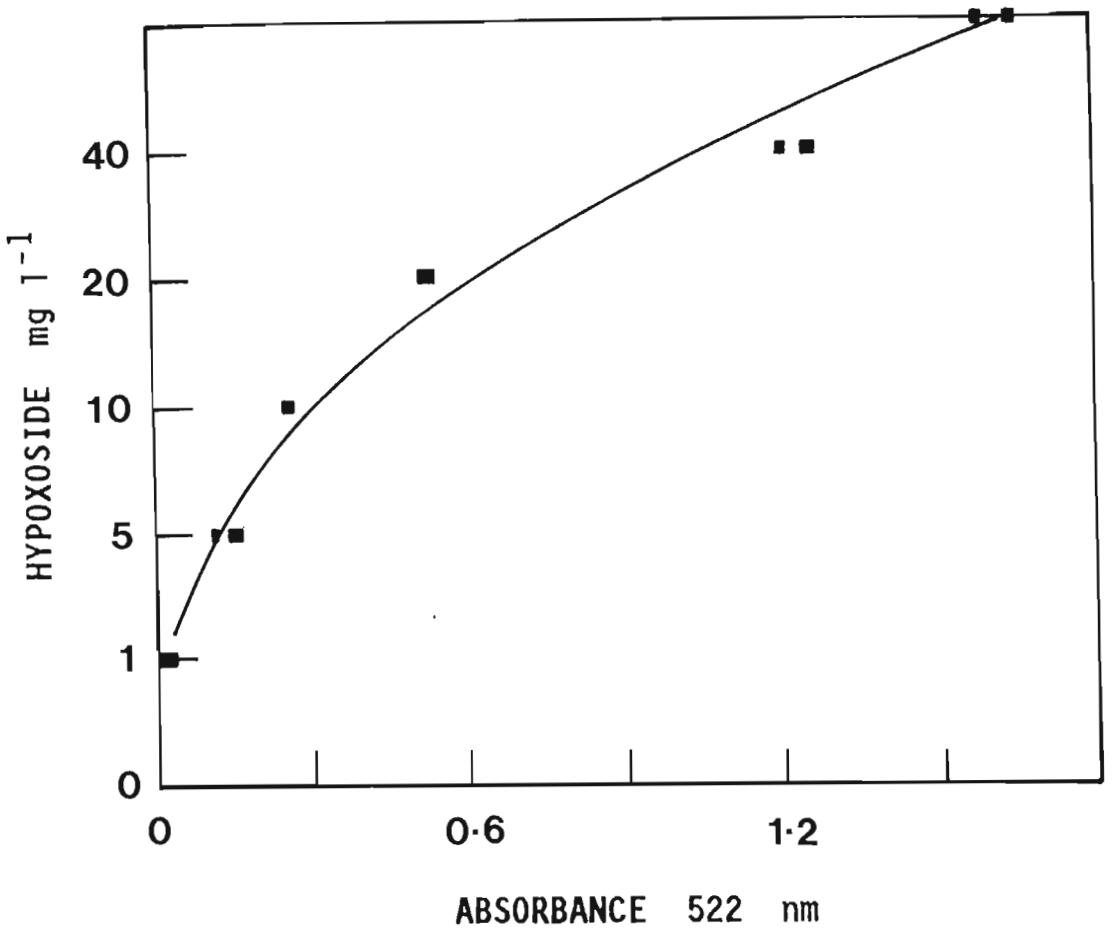


Figure 2.2.7: The calibration curve obtained for the hypoxoside / *p*-nitroaniline reagent reaction. The absorbance at 522 nm using different concentrations of hypoxoside was used to obtain a curve, $y=ae^{bx}$, (a= 4.88 and b= 0.41) with an $r^2=1.00$.

2.2.1.2. Application of ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-sodium acetate and ^{14}C-acetyl coenzyme A to root-producing callus

Since it was ascertained that root-producing callus contained the highest level of hypoxoside, this *in vitro* tissue was used for the biosynthetic studies. The aim of this experiment being, to determine the derivation of the aryl moieties of hypoxoside; whether of shikimate origin or of acetate or both.

Application of potential precursors

Root-producing callus initiated and maintained as described in 2.2.1.1 was subcultured onto fresh BM with the following inclusion. After autoclaving the medium the following radioisotopes were added under aseptic conditions and after filter sterilisation through a 0.22 μm Millpore filter:

2 x 10^6 dpm L[U-^{14}C]phenylalanine (specific activity 18.98 GBq mmol^{-1}),

2 x 10^6 dpm [3-^{14}C]*t*-cinnamic acid (spec. act. 1.77 GBq mmol^{-1}),

2 x 10^6 dpm [U-^{14}C]sodium acetate (spec. act. 2.15 GBq) and

2 x 10^6 dpm [1-^{14}C]acetyl coenzyme A (spec. act. 2.03 GBq mmol).

All radiochemicals were obtained from Amersham (UK). Radioisotopes were added to 50 ml BM in 100 ml conical flasks and three replicates were used per treatment. Approximately 1.5 g of root-producing callus was transferred into each flask.

The callus was incubated at 23 ± 2 ^{o}C in dim light (0.4 μE m^{-2} s^{-1}) for 8 weeks. At the end of this period the callus from each treatment was flash frozen with liquid nitrogen, freeze dried for 24 hours and stored at -20 ^{o}C until analysed.

Extraction, HPLC separation and assay for radioactivity

Homogeneously ground freeze dried callus (0.2 g) from each treatment was extracted for hypoxoside as described in 2.2.1.1. One milliliter samples from the filtered extracts were assayed for radioactivity by the addition of 4 ml Beckman Ready-Solv and the use of a Beckman LS 3000 scintillation counter. The extracts were separated on TLC and prepared for HPLC analysis as described in 2.2.1.1. Fractions of 1.5 ml were collected from the HPLC separations in Beckman mini-Poly-Q vials to which 4 ml of Beckman EP Ready Solv was added. The fractions were then assayed for radioactivity in a Beckman LS 3800 scintillation counter. Peaks of radioactivity co-eluting with authentic hypoxoside were noted.

2.2.1.3. The detection and quantification of the hydroxycinnamic acids in the cultured tissue

The data obtained from experiment 2.2.1.2 necessitated an investigation into the presence and quantity of the hydroxycinnamic acids in the root-producing callus in comparison to the shoot-producing callus.

Extraction of cinnamic acid and the hydroxycinnamic acids

Root- and shoot-producing callus initiated and maintained as described in section 2.2.1.1 was harvested at the end of an 8 week subculture, flash frozen with liquid nitrogen and freeze dried for 24 hours. The material was then milled to a homogenous powder and stored at -20 ^{o}C until used.

The extraction procedure used for the hydroxycinnamic acids was modified from the method of RIBÉREAU-GAYON (1972).

1. From each callus type 0.2 g freeze dried material was extracted with 50 ml 80 percent ethanol for 4 hours at 23 ^{o}C in the dark. Extracts were filtered, the residues washed with two volumes of 25 ml 80 percent ethanol and the combined volumes for each extract were reduced to dryness *in vacuo* at 30 ^{o}C.
2. The extracts were redissolved in 10 ml 2M sodium hydroxide and left to stand for 4 hours in the dark. The sodium hydroxide releases the free bases of cinnamic acid and the hydroxycinnamic acids from their natural state of esterification. Subsequently the extracts were acidified (pH 3.0) with 1M hydrochloric acid and partitioned three times against 50 ml ethylacetate.
3. The ethylacetate extracts were combined, reduced *in vacuo* at 30 ^{o}C to 1 ml and were then loaded onto Whatman No. 1 chromatography paper. The chromatograms were separated with the following solvent system, toluene: acetic acid: water (4:1:5, volume for volume, upper phase) for 3 hours, after an equilibration time of 6 hours.
4. After air drying at 23 ^{o}C the chromatograms were viewed under ultraviolet (UV) light at 254 nm and 366 nm respectively. UV fluorescent or absorbent bands co-chromatographing with the authentic standards (0.1 mg of each), *t*-cinnamic, *p*-coumaric, ferulic and sinapic acids were marked (Table 2.2.2).
5. Since caffeic acid remained at the origin in the above separation, a second separation was necessary. The origin of each chromatogram was eluted, loaded onto fresh Whatman No. 1 chromatography paper and was separated a second time with butan-1-ol: acetic acid: water (4:1:5, volume for volume, upper phase).
6. After air drying at 23 ^{o}C the chromatograms were viewed at 254 nm and 366 nm and UV fluorescent bands with a similar Rf to

Table 2.2.2: The colours obtained for the UV fluorescence or absorbance of cinnamic acid and the hydroxycinnamic acids at 254 nm and 366 nm and the Rf values of these acids on the paper chromatographic systems used for their separation.

STANDARDS	UV FLUORESCENCE/ ABSORBANCE		Rf
	254 nm	366 nm	
Chromatograph 1: toluene: acetic acid: water (4:1:5)			
caffeic acid	lilac	lilac	0.00
p-coumaric acid	purple	-	0.11
sinapic acid	light blue	light blue	0.19
ferulic acid	violet	violet	0.39
t-cinnamic acid	purple	-	0.88
Chromatograph 2: butan-1-ol: acetic acid: water (4:1:5)			
caffeic acid	lilac	lilac	0.81

authentic caffeic acid were marked (Table 2.2.2).

7. For each sample the UV fluorescent/absorbent bands coincident with the standards on the first and second chromatograms were eluted with two volumes of 50 ml 80 percent ethanol. The eluants from both chromatograms for each sample were then combined and reduced to dryness *in vacuo* at 30 ^{o}C. Samples were redissolved in 100 μl methanol prior to HPLC analysis.

HPLC analysis of the cinnamic acid and hydroxycinnamic acid extracts

The presence of cinnamic acid and the hydroxycinnamic acids in the extracts was tentatively confirmed with HPLC separation and by the coincidence of the retention times of peaks present in the extracts with the retention times of the authentic standards. UV absorbance was used as the method of detection.

For HPLC analysis 10 μl of each extract was injected onto a Micropak MCH-10 (4 x 300 mm, C_{18}) reverse phase column fitted to a Varian 5000 Liquid Chromatograph. The column was eluted at a flow rate of 1.5 ml min^{-1} with the solvents, 2 percent acetic acid (pH 2.8)(**A**) and methanol (**B**) which changed on a linear gradient from an initial 80 percent **A** and 20 percent **B** to 60 percent **A** and 40 percent **B** over 30 minutes. This was followed by a constant 50 percent **A** and 50 percent **B** for 5 minutes with the programme terminating in 100 percent **B** at 40 minutes. A Varian variable wavelength monitor set at 265 nm and fitted with an 8 μl flow through cell was used to detect compounds eluting from the column.

Authentic caffeic, *p*-coumaric, ferulic, sinapic and *t*-cinnamic acids (0.001 mg of each in 10 μl aliquots) eluted in this system with retention times of 12.5, 19.5, 22.7, 24.5 and 36.9 minutes respectively (Figure 2.2.8).

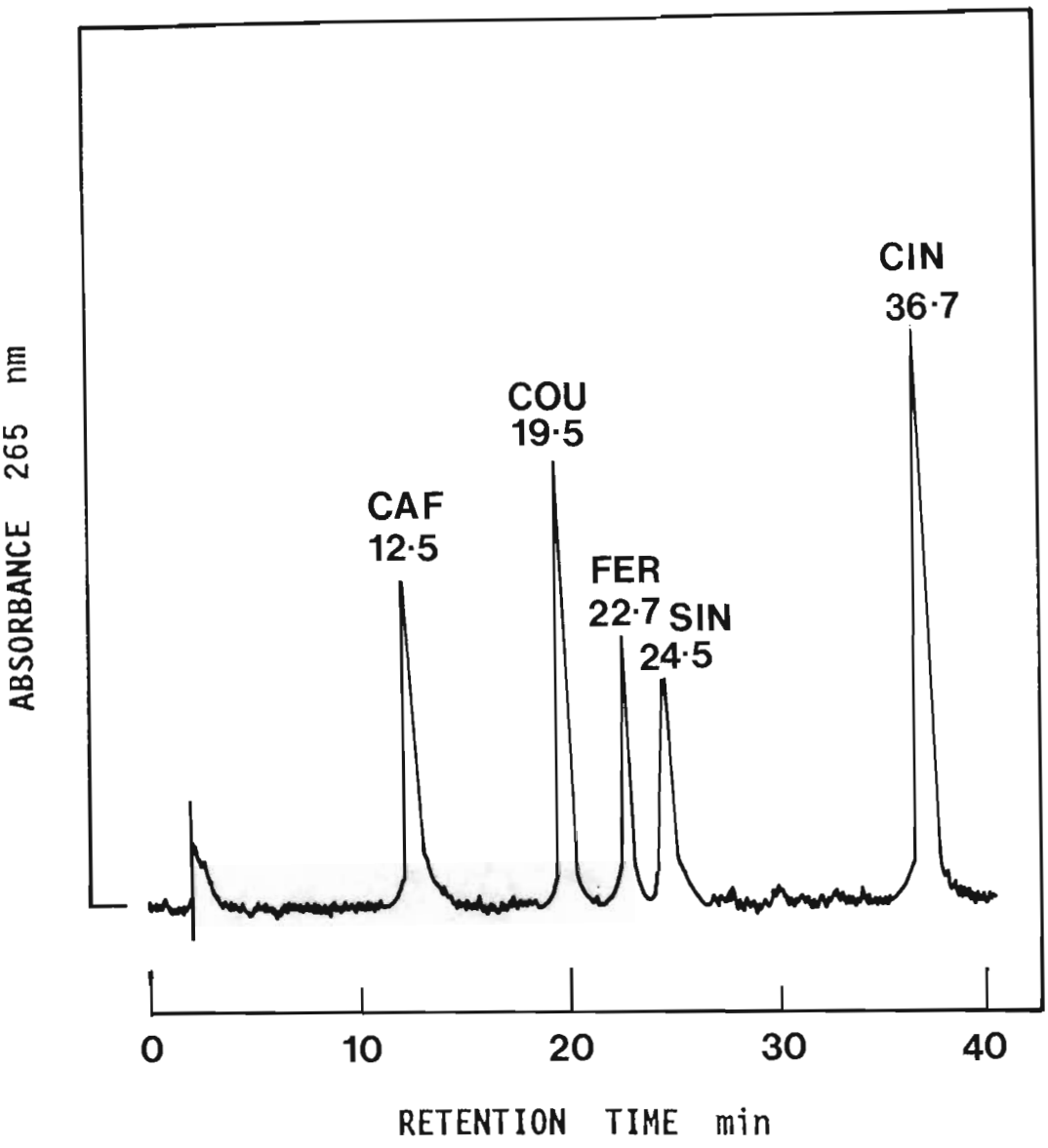


Figure 2.2.8: The UV absorbance at 265 nm obtained upon the HPLC separation of authentic caffeic, *p*-coumaric, ferulic, sinapic and *t*-cinnamic acids using a Varian 5000 high performance liquid chromatograph fitted with a Micropak MCH-10 column and a Varian variable wavelength monitor. The solvents used as eluants were a mix of 2 percent acetic acid and methanol the combination of which altered during the 40 minute running time. CAF= caffeic acid, COU= *p*-coumaric acid, FER= ferulic acid, SIN= sinapic acid and CIN= *t*-cinnamic acid.

Quantification of the hydroxycinnamic acids

For the purpose of quantification a second set of extraction and separations on paper were performed as described in section 2.2.1.3. Three replicates for each *in vitro* tissue type were used. The quantification method used employed the *p*-nitroaniline reagent made up as described in section 2.2.1.1. *t*-Cinnamic acid could not be quantified with this method as the reaction with *p*-nitroaniline requires the presence of phenolic hydroxyl groups which are absent from this molecule. The quantification procedure devised was as follows:

1. The bands of UV fluorescence/absorbance co-chromatographing with the standards on each chromatogram were eluted with 30 ml 80 percent ethanol. From these volumes 5 ml aliquots were withdrawn and 1 ml *p*-nitroaniline reagent was added. Samples were then vortexed and left to stand for 10 minutes in the dark.
2. The absorbance of each sample was measured in a Varian DMS 90 UV/visible spectrophotometer in quartz cuvettes against a blank sample containing only 80 percent ethanol and *p*-nitroaniline reagent.
3. The absorbance of *p*-coumaric acid samples were measured at 455 nm, the caffeic acid samples at 480 nm, the ferulic acid samples at 484 nm and the sinapic acid samples at 495 nm, respectively (Figure 2.2.9). The values obtained were compared to calibration curves that were derived for each of the standards using the concentration range; 150, 100, 75, 50 and 25 mg l^{-1} (Figure 2.2.10).

In each case, a mean percentage of compound present in each of the cultured tissue types, for each of the hydroxycinnamic acids, was calculated at a 95 percent confidence level from three replicates.

Figure 2.2.9: The absorbance of the *p*-coumaric acid / *p*-nitroaniline reagent (**A**), caffeic acid / *p*-nitroaniline reagent (**B**), ferulic acid / *p*-nitroaniline reagent (**C**) and sinapic acid / *p*-nitroaniline reagent (**D**) reactions measured between 350 nm and 750 nm in quartz cuvettes in a Varian DMS 90 UV/visible spectrophotometer. Standard solutions containing 50 mg l^{-1} of each acid were used. Maxima were detected at 455 nm (*p*-coumaric acid), 480 nm (caffeic acid), 484 nm (ferulic acid) and 495 nm (sinapic acid).

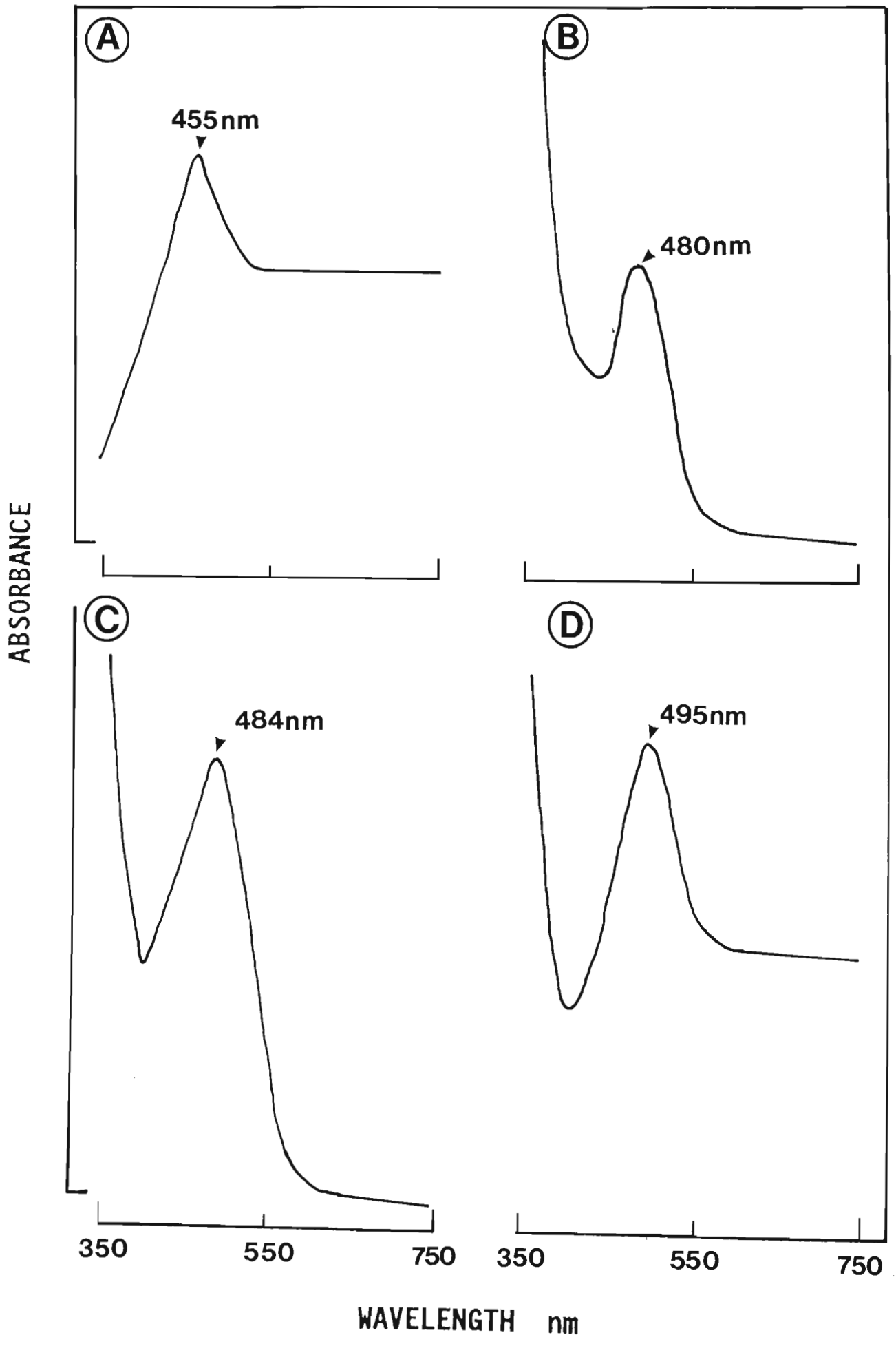
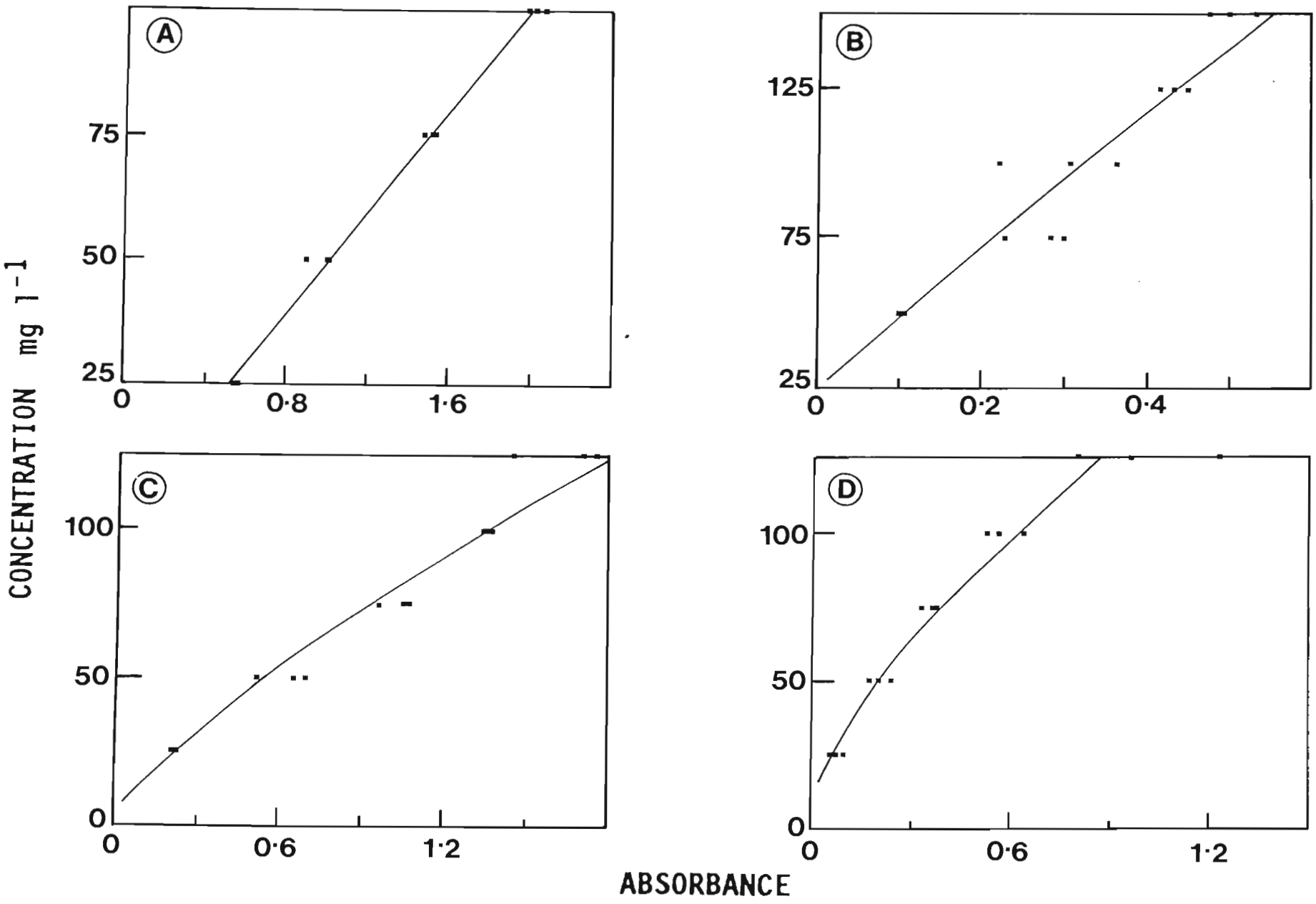

A
455nm
B
480nm
C
484nm
D
495nm
ABSORBANCE
350
550
750
350
550
750
WAVELENGTH nm

Figure 2.2.10: The calibration curves obtained for the *p*-coumaric acid / *p*-nitroaniline reagent (**A**), caffeic acid / *p*-nitroaniline reagent (**B**), ferulic acid / *p*-nitroaniline reagent (**C**) and sinapic acid / *p*-nitroaniline reagent (**D**) reactions. The absorbance at 455 nm, 480 nm, 484 nm and 495 nm using different concentrations of *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid was used to obtain curves $y=a+bx$ (r^2= 0.99, a= 0.04, b= 2.01), $y=ax^b$ (r^2= 0.92, a= 8.82, b= 0.96), $y=ax^b$ (r^2= 0.98, a=3.13, b= 0.77) and $y=ax^b$ (r^2= 0.97, a= 5.41, b= 0.63), respectively.


A
B
C
D
CONCENTRATION mg l-1
ABSORBANCE
25
50
75
125
0
0·8
1·6
0·2
0·4
100
0·6
1·2

calculated at a 95 percent confidence level from three replicates.

2.2.1.4. Extraction of cinnamic acid and the hydroxycinnamic acids from root-producing callus treated with ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-sodium acetate and ^{14}C-acetyl coenzyme A

Cinnamic acid and the hydroxycinnamic acids were extracted from the root-producing callus treated in experiment 2.2.1.2, in order to determine to what extent radioactive labelling of these had occurred, in the different treatments.

From each treatment 0.2 g freeze dried and homogeneously ground material was extracted, the extracts separated with paper chromatography and HPLC as described in section 2.2.1.3. Fractions of 1.5 ml, collected during the separation with HPLC, were assayed as outlined in section 2.2.1.2. Peaks of radioactivity co-eluting with the authentic standards were noted.

2.2.1.5. The synthesis of ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid

The results from the experiment carried out in 2.2.1.2 and the analyses in 2.2.1.3 and 2.2.1.4 necessitated the assessment of the role of *p*-coumaric and caffeic acids in the biosynthesis of hypoxoside in root-producing callus. This required that carbon-14 isotopically labelled *p*-coumaric and caffeic acids be synthesised as these radiochemicals could not be purchased commercially. The method described by BROWN, RIVETT and THOMPSON (1984), which is outlined below, was used.

1. [2-^{14}C]Sodium malonate (spec. act. 2.07 GBq mmol^{-1}, Amersham, UK) was dissolved in 1 ml distilled water and acidified (pH= 1.0)

with concentrated hydrochloric acid. This aqueous phase was partitioned against two 2.5 ml volumes of ethylacetate. The ethylacetate fractions were combined and reduced to dryness *in vacuo* at 30 ^{o}C. The residue was redissolved in 800 μl anhydrous pyridine.

2. Anhydrous pyridine was obtained by stirring 5 to 6 pieces of calcium hydride with 10 ml pyridine for 1 hour. The pyridine was then distilled off using and oil bath at 120 ^{o}C and a calcium chloride trap.

3. [2-^{14}C]*p*-Coumaric acid was prepared by dissolving 61 mg 4-hydroxybenzaldehyde (0.5 mmols) in 300 μl of [2-^{14}C]malonic acid (2.5 MBq) in pyridine plus 10 μl aniline. After a reaction time of 6 hours, during which the temperature was kept at 55 ^{o}C with an oil bath, 52 mg malonic acid (0.5 mmols) was added and the reaction was left for a further 12 hours. The reaction was quenched by pouring the mixture into 1.5 ml concentrated hydrochloric acid and 0.5 ml distilled water. The precipitate was filtered off, dissolved in acetone, dried and recrystallised from 2 ml hot distilled water. The purity of the filtered crystals was ascertained by HPLC using the method described in section 2.2.1.3 and 1.5 ml fractions were collected from the HPLC separation and these were assayed for radioactivity (Figure 2.2.11 A).

4. [2-^{14}C]Caffeic acid was prepared by dissolving 72 mg 3,4-dihydroxybenzaldehyde in 300 μl [2-^{14}C]malonic acid (2.5 MBq) in pyridine plus 10 μl aniline. The reaction and method of purification was carried out as described above. The purity of the obtained crystals was ascertained with HPLC (section 2.2.1.3) and 1.5 ml fractions were collected to assay for radioactivity (Figure 2.2.11 B).

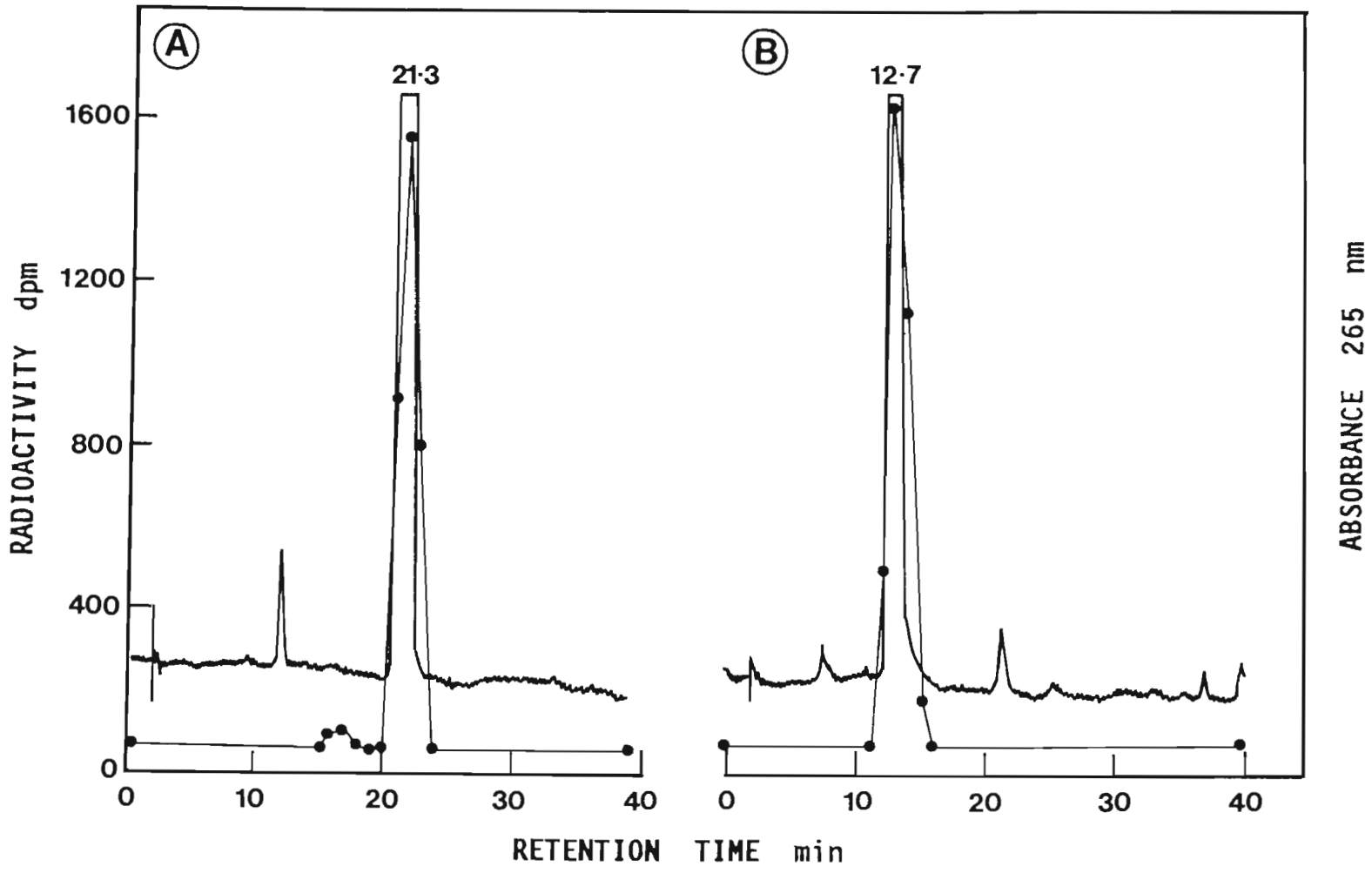


Figure 2.2.11: The separation of the synthesised (2-^{14}C)*p*-coumaric acid (A) and (2-^{14}C)caffeic acid (B) by HPLC. The graphs show the UV absorbance (265 nm) and radioactive (●—●) profiles obtained.

The data obtained for the yield of radioactivity and the purity of the prepared [2-^{14}C]*p*-coumaric acid and [2-^{14}C]caffeic acid are given in Table 2.2.3.

2.2.1.6. The treatment of root-producing callus with ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid

The role of *p*-coumaric acid and caffeic acid in the biosynthesis of hypoxoside in root-producing callus and the efficiency of incorporation of these potential precursors in comparison to phenylalanine and *t*-cinnamic acid was investigated.

Application of radioactivity

Root producing callus initiated and maintained as described in 2.2.1.1 was subcultured onto fresh BM. After autoclaving the BM the filter sterilised radioisotopes were added under aseptic condition in the following quantities:

2×10^6 dpm L[U-^{14}C]phenylalanine (spec. act. 18.98 GBq mmol^{-1}),

2×10^6 dpm [3-^{14}C]*t*-cinnamic acid (spec. act. 1.77 GBq mmol^{-1}),

1.4×10^6 dpm [2-^{14}C]*p*-coumaric acid (spec. act. 3.84 MBq mmol^{-1}) and

0.99×10^6 dpm [2-^{14}C]caffeic acid (spec. act. 1.49 MBq mmol^{-1}).

All the radioisotopes were added to the BM in 1 ml volumes and in the case of the ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid the solutions were made slightly alkaline (pH= 8.5) with dilute sodium hydroxide to affect dissolution. Three replicates were used per treatment and upon solidification of the medium, approximately 1.5 g of callus was aseptically transferred to each flask.

Table 2.2.3: The yield of radioactivity and purity obtained from HPLC analysis of the synthesised [2-^{14}C]*p*-coumaric acid and [2-^{14}C]caffeic acid.

RADIOCHEMICAL	YIELD OF RADIOACTIVITY %	SPECIFIC ACTIVITY MBq mmol^{-1}	PURITY %
[2-^{14}C]*p*-coumaric acid	58.0	3.84	99.2
[2-^{14}C]caffeic acid	15.2	1.49	97.1

The callus was incubated at 23 ± 2 ^{o}C in dim light (0.4 μE m^{-2} s^{-1}) for 8 weeks. At the end of this period the callus for each treatment was flash frozen with liquid nitrogen and freeze dried for 24 hours. The material was then homogeneously ground and stored at -20 ^{o}C until analysed.

Assay for incorporation of radioactivity into hypoxoside

Half of the root-callus material for each treatment (± 0.25 g) was extracted for hypoxoside and 1 ml aliquots of each of the filtered extracts were assayed for radioactivity. The extraction procedure, separation of the extracts with TLC and HPLC and the assaying of radioactivity present in 1.5 ml fraction collected from the HPLC separation were undertaken as described in 2.2.1.1 and 2.2.1.2. The presence and size of peaks of radioactivity co-eluting with authentic hypoxoside were noted.

Assay for incorporation of radioactivity into cinnamic acid and the hydroxycinnamic acids

The remaining half of the root-producing callus from each treatment (± 0.25 g) was extracted for cinnamic acid and the hydroxycinnamic acids, extracts were separated with paper chromatography and HPLC as described in 2.2.1.3. Fractions of 1.5 ml collected during separation with HPLC were assayed for radioactivity. Peaks of radioactivity co-eluting with the authentic standards were noted. Thus incorporation of radioactivity and the quantity thereof, was ascertained for each sample.

2.2.2. INVESTIGATIONS WITH INTACT PLANTS

An investigation of the incorporation of radioactivity into hypoxoside and the hydroxycinnamic acids in intact plants of *H. hemerocallidea*,

when radioisotopically labelled potential precursors were applied, was undertaken to determine if the *in vitro* findings could be extrapolated to the situation in the intact plant.

2.2.2.1. The application of ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid to intact plants

Plant material

Eighteen-month-old clonal plants propagated using *in vitro* methods were used. The plants were obtained and "hardened-off" as described by PAGE (1984). Shoots, obtained from the shoot-producing callus derived and maintained as described in 2.2.1.1, were subcultured onto 100 ml fresh BM in 250 ml glass jars with plastic screw top lids. The hormonal supplement was omitted from the BM. After 6 weeks, during which time the shoots were maintained at 23 ± 2 ^{o}C, 35 μE m^{-2} s^{-1}, and 16 hours photoperiod, rooting occurred. The plastic lids of the glass jars were then replaced with sterile plastic bags, the corners of which were cut off after 2 weeks. After a further 2 weeks the rooted plantlets were potted out into small plastic pots containing a mixture of sand: organic fertiliser (Gromore) (2:1). The plantlets were maintained under greenhouse conditions until use at eighteen months. At no time did these enter a period of dormancy.

Application of radiolabelled potential precursors

The radiolabelled precursors were applied to the intact plants by the insertion of the needle (0.45 mm diameter) of a micro syringe, carrying the radioactivity, into a needle (0.8 mm diameter) cut to a 2 cm length which was inserted in the shoot apical region of each plant (Figure 2.2.12).

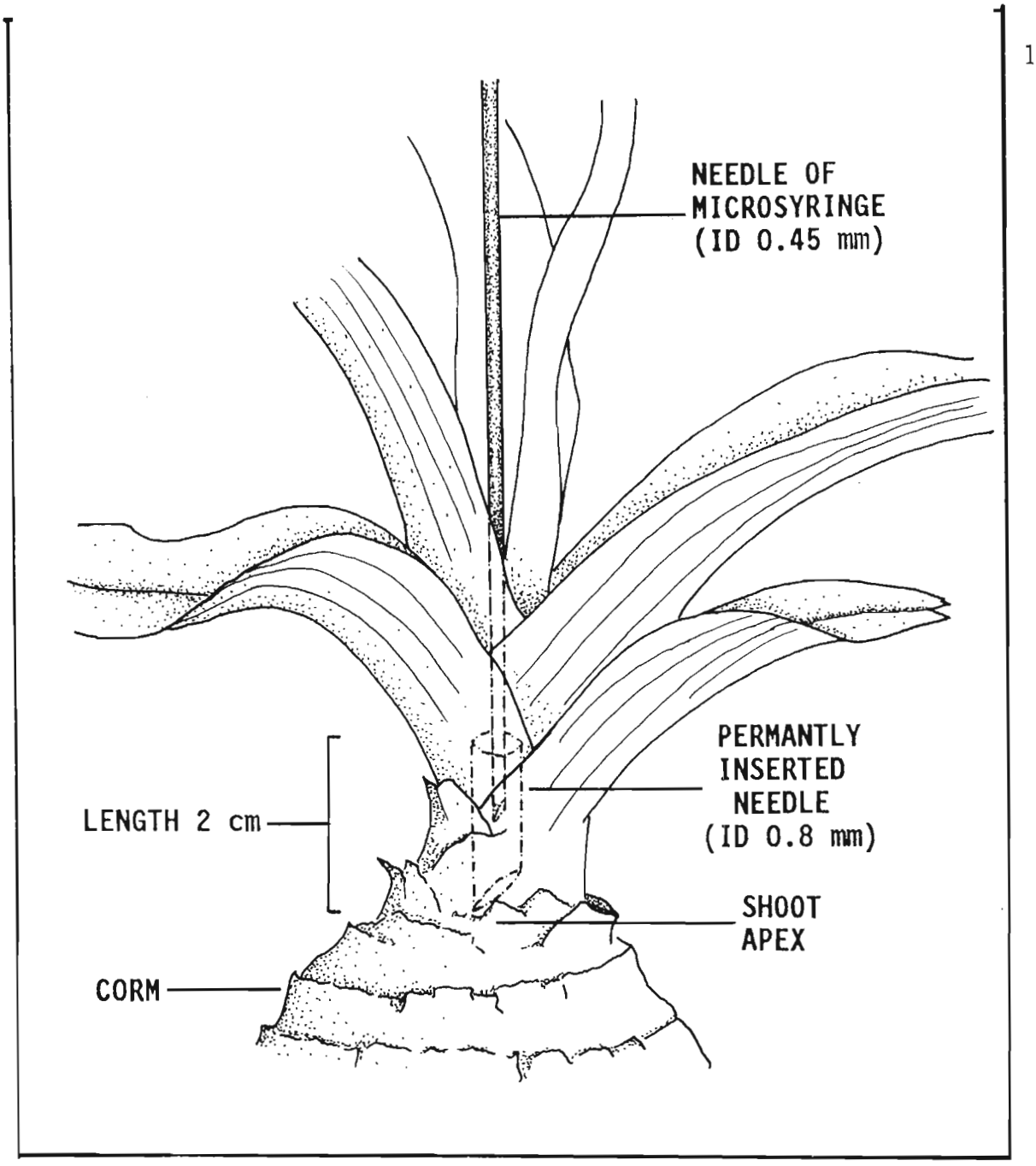


Figure 2.2.12: Diagrammatic representation of the method used to apply radioactive compounds to the shoot apical region of intact plants of *H. hemerocallidea*.

The following quantities of radioactivity were applied:
1.11×10^6 dpm L[U-^{14}C]phenylalanine (spec. act. 18.98 GBq mmol^{-1}),
1.11×10^6 dpm [3-^{14}C]*t*-cinnamic acid (spec. act. 1.77 GBq mmol^{-1}),
1.11×10^6 dpm [2-^{14}C]*p*-coumaric acid (spec. act. 3.84 MBq mmol^{-1}) and
0.47×10^6 dpm [2-^{14}C]caffeic acid (spec. act. 1.49 MBq mmol^{-1}).
In each case the radioactivity was administered in a 10 μl volume.

The above quantities of radioactivity were administered to the plants at weekly intervals for a period of 8 weeks and three plants were used per treatment. During this time the plants were maintained under the following conditions: temperature, 23 ± 2 ^{o}C; light intensity, 35 μE m^{-2} s^{-1}; photoperiod of 16 hours.

The experiment was completed by harvesting the plants, excising the corm from each plant and flash freezing it with liquid nitrogen. The corms were then freeze dried for 24 hours, homogeneously ground and stored at -20 ^{o}C until used.

Assay for the incorporation of radioactivity into hypoxoside

Half of the dry mass of the corms (± 1.00 g) for each treatment was extracted for hypoxoside. Only the corms were extracted since these organs contained the greatest quantity of hypoxoside in the intact plant (section 3.3.1.1). Aliquots of 1 ml were withdrawn from the filtered extracts and were assayed for radioactivity. The extracts were then separated on TLC and with HPLC as described in section 2.2.1.1. The 1.5 ml fractions collected from the HPLC separation were assayed for radioactivity according to the method described in 2.2.1.3. Incorporation of radioactivity into peaks co-eluting with hypoxoside was noted.

Assay for radioactivity incorporated in cinnamic acid and the hydroxycinnamic acids

The remaining half of the dry mass of the corms (± 1.00 g) for each treatment was extracted for cinnamic acid and the hydroxycinnamic acids. The extraction and subsequent separation with paper chromatography and HPLC were executed as described in 2.2.1.1. Fractions of 1.5 ml that were collected during the separations with HPLC were assayed for radioactivity and peaks of radioactivity that coincided with the retention time of *t*-cinnamic acid and the hydroxycinnamic acids were noted.

2.3. RESULTS

2.3.1. *IN VITRO* INVESTIGATIONS

2.3.1.1. Hypoxoside concentrations present in *in vitro* tissues

HPLC analysis

UV absorbent peaks co-eluting with authentic hypoxoside were obtained for the extracts from the root-producing callus, shoot-producing callus and the malformed root-producing callus (Figure 2.3.1.). The extracts from all three callus types were relativity clean with 77.7 percent of the optical activity in the root-producing callus extract being accounted for by a UV absorbent peak at 12.9 minutes. A similar peak with a retention time of 12.8 minutes accounted for 81.3 percent in the malformed root-producing callus.

Quantification of hypoxoside

Ten months after initiation the root-producing callus contained the highest percentage of hypoxoside followed by the shoot-producing callus (Table 2.3.1). The levels contained in the malformed root producing callus were less, being significantly lower than the root-producing callus but not the shoot-producing callus. Thus for further *in vitro* investigations of hypoxoside biosynthesis the root-producing callus was used as the experimental material.

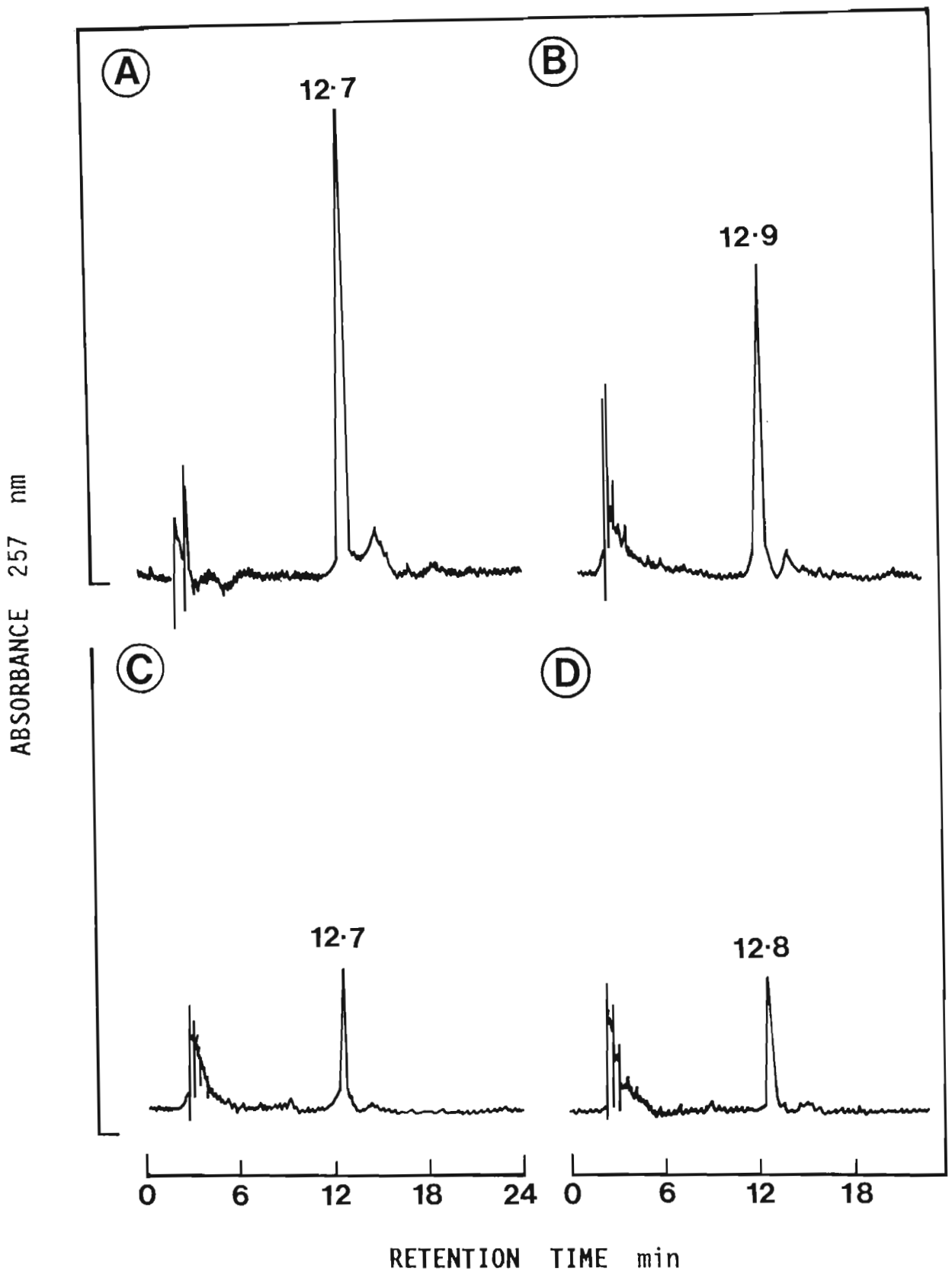


Figure 2.3.1: The HPLC separation of authentic hypoxoside (A) and hypoxoside extracts from root-producing callus (B), shoot-producing callus (C) and malformed-root producing callus (D) of *H. hemerocallidea* obtained from corm explants 10 months after initiation.

Table 2.3.1: The percentage hypoxoside (dry mass) detected in the three types of cultured *H. hemerocallidea* tissue 10 months after initiation, using *p*-nitroaniline reagent and 15 percent sodium carbonate. Three replicates were used to obtain each mean value and 95 percent confidence limits were applied.

SAMPLE	HYPOXOSIDE %	
Root-producing callus	0.135 ± 0.034	*
Shoot-producing callus	0.085 ± 0.006	* *
Malformed root-producing callus	0.051 ± 0.004	*

* indicates treatments that are the same, calculated using the multiple range test at $p < 0.05$.

2.3.1.1. Application of ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-sodium acetate and ^{14}C-acetyl coenzyme-A to root-producing callus

Uptake of radioactivity

The efficiency with which the root-producing callus took up the various precursors was not significantly different (Table 2.3.2). ^{14}C-Phenylalanine, ^{14}C-*t*-cinnamic acid and ^{14}C-sodium acetate were taken up by the callus slightly more efficiently than the ^{14}C-acetyl coenzyme-A was.

HPLC analysis

The exogenous application of ^{14}C-phenylalanine produced a root callus extract that yielded a peak of radioactivity with the same retention time as authentic hypoxoside when separated with HPLC (Figure 2.3.2. A). This peak of radioactivity was considerably smaller and less distinct than that obtained after the separation of the ^{14}C-*t*-cinnamic acid treated callus extract (Figure 2.3.2. B). When compared, the level of radioactivity incorporated into this peak that co-eluted with authentic hypoxoside, was greater, but not significantly so, when ^{14}C-phenylalanine was applied than when ^{14}C-*t*-cinnamic acid was used as a precursor (Table 2.3.3). Thus phenylalanine is tentatively implicated as a precursor for hypoxoside while the role of cinnamic acid remains less definite.

HPLC analysis carried out in this feeding experiment, for the extracts of ^{14}C-acetate and ^{14}C-acetyl coenzyme-A fed root callus, did not yield any peaks of radioactivity that co-eluted with authentic hypoxoside (Figure 2.3.2 C and D). This result indicates that the

Table 2.3.2: The radioactivity recovered (expressed as a percentage of the total radioactivity applied) from root-producing callus of *H. hemerocallidea* 8 weeks after treatment with ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-sodium acetate and ^{14}C-acetyl coenzyme A. Three replicates were used to obtain each mean and 95 confidence limits were applied.

RADIOACTIVE COMPOUND APPLIED	RADIOACTIVITY RECOVERED %
L(U-^{14}C)phenylalanine	40.9 ± 27.4 *
(3-^{14}C)*t*-cinnamic acid	37.8 ± 22.6 *
(U-^{14}C)sodium acetate	38.4 ± 7.6 *
(1-^{14}C)acetyl coenzyme A	26.2 ± 0.5 *

* indicates treatments that are the same, calculated using the multiple range test at $p < 0.05$.

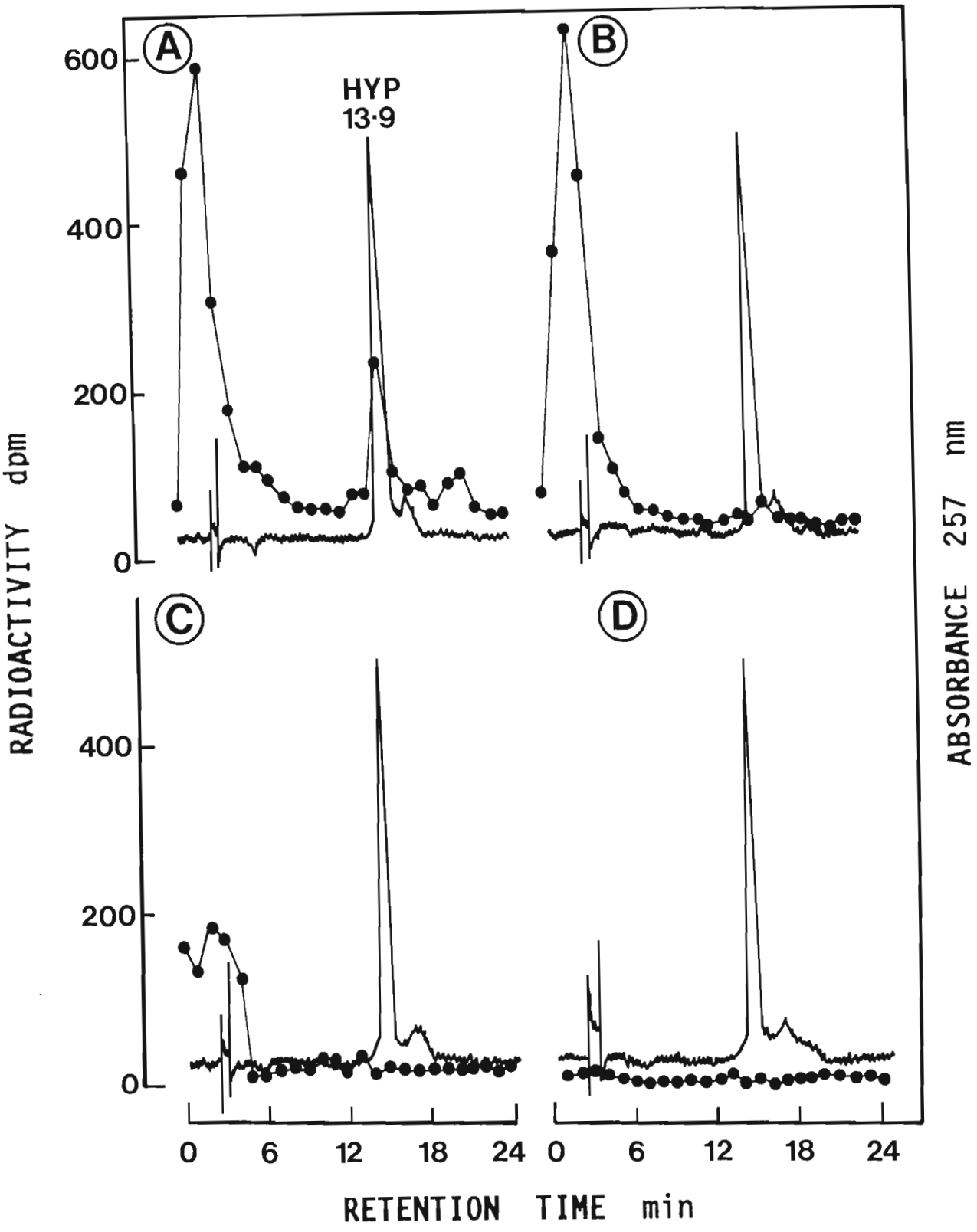


Figure 2.3.2: The HPLC separation of the radioactive hypoxoside extracts (●—●) obtained from root-producing callus of *H. hemerocallidea* after treatment with L(U-^{14}C)phenylalanine (**A**), (3-^{14}C)*t*-cinnamic acid (**B**), (U-^{14}C)sodium acetate (**C**) and (1-^{14}C)acetyl coenzyme-A (**D**) for 8 weeks. HYP= hypoxoside.

Table 2.3.3: The radioactivity (expressed as a percentage of the total radioactivity applied) incorporated into hypoxoside when ^{14}C- phenylalanine and ^{14}C-*t*-cinnamic acid were applied to root-producing callus of *H. hemerocallidea*. Three replicates were used in each case and 95 percent confidence limits were applied.

RADIOACTIVE COMPOUND APPLIED	INCORPORATION OF RADIOACTIVITY %
L(U-^{14}C)phenylalanine	0.057 ± 0.035 *
(3-^{14}C)*t*-cinnamic acid	0.019 ± 0.021 *

* indicates treatments that are the same, calculated using the multiple range test at $p < 0.05$.

acetate pathway is, apparently not, a source of precursors for hypoxoside biosynthesis.

2.3.1.3. The presence and concentrations of the hydroxycinnamic acids.

UV absorbent peaks co-eluting with the retention times of *t*-cinnamic acid and the hydroxycinnamic acids, caffeic, *p*-coumaric, ferulic and sinapic acids were detected in both root- and shoot-producing callus extracts on separation with HPLC (Figure 2.3.3).

The results of the quantitative analysis with *p*-nitroaniline of the bands co-eluting with the hydroxycinnamic acids on paper for both root- and shoot-producing callus are shown in Table 2.3.4. In all cases the shoot-producing callus contained higher percentages, on a dry mass basis, of the hydroxycinnamic acids than the root-producing callus (Table 2.3.4). These levels were all significantly different. In the shoot-producing callus the level of caffeic acid was the highest followed by sinapic and ferulic acids. *p*-Coumaric acid was present at the lowest level for the hydroxycinnamic acids detected in shoot-producing callus.

In the root-producing callus a similar trend was observed, with caffeic acid present in the greatest quantity, followed by sinapic acid. However, in the root-producing callus the level of ferulic acid detected was very low, with the *p*-coumaric acid level being greater.

t-Cinnamic acid could not be assayed with this technique as the reaction with the *p*-nitroaniline reagent requires the presence of a

Figure 2.3.3: The HPLC separation of authentic *t*-cinnamic acid and the hydroxycinnamic acids (**A**) and the extracts obtained from shoot-producing callus (**B**) and root-producing callus (**C**) of *H. hemerocallidea* 10 months after generation from corm explants. CAF= caffeic acid, COU= *p*-coumaric acid, FER= ferulic acid, SIN= sinapic acid and CIN= *t*-cinnamic acid.

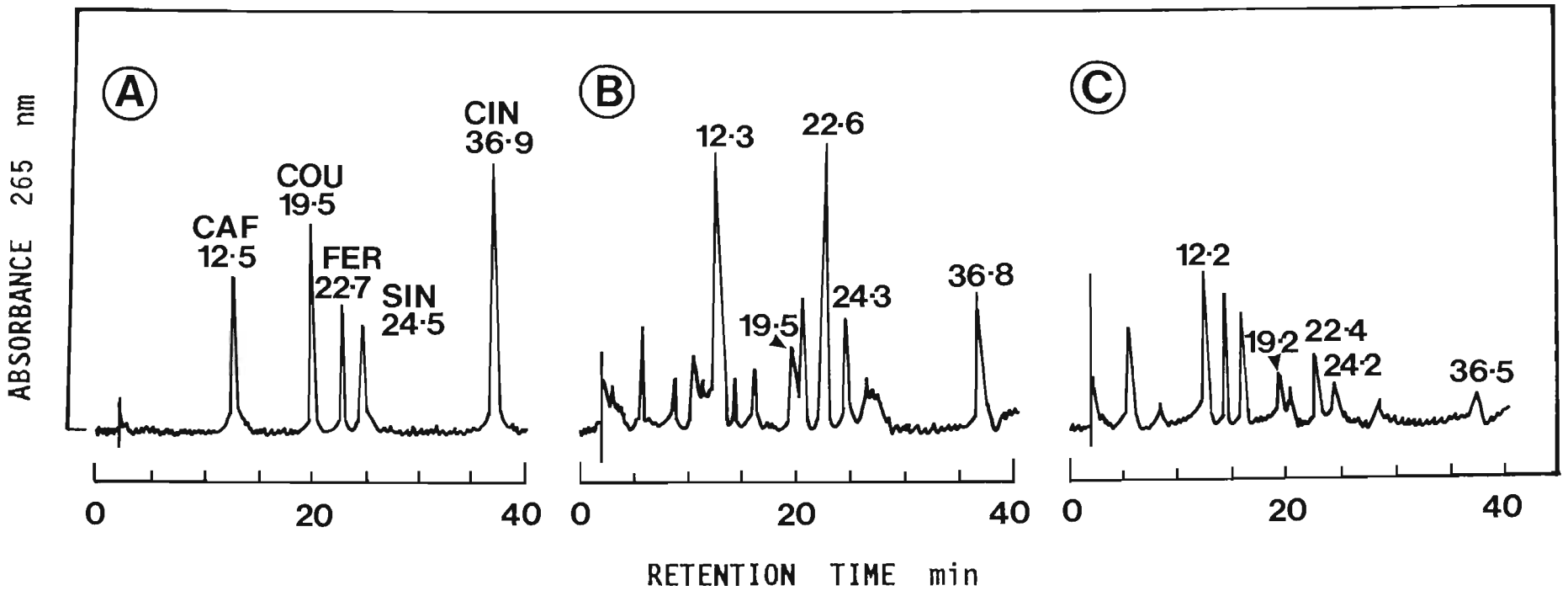

A
CAF
12·5
COU
19·5
FER
22·7
SIN
24·5
CIN
36·9
B
12·3
19·5
22·6
24·3
36·8
C
12·2
19·2
22·4
24·2
36·5
ABSORBANCE 265 nm
0
20
40
RETENTION TIME min

Table 2.3.4: The percentage hydroxycinnamic acids (dry mass) detected in shoot-producing and root-producing callus of *H. hemerocallidea* 10 months after initiation at the end of an 8 week culture period. Three replicates were used and 95 percent confidence limits applied.

IN VITRO TISSUE	HYDROXYCINNAMIC ACIDS %			
	Cou	Caf	Sin	Fer
Shoot-producing callus	0.069±0.003*	0.282±0.016*	0.131±0.010*	0.123±0.014*
Root-producing callus	0.013±0.004 *	0.158±0.044 *	0.068±0.008 *	0.001±0.001 *

cou= *p*-coumaric acid; caf= caffeic acid, sin= sinapic acid and fer = ferulic acid.

* indicates values that are not significantly different calculated by using multiple variance analysis and least significant differences.

hydroxyl substituent on the benzene ring, as stated earlier (RIBÉREAU-GAYON, 1972).

2.3.1.4 The extraction of the hydroxycinnamic acids from root-producing callus fed ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-sodium acetate and ^{14}C-acetyl coenzyme-A.

HPLC analysis of the hydroxycinnamic acids extracted from root-producing callus fed ^{14}C-sodium acetate and ^{14}C-acetyl coenzyme-A did not yield any radioactive peaks that co-eluted with the retention times of the authentic hydroxycinnamic acids or cinnamic acid (Figure 2.3.4 C and D).

Although small in some cases, both the ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acid treated root-callus produced extracts that contained peaks of radioactivity, which co-eluted with all of the standards (Figure 2.3.4 A and B). However, the distribution of the radioactivity between *t*-cinnamic acid and the hydroxycinnamic acids, differed in the extracts from the two treatments (Table 2.3.5).

In root callus fed ^{14}C-phenylalanine most of the radioactivity was associated with the retention time of caffeic acid while in the ^{14}C-*t*-cinnamic acid treated callus, this association occurred at the retention time of sinapic acid. The latter treatment also yielded a large radioactive peak with a retention time similar to that of *t*-cinnamic acid. This was obviously due to exogenously applied ^{14}C-*t*-cinnamic acid which remained unmetabolised. The latter peak represented 0.48 ± 0.14 percent of the total radioactivity taken up in the treatment.

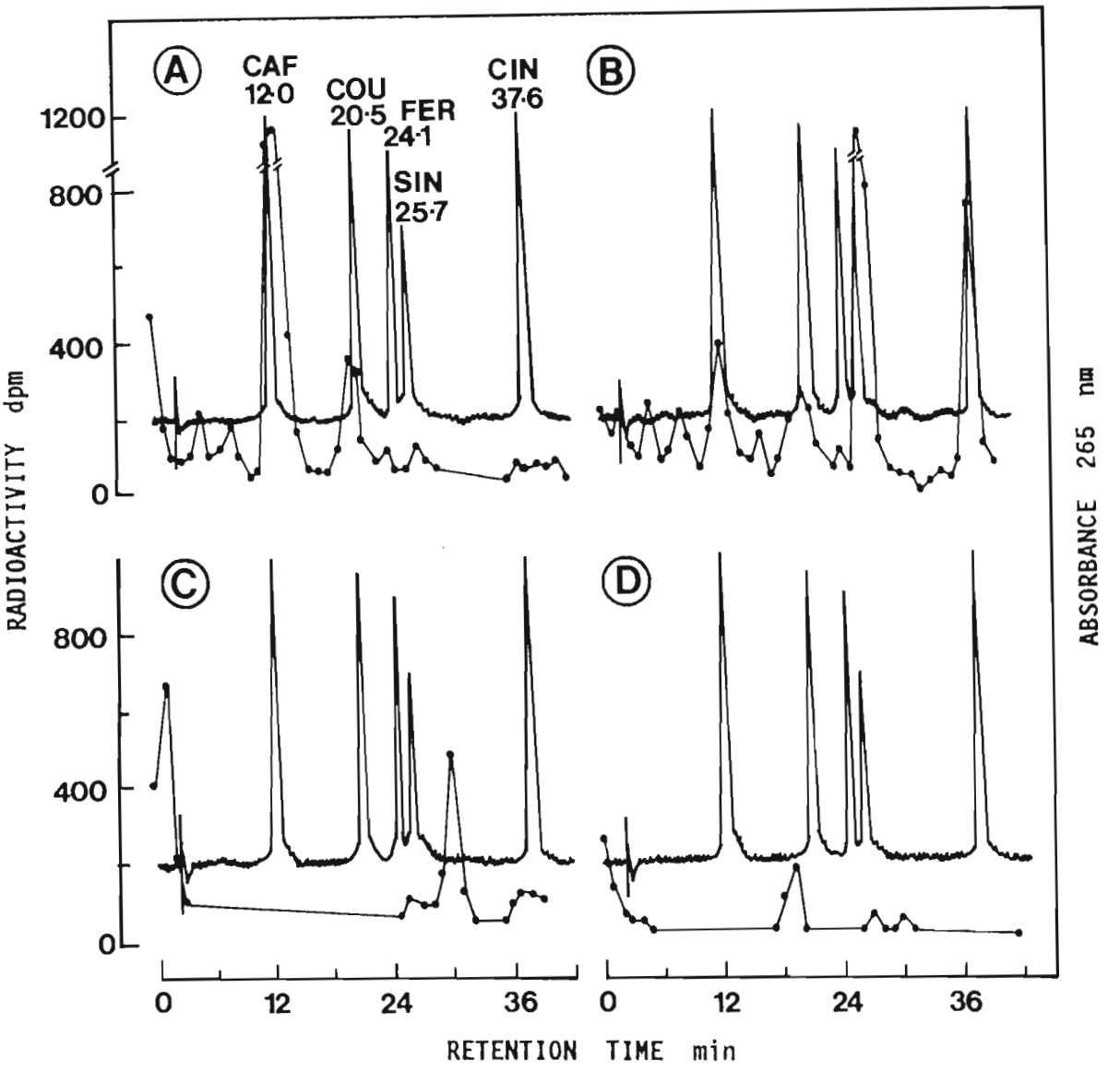


Figure 2.3.4: The HPLC separation of the radioactive hydroxycinnamic acid extracts (●—●), obtained from root-producing callus of *H. hemerocallidea* after treatment with L(U-^{14}C)phenylalanine (**A**), (3-^{14}C)*t*-cinnamic acid (**B**), (U-^{14}C)sodium acetate (**C**) and (1-^{14}C)acetyl coenzyme-A (**D**) for 8 weeks. CAF= caffeic acid, COU= *p*-coumaric acid, FER= ferulic acid, SIN= sinapic acid and CIN= *t*-cinnamic acid.

Table 2.3.5: The radioactivity incorporated (as a percentage of the total radioactivity recovered) into the hydroxycinnamic acids and cinnamic acid in root-producing callus of *H. hemerocallidea* to which (^{14}C)-phenylalanine and ^{14}C-*t*-cinnamic acid was applied. Three replicates were used and 95 percent confidence limits applied.

RADIOACTIVE COMPOUND APPLIED	RADIOACTIVITY INCORPORATED %				
	Cin	Cou	Caf	Sin	Fer
L(U-^{14}C)phenyl-alanine	0.05±0.02^{a}	0.47±0.17^{b}	2.07±0.83^{c}	0.08±0.04^{a}	0.06±0.01^{a}
(3-^{14}C)*t*-cinnamic acid	0.48±0.14^{b}	0.26±0.06^{d}	0.29±0.14^{d}	1.26±0.43^{e}	0.66±0.01^{f}

cin= *t*-cinnamic acid; cou= *p*-coumaric acid; caf= caffeic acid, sin= sinapic acid and fer= ferulic acid.

a,b,c,d,e,f, indicate values that are not significantly different calculated by using multiple variance analysis and least significant differences at $p < 0.05$.

Since the incorporation of radioactivity into the hydroxycinnamic acids, *p*-coumaric and caffeic acids, particularly the latter, was high in the treatment that yielded radioactivity at the retention time of hypoxoside, the role of these acids in the biosynthesis of hypoxoside was investigated.

2.3.1.5. Application of ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid to root-producing callus.

Incorporation of radioactivity

^{14}C-Phenylalanine and ^{14}C-*t*-cinnamic acids were taken up by the root-producing callus approximately ten times more efficiently than the ^{14}C-*p*-coumaric and ^{14}C-caffeic acids (Table 2.3.6). The uptake for the latter two acids was very low while the uptake of the phenylalanine and *t*-cinnamic acid was lower than that obtained previously (Table 2.3.2).

Incorporation of radioactivity into hypoxoside

Only the extracts from root-producing callus, to which ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acid was applied, contained substantial peaks of radioactivity with retention times similar to that of hypoxoside, when separated with HPLC (Figure 2.3.5 A and B).

Incorporation of radioactivity into such a peak in ^{14}C-*p*-coumaric and ^{14}C-caffeic acid treated callus was very low (Figure 2.3.5 C and D). Comparisons of incorporation of radioactivity into hypoxoside (Table 2.3.7) show that, the efficiency with which ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acid were utilised, was very similar while, the incorporation of ^{14}C-*p*-coumaric and ^{14}C-caffeic acids was negligible.

Table 2.3.6: The radioactivity recovered (expressed as a percentage of the total radioactivity applied) from root-producing callus of *H. hemerocallidea* 8 weeks after the application of ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid. Three replicates were used and 95 percent confidence limits were applied.

RADIOACTIVE COMPOUND APPLIED	RADIOACTIVITY RECOVERED %		
L(U-^{14}C)phenylalanine	23.53 ± 4.48	*	
(3-^{14}C)t-cinnamic acid	19.40 ± 4.05	*	
(2-^{14})*p*-coumaric acid	3.82 ± 0.41		*
(2-^{14}C)caffeic acid	2.93 ± 0.57		*

* indicates treatments that are the same, determined with a multiple range test at $p < 0.05$

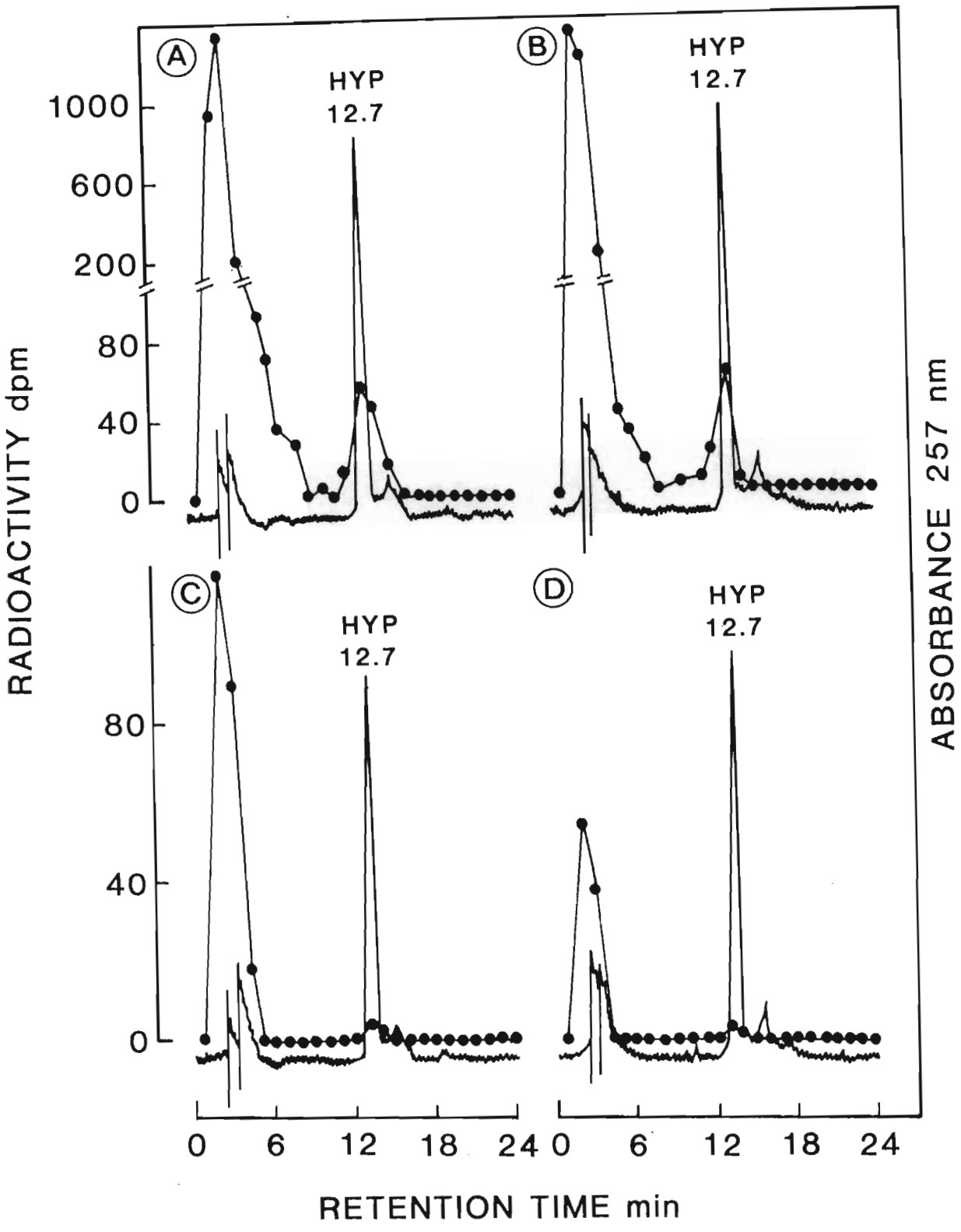


Figure 2.3.5: The HPLC separation of the radioactive hypoxoside extracts (●—●) obtained from root-producing callus of *H. hemerocallidea* treated with L(U-^{14}C)phenylalanine (**A**), (3-^{14}C)*t*-cinnamic acid (**B**), (2-^{14}C)*p*-coumaric acid (**C**) and (2-^{14}C)caffeic acid (**D**) for 8 weeks.

Table 2.3.7: The radioactivity (expressed as a percentage of the total applied) incorporated into hypoxoside when ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid were applied to root-producing callus of *H. hemerocallidea*. Three replicates were used and 95 percent confidence limits applied.

RADIOACTIVE COMPOUND APPLIED	RADIOACTIVITY INCORPORATED %	
L(U-^{14}C)phenylalanine	0.006 ± 0.005	*
(3-^{14}C)t-cinnamic acid	0.005 ± 0.003	*
(2-^{14}C)*p*-coumaric acid	0.001 ± 0.0007	*
(2-^{14}C)caffeic acid	0.0004 ± 0.0002	*

* indicates treatments that are the same, determined with a multiple range test at $p < 0.05$

Incorporation of radioactivity into the hydroxycinnamic acids

The hydroxycinnamic acid extracts from the root-producing callus to which either ^{14}C-phenylalanine or ^{14}C-*t*-cinnamic acid was applied, displayed a similar array of peaks of radioactivity when analysed with HPLC (Figure 2.3.6 A and B). Both extracts contained peaks of radioactivity that co-eluted with caffeic and *p*-coumaric acids. When the distribution of radioactivity between these peaks is compared for the two treatments (Table 2.3.8), in both cases approximately twice as much of the radioactivity present in *p*-coumaric acid is present in caffeic acid. The ^{14}C-phenylalanine treatment yielded more radioactivity in association with caffeic acid than did the ^{14}C-*t*-cinnamic acid application, while the reverse holds for incorporation of radioactivity into *p*-coumaric acid.

The root-producing callus treated with ^{14}C-*p*-coumaric acid yielded peaks of radioactivity that co-eluted with the retention times of *p*-coumaric and caffeic acids (Figure 2.3.6 C). The former peak of radioactivity was obviously due to the presence of applied ^{14}C-*p*-coumaric acid that remained unmetabolised. The extract from the ^{14}C-caffeic acid treatment produced similar results (Figure 2.3.6 A); in this case the peak of radioactivity coincident with caffeic acid representing the unmetabolised applied compound.

An examination of the levels of radioactivity present in the peaks, obtained for the ^{14}C-*p*-coumaric and ^{14}C-caffeic treated root-callus (Table 2.3.8), showed that applied ^{14}C-*p*-coumaric acid was metabolised to caffeic acid, resulting in approximately six times more radiolabelled caffeic acid being present than *p*-coumaric acid, while applied ^{14}C-caffeic acid was not converted as readily to *p*-coumaric acid, indicating that the reverse process does not occur as readily.

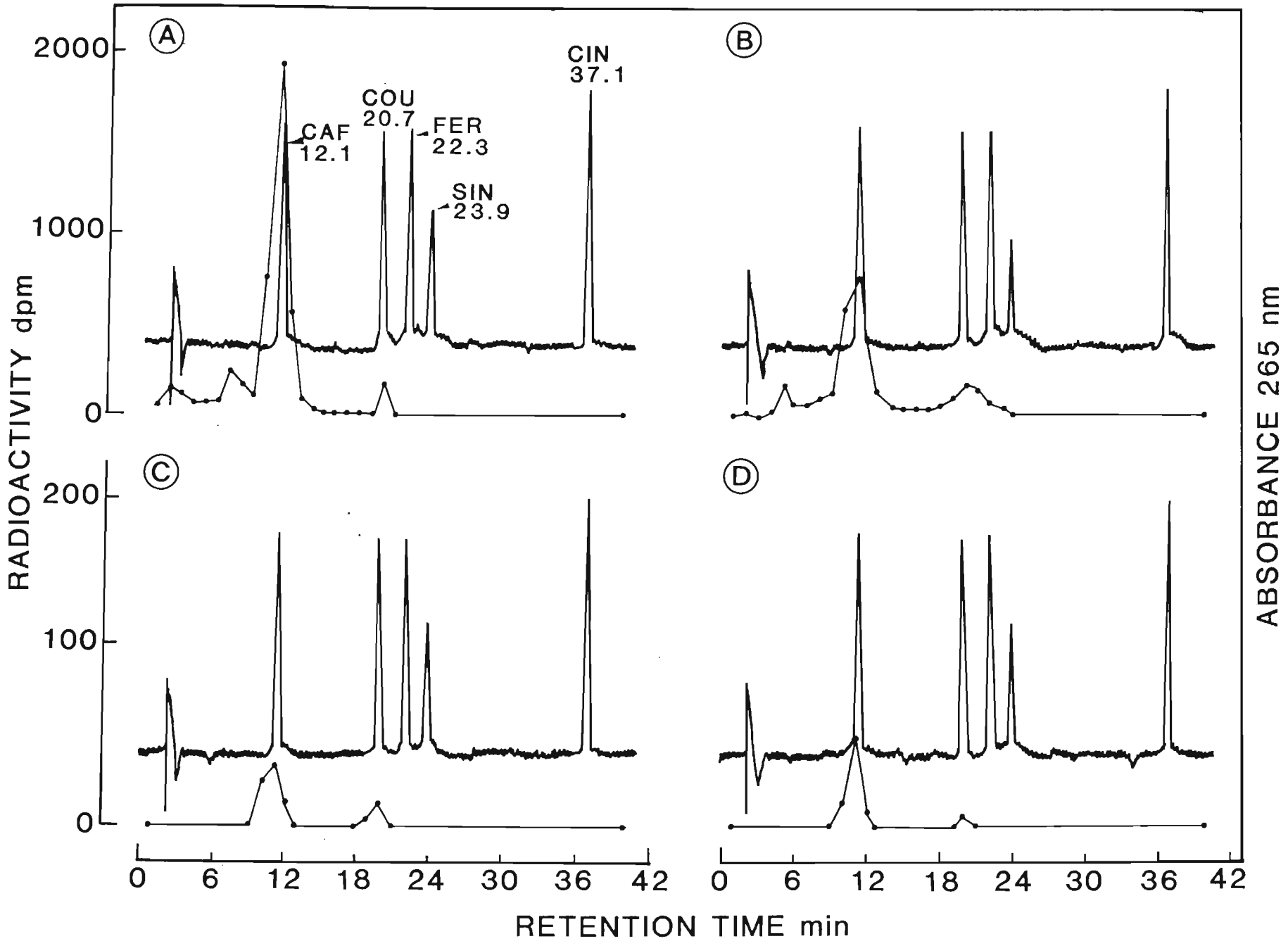

A
B
C
D
CIN
37.1
COU
20.7
FER
22.3
CAF
12.1
SIN
23.9
2000
1000
0
200
100
0
RADIOACTIVITY dpm
ABSORBANCE 265 nm
0 6 12 18 24 30 36 42
RETENTION TIME min

Table 2.3.8: Radioactivity incorporated (expressed as a percentage of the total radioactivity recovered) into cinnamic acid and the hydroxycinnamic acids in ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid treated root callus. In each case three replicates were used and 95 percent confidence limits were applied.

RADIOACTIVE COMPOUND APPLIED	RADIOACTIVITY INCORPORATED %				
	Cin	Cou	Caf	Fer	Sin
L(U-^{14}C)phenylalanine	-	0.22±0.14^{a}	6.06±0.84^{c}	-	-
(3-^{14}C)t-cinnamic acid	-	0.94±0.17^{b}	1.90±0.90^{d}	-	-
(2-^{14}C)*p*-coumaric acid	-	0.23±0.11^{a}	1.25±0.82^{d}	-	-
(2-^{14}C)caffeic acid	-	0.09±0.06^{a}	1.48±0.59^{d}	-	-

cin= *t*-cinnamic acid,cou= *p*-coumaric acid, caf= caffeic acid, fer= ferulic acid and sin= sinapic acid.

^{a,b,c,d} indicate values that are not significantly different determined from least significant difference calculations at $p < 0.05$.

In all of the treatments it is apparent that the applied radioisotopes were either, not readily converted to *t*-cinnamic, ferulic or sinapic acid, or conversion to these acids was transient.

2.3.1.6. Exogenous application of ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid to intact plants.

Radioactivity recovered

The radioactivity recovered from corms of ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acid treated plants was greater than that recovered from those to which ^{14}C-*p*-coumaric and ^{14}C-caffeic acid was applied (Table 2.3.9). The recovery from the ^{14}C-*t*-cinnamic acid treatment was significantly different from the ^{14}C-*p*-coumaric and ^{14}C-caffeic acid treatments, while the recovery from the ^{14}C-phenylalanine application was only significantly different from the ^{14}C-caffeic acid treatment.

This indicated that either the ^{14}C-*p*-coumaric and ^{14}C-caffeic acids were taken up less efficiently and/or that they were less effectively transported from the shoot apical region to the corm.

Incorporation of radioactivity into hypoxoside

Peaks of radioactivity co-eluting with authentic hypoxoside were detected in all treatments when corm extracts were separated with HPLC (Figure 2.3.7). When these peaks of radioactivity are compared quantitatively, as percentages of the total radioactivity fed per plant (Table 2.3.10), the effectiveness of the applied potential precursors differs. ^{14}C-Phenylalanine and ^{14}C-*t*-cinnamic acid were more efficiently incorporated into hypoxoside, with the latter

Table 2.3.9: Radioactivity recovered from corms (expressed as a percentage of the total radioactivity applied) from ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid treated plants. Three replicates were used to obtain means and 95 percent confidence limits were applied.

RADIOACTIVE COMPOUND APPLIED	RADIOACTIVITY RECOVERED FROM CORM %	
L(U-^{14}C)phenylalanine	7.38 ± 6.15	* *
(3-^{14}C)*t*-cinnamic acid	12.78 ± 5.67	*
(2-^{14}C)*p*-coumaric acid	2.16 ± 0.79	* *
(2-^{14}C)caffeic acid	1.57 ± 0.27	*

* indicate treatments that are the same calculated using the multiple range test at $p < 0.05$.

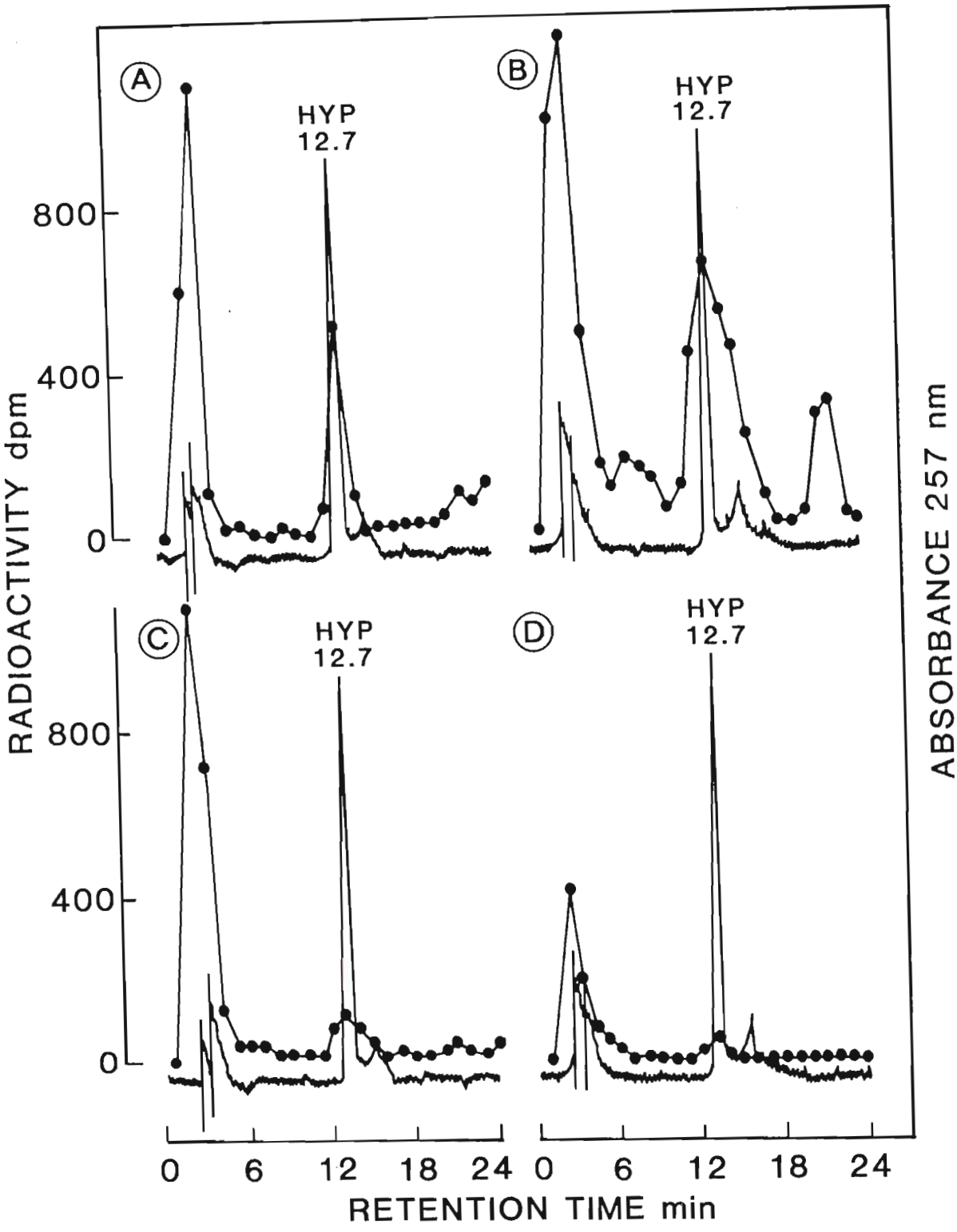
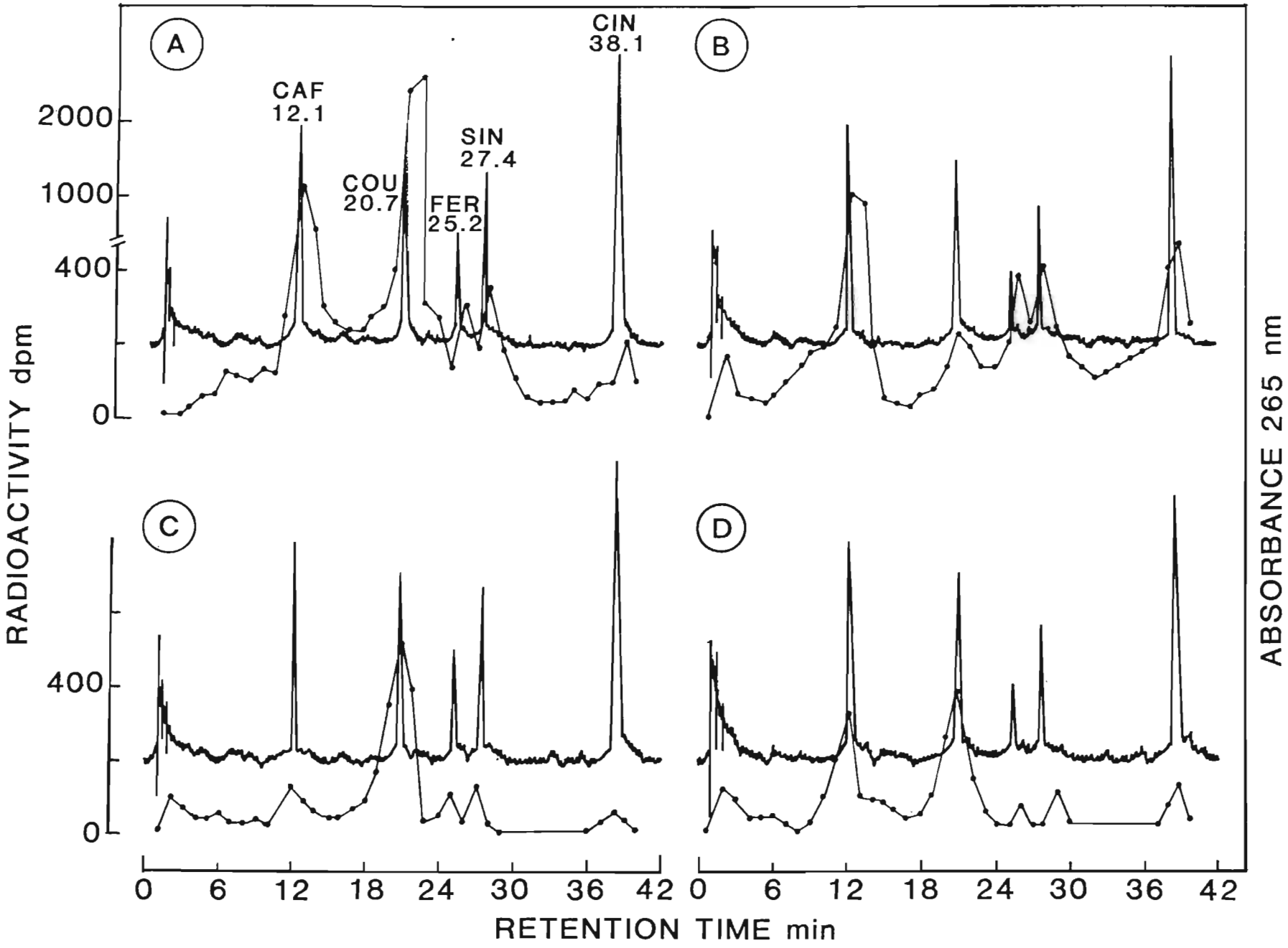


Figure 2.3.7: The HPLC separation of the radioactive hypoxoside extracts (●—●) obtained from the corms of intact plants of *H. hemerocallidea* treated with L(U-^{14}C)phenylalanine (**A**), (3-^{14}C)*t*-cinnamic acid (**B**), (2-^{14}C)*p*-coumaric acid (**C**) and (2-^{14}C)caffeic acid (**D**) for 8 weeks. HYP= hypoxoside.

Table 2.3.10 Incorporation of radioactivity into hypoxoside (expressed as a percentage of the total radioactivity applied and recovered) when ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid were applied to intact plants of *H. hemerocallidea*. The means were obtained from three replicates at a 95 percent confidence level.

RADIOACTIVE COMPOUND APPLIED	RADIOACTIVITY INCORPORATED %	
	TOTAL	RECOVERED
L(U-^{14C} phenylalanine	0.089 ± 0.051 * *	0.95 ± 0.50 *
(3-^{14}C)*t*-cinnamic acid	0.176 ± 0.102 *	0.74 ± 0.57 * *
(2-^{14}C)*p*-coumaric acid	0.010 ± 0.005 *	0.22 ± 0.06 *
(2-^{14}C)caffeic acid	0.001 ± 0.001 *	0.04 ± 0.02 *

* indicates treatments that are the same calculated using the multiple range test at $p < 0.05$.


A
B
C
D
CAF
12.1
COU
20.7
FER
25.2
SIN
27.4
CIN
38.1
2000
1000
400
0
400
0
RADIOACTIVITY dpm
ABSORBANCE 265 nm
0 6 12 18 24 30 36 42
0 6 12 18 24 30 36 42
RETENTION TIME min

precursor being utilised more effectively than the former, but not significantly so.

^{14}C-*p*-Coumaric acid and ^{14}C-caffeic acid were poor precursors for this peak of radioactivity, although the incorporation of ^{14}C-*p*-coumaric acid was not significantly less than that of ^{14}C-phenylalanine. When the results were expressed as a percentage of the radioactivity recovered, similar trends were observed, although in this case ^{14}C-phenylalanine is more efficiently incorporated than ^{14}C-*t*-cinnamic acid. Thus all applied compounds served as precursors for a compound that co-eluted with hypoxoside, albeit to different degrees.

Incorporation of radioactivity into the hydroxycinnamic acids

HPLC separation of the corm extracts from all treatments, yielded peaks of radioactivity that co-eluted with all the authentic standards (*t*-cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid) although in some cases the peaks were smaller than in others (Figure 2.3.8.).

Quantification of the peaks of radioactivity, recovered at the retention times of the standards, expressed as a percentage of the total radioactivity recovered per corm, reveal that most of the peaks co-incident with *p*-coumaric and caffeic acids were greater than those obtained for the other standards (Figure 2.3.9). The ^{14}C-phenylalanine treated corms contained high levels of labelled *t*-cinnamic *p*-coumaric and caffeic acids, whilst the levels of radioactive ferulic and sinapic acids were lower.

In the ^{14}C-*t*-cinnamic acid treated corm the levels of radioactivity incorporated into *p*-coumaric and caffeic acid were higher than those

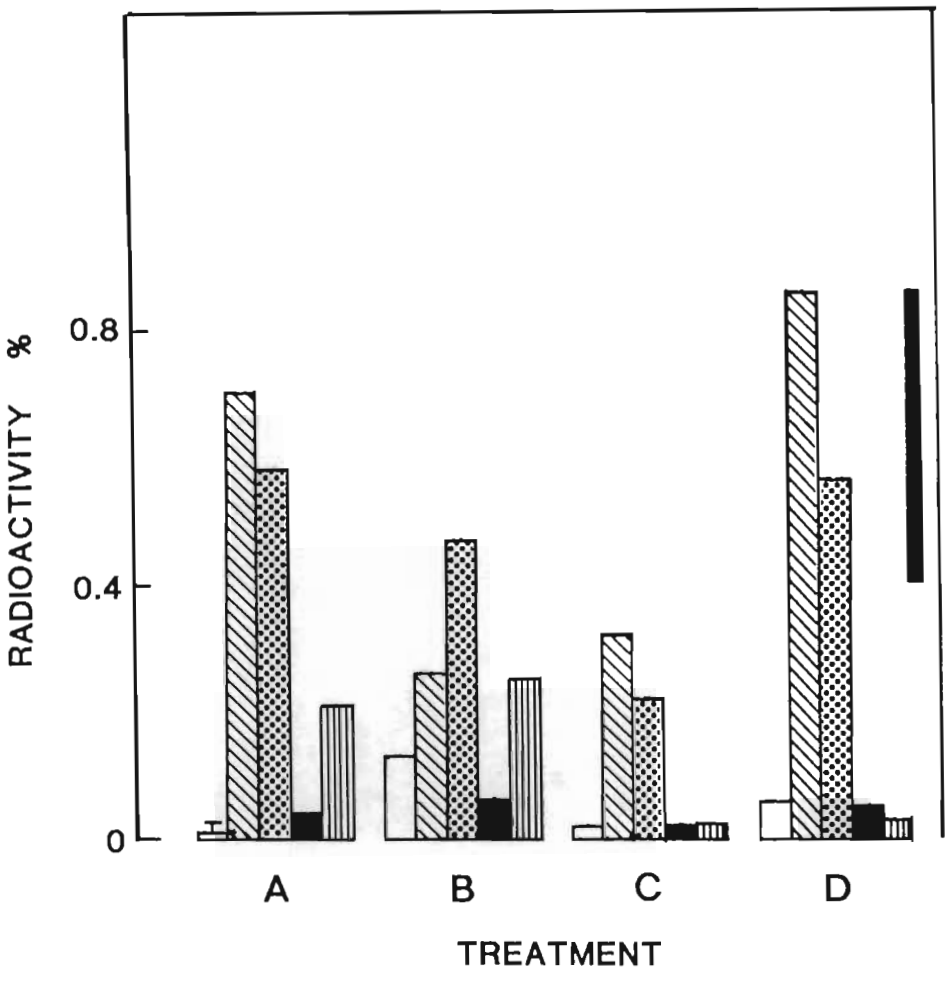


Figure 2.3.9: The incorporation of radioactivity into *t*-cinnamic acid □, *p*-coumaric acid ▨, caffeic acid ▩, ferulic acid ■ and sinapic acid ▥ hydroxycinnamic acid extracts obtained from the corms of intact plants of *H. hemerocallidea* treated with L(U-^{14}C)phenylalanine (**A**), (3-^{14}C)*t*-cinnamic acid (**B**), (2-^{14}C)*p*-coumaric acid (**C**) and (2-^{14}C)caffeic acid (**D**) for 8 weeks. The solid bar represents the least significant difference calculated using multiple variance analysis at $p < 0.05$.

observed for *t*-cinnamic, ferulic and sinapic acids but this difference was not as marked as that observed for the levels of incorporation observed for the ^{14}C-phenylalanine treated corm.

In both the ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acid treated corms the incorporation of radioactivity into *t*-cinnamic acid was low indicating rapid metabolism of the precursor.

In the ^{14}C-*p*-coumaric acid treated corms most of the radioactivity was present in *p*-coumaric acid with conversion mainly to caffeic acid. The radioactivity in extracts from the ^{14}C-caffeic acid treated corms was associated predominantly with *p*-coumaric and caffeic acids.

2.4. DISCUSSION

2.4.1. *IN VITRO* INVESTIGATIONS

The finding that compounds co-eluting with authentic hypoxoside were detected only in tissues displaying some type of root differentiation agrees with the report of PAGE and VAN STADEN (1987). The level of hypoxoside detected in malformed root tissue, generated from corm explants, after 10 months of culture agrees with the results obtained by PAGE (1984). She determined that, while this tissue initially had a high level of hypoxoside compared to the other *in vitro* grown tissues, it decreased to approximately 0.005 percent (fresh mass) after 10 months; this being similar to the level she detected in root-producing callus. The conclusion reached by PAGE (1984) was that subculturing and enhancement of growth of the callus were detrimental to hypoxoside formation.

When the level of hypoxoside, detected in root-producing callus, generated from corm explants in the current study, was approximated to a fresh mass value (assuming an 80 percent water content), the value obtained was three times higher than that detected by PAGE and VAN STADEN (1987). This discrepancy could be due to the different method of quantification used in the present study. However, since the levels detected for malformed root tissue agree with those of PAGE and VAN STADEN (1987), the reason more likely rests with the fact that a better line for producing hypoxoside was obtained in the present investigation. It is well documented that the proficiency of secondary metabolite production can vary considerably between cell lines generated from different plants of the same species (KURZ and CONSTABEL, 1985; BERLIN, 1988).

The incorporation of radioactivity into a compound that co-eluted with hypoxoside in extracts from root callus to which ^{14}C-phenylalanine had been applied is at a level considered to be meaningful by other researchers (ROUGHLEY and WHITING, 1971; BANERJI and CHINTALWAR, 1984; INOUE, KENMOCHI, FURUKAWA, and FUJITA, 1987). ROUGHLEY and WHITING (1971) in studies on the biosynthesis of the diarylheptanoid, curcumin, obtained a level of 0.051 percent incorporation of total radioactivity applied into curcumin in whole plants of *Curcuma longa*. In the current study the level of ^{14}C-phenylalanine incorporated into hypoxoside, expressed as a percentage of the total radioactivity applied, was 0.057 percent. BANERJI and CHINTALWAR (1984) studying the biosynthesis of bakuchinol, an aryl group with an isoprenoid side chain, in seeds of *Psoralea corylifolia* L. obtained a level of 0.04 percent incorporation of total radioactivity applied. This they considered to be an efficient level of incorporation. INOUE, KENMOCHI, FURUKAWA and FUJITA (1987) obtained levels of incorporation of 0.036 and 0.052 percent, of applied L(1-^{14}C)phenylalanine and DL(3-^{14}C)phenylalanine respectively, into acerogenin, a diarylheptanoid, in the young shoots of *Acer nikoense*.

All the mentioned examples apply to *in vivo* systems while the current study was conducted in an *in vitro* system. In studies conducted with *Lachnanthes tinctoria* concerning the biosynthesis of the 9-phenylphenalenone, lachnanthoside, an incorporation of 3.49 percent of the DL(U-^{14}C)phenylalanine applied was obtained (EDWARDS, SCHMITT and WEISS, 1972) whilst in the callus suspension cultures, derived from roots of *L. tinctoria*, a level of 2.2 percent incorporation into the methyl esters of all the 9-phenylphenalenone pigments present, was obtained (BEECHER, SARG and EDWARDS, 1983). The levels of incorporation obtained in these systems are described as being

exceptionally good. Nevertheless it is obvious that better incorporation is obtained *in vivo* than *in vitro*.

A level of incorporation below 0.01 percent, it seems, is regarded as unacceptable as far as precursor studies are concerned (UNDERHILL, WATKIN and NEISH, 1957; ROUGHLEY and WHITING, 1973; BROWN, RIVETT and THOMPSON, 1984). Thus the level of ^{14}C-cinnamic acid incorporated in the current study, 0.019 percent, is close to the lowest level acceptable and thus the role of this acid as a normal endogenous precursor for hypoxoside in root-producing callus was uncertain.

In the studies with the feeding of (U-^{14}C)sodium acetate and (1-^{14}C)acetyl coenzyme-A, the uptake of the former and the latter did not differ from the uptake of the L(U-^{14}C)phenylalanine and (3-^{14}C)*t*-cinnamic acid applied. Thus while the callus took up the acetate with apparent efficiency, it was not utilised at all for hypoxoside biosynthesis.

The results suggest the involvement of phenylalanine and the shikimate pathway in the process of hypoxoside production and the non-involvement of the acetate pathway.

In the studies of ROUGHLEY and WHITING (1971) and INOUE, KENMOCHI, FURUKAWA and FUJITA (1987) the levels of radioactivity incorporated when ^{14}C-phenylalanine was fed are comparable with the results obtained in this study. However, the levels of radioactive acetate incorporated in the above investigation, were not much lower than those obtained for phenylalanine in these studies. ROUGHLEY and WHITING (1971) obtained 0.013 percent incorporation of (2-^{14}C)sodium acetate into curcumin in *Curcuma longa* whilst INOUE, KENMOCHI,

FURUKAWA and FUJITA (1987) obtained a 0.039 and 0.001 percent incorporation of radioactivity into acerogenin in young shoots of *Acer nikoense* when (2-^{14}C)sodium acetate and (1-^{14}C)sodium acetate were applied. While the levels of acetate incorporated into the end product were lower in both studies than those obtained with ^{14}C-phenylalanine applications, they were still detectable. In this study the level of phenylalanine incorporated was better than obtained in the above two studies, yet no acetate was incorporated. This implies that acetate is not operable in hypoxoside biosynthesis.

This conclusion supports the original postulation by MARINI-BETTOLO, PATAMIA, NICOLETTI, GALEFFI and MESSANA (1982) that the hypoxoside molecule is derived purely from the shikimate pathway via the conjugation of two phenylalanine units with the loss of one carbon. MARINI-BETTOLO, NICOLETTI, MESSANA, GALEFFI, MSONTHI and CHAPYA (1985) refine their theory further with the postulation that hypoxoside is formed by the junction of a acetylenic, C_6-C_2, unit on a propenylic, C_6-C_3, moiety at the *Y* position. It is clear that the propenylic moiety could originate from phenylalanine or any of the hydroxycinnamic acids, particularly *p*-coumaric and caffeic acids quite easily. However, the derivation of the acetylenic moiety from phenylalanine is not so obvious. Therefore, while the results of the current study have indicated that at least one of the aryl units is derived from phenylalanine, how the other aryl unit is generated from the shikimate pathway, is open to speculation.

The observation that the hydroxyl substitution of the hydroxycinnamic acid, caffeic acid, is identical to the pattern present on the aryl groups of hypoxoside, namely *ortho* with respect to one another, prompted an investigation into the role of the hydroxycinnamic acids

in hypoxoside biosynthesis. The fact that the shoot-producing callus contained higher levels of the hydroxycinnamic acids compared to the root-producing callus, was probably a reflection of the fact that this tissue was more photosynthetically active than the root-producing callus, rather than an ability to synthesise hypoxoside. Where root callus had been fed ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acid, peaks of radioactivity were detected at the retention times of all the hydroxycinnamic acids, *p*-coumaric, caffeic, sinapic and ferulic acids. The fact that such peaks of radioactivity were obtained in the ^{14}C-sodium acetate and ^{14}C-acetyl coenzyme-A callus extracts was not surprising. The only way in which such an incorporation could have occurred, in this case, would be by the eventual participation of acetate, through pyruvate, in phenylalanine metabolism or through degradation to carbon dioxide and reutilisation through photosynthesis. Both possibilities would result in such a dilution of radioactivity as to render any eventual incorporation into the hydroxycinnamic acids, undetectable.

Thus the evidence obtained in the present study favours the first hypothesis for the derivation of hypoxoside which is presented in 2.1 of this chapter; namely that the molecule is derived from the joining of two cinnamate units with the loss of a carbon atom. At present the mechanism for such a reaction has not been investigated.

In the root-producing callus, to which ^{14}C-phenylalanine was applied, the peak of radioactivity co-eluting with caffeic acid was large, and to a lesser extent so was that co-eluting with *p*-coumaric acid. This suggests an accumulation of radioactivity at the *p*-coumaric/caffeic acid point of the hydroxycinnamic acid conversion. Since this treatment also yielded labelled hypoxoside, the temptation to

postulate that these compounds are involved in the biosynthetic route for hypoxoside, is strong. It must be mentioned, however, that the endogenous levels of caffeic acid were high in the root-producing callus and shoot-producing callus. Therefore it may be that the *in vitro* tissues of *H. hemerocallidea* are just more tolerant to higher levels of caffeic acid than to the other hydroxycinnamic acids investigated.

However, when labelled *t*-cinnamic acid was applied, this accumulation occurred in sinapic acid, while peaks coincident with *p*-coumaric and caffeic acid were still well labelled but not to the extent obtained for the ^{14}C-phenylalanine treatment. The ^{14}C-*t*-cinnamic acid treatment did not give very good incorporation of radioactivity into hypoxoside. Thus the probable involvement of *p*-coumaric and caffeic acids in hypoxoside biosynthesis became an attractive possibility. Why the feeding of ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acids should result in radioactivity accumulating in different hydroxycinnamic acids, is not known and whether this difference accounts for the different efficiencies obtained for these compounds, as precursors for hypoxoside, can only be pondered upon.

In a later experiment, when ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric and ^{14}C-caffeic acids were applied to root-producing callus, the results for the incorporation of radioactivity into a peak co-incident with authentic hypoxoside for the first two treatments, were almost identical. The distribution of the radioactivity incorporated into the hydroxycinnamic acids in these two treatments differed slightly although most of the accumulation of radioactivity occurred in caffeic acid and in *p*-coumaric acid. Why these results differ from the previous data is not known as both

sets of experiments were conducted under identical conditions. In fact the percentage incorporation into hypoxoside achieved in feeding ^{14}-phenylalanine and ^{14}C-*t*-cinnamic acid in this experiment were below 0.01 percent of the total radioactivity applied and therefore it is not acceptable to consider these compounds to be precursors. In the previous experiment phenylalanine could at least be considered as such. The possible reason for this discrepancy can probably be explained by the fact that uptake of radioactivity in this later experiment was much less efficient in comparison to the previous experiment.

In this later experiment, the incorporations of ^{14}C-caffeic and ^{14}C-*p*-coumaric acids into a peak co-eluting with hypoxoside were so small as to be insignificant. Whether these compounds could be excluded as precursors for hypoxoside is debatable, however, as the recovery of radioactivity from callus in these treatments was much lower(ten times less) than that obtained with ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acid application suggesting either poor uptake or binding of the radioactivity to alcohol insoluble components of the tissue. The distribution of applied radioactivity amongst the hydroxycinnamic acids in these two treatments, also showed an accumulation of radioactivity into caffeic acid and, to a lesser extent, into *p*-coumaric acid. The conversion of *p*-coumaric acid to caffeic acid was greater than the reciprocal reaction, suggesting that caffeic acid is the more stable form. This agrees with the findings for the endogenous levels of the hydroxycinnamic acids.

Because of the variability of the *in vitro* system and the fact that the roles of *p*-coumaric, caffeic acid and *t*-cinnamic acid in

hypoxoside biosynthesis could not be clearly elucidated, it was decided to try and extrapolate these efforts to an *in vivo* system.

2.4.2. *IN VIVO* INVESTIGATIONS

The degree of incorporation of radioactivity into a peak coincident with hypoxoside in the corm extracts from ^{14}C-phenylalanine and ^{14}C-*t*-cinnamic acid treatments, 0.089 and 0.176 percent respectively, make it reasonable to consider that both these compounds are precursors for hypoxoside, under natural conditions.

The level of radioactivity incorporated into hypoxoside when ^{14}C-phenylalanine was applied, compare favourably with the values quoted by other researchers using this precursor in the biosynthetic studies of structurally related compounds. Exceptionally high levels of incorporation of ^{14}C-phenylalanine into lachnanthoside, in the roots of *Lachnanthes tinctoria*, have been obtained by EDWARDS, SCHMITT and WEISS (1972). Values of 0.449 percent, 3.48 percent and 3.8 percent incorporation have been obtained by these authors with applications of DL(U-^{14}C)phenylalanine, DL(3-^{14}C)phenylalanine and DL(1-^{14}C)phenylalanine, respectively.

THOMAS (1971) reports an incorporation of 0.3 percent and 0.29 percent into the aglycone of haemocorin, a 9-phenylphenalenone, when (1-^{14}C)phenylalanine and (2-^{14}C)phenylalanine were administered to the rhizome of *Haemodorum corymbosum*. ROUGHLEY and WHITING (1971) obtained 0.103 and 0.051 percent incorporation into curcumin in *Curcuma longa* plants, when (1-^{14}C)phenylalanine and (3-^{14}C)phenylalanine were applied. At a latter stage these authors (ROUGHLEY and WHITING, 1973) reported levels of 0.013 and 0.354

percent incorporation into curcumin in the same system when (1-^{14}C)phenylalanine was applied. This difference emphasises the variability of response of plant tissues to exogenous application which is most probably the result of differences in the physiological status prevailing in the plant tissue at the time of each experiment.

INOUE, KENMOCHI, FURUKAWA and FUJITA (1987), studying acerogenin production in young shoots of *Acer nikoense*, obtained incorporation levels of 0.036 percent, 0.052 percent and 0.033 percent when L(1-^{14}C)phenylalanine, DL(3-^{14}C)phenylalanine and (3-^{14}C)cinnamic acid were applied. BANERJI and CHINTALWAR (1984) reported incorporations of 0.04 percent and 0.01 percent for L(U-^{14}C)phenylalanine and (U-^{14}C)cinnamic acid into bakuchinol in *Psoralea corylifolia*.

These examples of the levels of incorporation of applied radioactivity into the studied end product, indicate that it is reasonable to assume, from the results obtained in the current study, that both phenylalanine and *t*-cinnamic acid are efficient precursors for hypoxoside in *H. hemerocallidea* corms. In the study with the intact plant, the *t*-cinnamic acid appeared to be a slightly better precursor for hypoxoside than phenylalanine; a finding which contradicts the *in vitro* results.

There are numerous reports where phenylalanine has been found to be a better precursor for compounds of phenylpropanoid derivation than cinnamic acid. Examples include bakuchinol biosynthesis in *Psoralea corylifolia*, (BANERJI and CHINTALWAR, 1984), acerogenin biosynthesis in *Acer nikoense* (INOUE, KENMOCHI, FURUKAWA and FUJITA, 1987) quercetin (a flavonoid) biosynthesis in *Fagopyrum tataricum* Gaertn. (UNDERHILL, WATKIN and NEISH, 1957), and biosynthesis of the tropic

acid moiety of the tropane alkaloids hyoscyamine and scopolamine in *Datura inoxia* Mill.(LEETE, 1983).

Reports where cinnamic acid had served as a better precursor than phenylalanine, for phenylpropanoid derivatives related to hypoxoside, could not be found. CO and MARKAKIS (1966) reported better incorporation for cinnamic acid into the anthocyanin, pelargonidin-3-glucoside in strawberry fruit.

The 0.010 and 0.001 percent incorporation of total radioactivity into peaks co-incident with hypoxoside in the (2-^{14}C)*p*-coumaric acid and (2-^{14}C)caffeic acid applications, indicate that these compounds cannot be considered to usually play a major part in hypoxoside biosynthesis. The efficiency of *p*-coumaric acid and caffeic acid as precursors for compounds structurally related to hypoxoside, vary. ROUGHLEY and WHITING (1973) determined that *p*-coumaric and caffeic acids (giving 0.022 percent and 0.005 percent incorporation respectively) were much poorer precursors for curcumin in *Curcuma longa* plants than phenylalanine (0.354 percent incorporation). Ferulic acid gave favourable results in the above experiment (0.079 percent). However, BANERJI and CHINTALWAR (1984) obtained very efficient incorporation of (2-^{14}C)*p*-coumaric acid into bakuchinol in *Psoralea corylifolia*. This incorporation was more efficient than when phenylalanine was fed. KLISCHIES and ZENK (1978) obtained better incorporation of caffeic acid (1.32 percent and 0.78 percent) than *p*-coumaric acid (0.34 percent and 0.22 percent) into rhododendrin in *Alnus glutinosa* Gaertn. and *Betula alba* L..

The results obtained for the incorporation of the hydroxycinnamic acids into other phenolic compounds also vary. UNDERHILL, WATKIN and

NEISH (1957) determined that with increasing hydroxylation and methylation substitution on the aryl moieties of the phenylpropanoids, the less efficient the compounds became as precursors for the B ring of quercetin in *Fagopyrum tataricum*. ^{14}C-Phenylalanine and ^{14}C-cinnamic acid gave incorporations of 1.512 percent and 0.670 percent, while ^{14}C-*p*-coumaric, ^{14}C-caffeic, ^{14}C-sinapic and ^{14}C-ferulic acids gave incorporations of 0.539, 0.078, 0.024 and 0.0001 percent, respectively.

SATO and HASEGAWA (1972) investigated the biosynthesis of the dihydroxycoumarin, daphnin, and the daphnetin-8-glucoside in *Daphne odora* Thundg.. They found that, while *p*-coumaric acid was a better precursor for these than cinnamic acid, caffeic acid was a much poorer precursor. BROWN, RIVETT and THOMPSON (1984) also found that *p*-coumaric acid was a better precursor for the coumarin, puberulin, in *Agathosma puberula* (Steud.) Fourc..

Thus, generally the results obtained in precursor studies with *p*-coumaric and caffeic acids for hypoxoside in *H. hemerocallidea*, agree with the findings of researchers working with related and unrelated phenolic compounds, in that *p*-coumaric and caffeic acids are not usually as efficient as phenylalanine and cinnamic acid as precursors.

Why (2-^{14}C)*p*-coumaric and (2-^{14}C)caffeic acids are poor precursors for hypoxoside is open to debate. Firstly the radioactivity recovered in these treatments was much lower than that recovered in the phenylalanine and *t*-cinnamic acid treatments. This could be the result of:

(i) poor uptake of the shoot apically applied radio labelled compound in each case;

(ii) poor transport of the radioactivity taken up within the plant; or

(iii) a poor recovery due to the incorporation of a large percentage of the radioactivity taken up into the alcohol insoluble wall components of the plant.

SHANN and BLUM (1987) have shown in experiments with excised roots of *Cucumis sativus* L., that uptake of ferulic acid was pH dependent; this process being most efficient at a low pH. In this study the *p*-coumaric and caffeic acids were fed in slightly alkaline solutions which may have hindered their uptake. In higher plants the hydroxycinnamic acids are present endogenously as glucose esters (ZENK, 1979; HARBORNE, 1980; MÖLLER and HERMANN, 1983; SCHUSTER and HERMANN, 1985). Since both *p*-coumaric and caffeic acids were applied as the free bases in this investigation, the possibility exists that their unconjugated state may have affected their uptake and transport by the *H. hemerocallidea* plants. However, it is believed that upon application of these free bases of the hydroxycinnamic acids and other plant phenols to plant tissue, rapid conjugation of the molecules occurs (RIBÉREAU-GAYON, 1972).

Poor transport within the tissue of *H. hemerocallidea* may not have been the only reason for the lack of effectiveness of *p*-coumaric acid and caffeic acids in the biosynthesis of hypoxoside. It is possible that incorporation into the cell walls of the cells of the plant did occur over the 8 week period.

Lignins are built from three basic phenylpropanoid units, namely *p*-coumaryl, coniferyl and sinapoyl alcohols which are derived from *p*-coumaric, ferulic and sinapic acids, respectively (BROWN and NEISH, 1955; GROSS, 1979; HASLAM, 1985). In *H. hemerocallidea* lignification

is associated with the vascular tissue of the plant and the bundles of sclerenchymatous tissues which are found particularly in the leaves, while both the roots and corms possess a heavily suberised root and corm hypodermis (THOMPSON, 1972). The phenylpropanoids, apart from their involvement in lignification, are also implicated in the formation of suberin (COTTLE and KOLATTUKUDY, 1982). The phenolic acids are also present as a wall bound fraction, which is described as cinnamic, *p*-coumaric and ferulic acid esters that are bound to the carbohydrate moieties of primary cell walls, where they are thought to be involved in the formation and expansion of the cell walls (HARTLEY, JONES and WOOD, 1976; FRY, 1982).

Thus the poor recovery of radioactivity from *p*-coumaric and caffeic acid treated corms, may have been due to a lack of transport or a binding to, or utilisation for cell wall formation. However, when this possibility is taken into account and the radioactivity incorporated into hypoxoside is calculated as a percentage of the radioactivity recovered, both *p*-coumaric and caffeic acids are still much poorer precursors for hypoxoside when compared to the efficiency of phenylalanine and *t*-cinnamic acid. There are two possible explanations for this lack of efficiency.

(i) The applied labelled *p*-coumaric and caffeic acids may not be converted with efficiency to an active form that is required for the formation of hypoxoside.

(ii) The biosynthesis of hypoxoside may not involve the direct incorporation of these hydroxycinnamic acids; hydroxylation of the aryl moieties occurring subsequent to the linkage of these moieties by the five carbon chain.

With consideration of the first proposal there are a number of reports on the involvement of the conjugated forms of the hydroxycinnamic acid in plant metabolism. HARBORNE and CORNER (1961) proposed that the phenolic glucose esters are essential intermediates in plant phenylpropanoid metabolism and do not merely accumulate as end products. Subsequent to this, glucose esters of hydroxycinnamic acids have been found to be metabolically active in many systems. (HARBORNE and CORNER, 1961; KOJIMA and URITANI, 1972, 1973; STRACK, 1977; MOLDEREZ, NAGELS and PARMENTIER, 1978; SCHLEPPHORST and BARZ, 1979). Whether transformation of *p*-coumaric and caffeic acid to an active form, is required for inclusion in hypoxoside biosynthesis is not known. If the transformation does occur what the nature of the activated forms are, whether they are glucose esters, alcohols or coenzyme-A activated acids, can only be speculated upon.

KLISCHIES and ZENK (1978) proposed that formation of rhododendrin in *Alnus glutinosa* and *Betula alba*, proceeds via the formation of *p*-coumaryl alcohol, followed by dihydro-*p*-coumaryl alcohol. Participation of *p*-coumaric, ferulic and sinapic acids in lignin biosynthesis requires their conversion to *p*-coumaryl, coniferyl and sinapyl alcohols (HASLAM, 1985; BROWN and NEISH, 1955; GROSS, 1979). STOTZ, SPRIBILLE and FORKMANN (1984) showed that all *Verbena hybrida* strains tested were able to utilise *p*-coumaryl coenzyme-A and caffeoyl coenzyme-A as substrates for the flavonoid skeleton of the flavonoids present in flowers of this species. The former was a better precursor than the latter.

There are also a number of reports which indicate that the second possibility should receive consideration. Numerous studies have indicated that the 3'-hydroxyl group of the B ring is introduced at

the flavone or dihydroflavonol stage (FORKMANN, HELLER and GRISEBACH, 1980; FORKMANN and STOTZ, 1981; SPRIBILLE and FORKMANN, 1982; HAGMANN, HELLER and GRISEBACH, 1983). There are also reports that the 3', 4'-hydroxylated flavonoids are more effectively formed from caffeic acid than from *p*-coumaric acid (SÜTFELD, KEHREL and WIERMANN, 1978; KAMSTEEG, VAN BREDERODE and VAN NIGTEVECHT, 1980; SÜTFELD and WIERMANN, 1981). In this case the 3'- and 4'-hydroxylation represents the *meta* and *para* hydroxyl substitution present in caffeic acid. Observations such as these, which indicate that hydroxylation may occur subsequent to condensation of the aryl moieties, are a possibility for hypoxoside biosynthesis.

SATO and HASEGAWA (1972) propose that the natural dihydroxycoumarin, daphnetin, which has two adjacent hydroxyl groups is derived from *p*-coumaric acid in *Daphne odora* and that hydroxylation in the second position (the 6 position) occurs subsequent to the inclusion of the phenylpropanoid unit into the molecule. These authors obtained similar results for cinchorin biosynthesis in *Cichorium intybus* L..

BROWN (1985) substantiates the above authors' findings as far as the biosynthesis of the aglycone, cinchorin, in *Cichorium intybus* is concerned. This researcher states that the 6-oxygenation of the 6, 8 oxygenation of this dihydroxycoumarin takes place at the coumarin stage, rather than the cinnamic acid stage of biosynthesis.

If in fact, oxygenation on either of the aryl rings only takes place subsequent to linkage of these rings, then *p*-coumaric and caffeic acids would participate in hypoxoside biosynthesis through their conversion to *t*-cinnamic acid and possibly phenylalanine. In corms treated with *p*-coumaric acid conversion to *t*-cinnamic acid did occur

but only a small amount of radioactivity was detected in association with this acid. Compared to the other hydroxycinnamic acids applied *t*-cinnamic was rapidly depleted/converted in the system implying that this acid is highly active and metabolised rapidly by the plant. Thus the apparently minor conversion of *p*-coumaric acid to *t*-cinnamic acid may be an erroneous conclusion. The applied *p*-coumaric acid was also converted to caffeic acid but to a lesser extent than the reciprocal conversion. In addition in the caffeic acid treatment labelled *t*-cinnamic acid was also detected.

Thus the indications are that, in the *in vivo* situation *t*-cinnamic acid and phenylalanine are both precursors for hypoxoside but the former proved to be the most efficient. *p*-Coumaric and caffeic acids participated in this process but not at a level where the applied components could be considered to be precursors. However their poor participation in this process could be due to many factors, such as poor transport within the tissue, lack of a ready conversion to active forms as phenylalanine as *t*-cinnamic acid may be or their participation may have occurred via conversion to *t*-cinnamic acid. It is therefore obvious that the role played by *p*-coumaric and caffeic acids in the process of hypoxoside biosynthesis, is not clear.

CHAPTER 3

HYPOXOSIDE PRODUCTION: SITE, PRECURSOR MOBILITY AND SEASONAL INVESTIGATIONS

3.1 INTRODUCTION

Presently, reports only on the presence of hypoxoside in the rootstocks of *Hypoxis* species are available. MARINI-BETTOLO, PATAMIA, NICOLETTI, GALEFFI and MESSANA (1982) detected levels of up to 3.7 percent of the phytochemical in the rhizomes of *H. obtusa*. This was followed by reports of 3.5 to 4.5 percent of the compound as a constituent of *H. hemerocallidea* corms (DREWES, HALL, LEARMONTH and UPFOLD, 1984). The occurrence and concentration of this compound in other organs of *H. hemerocallidea* had not been investigated. Thus before postulations as to the possible site of hypoxoside biosynthesis could be made, it was obvious that its presence and quantity in the various organs of *H. hemerocallidea* had to be determined.

It is undoubtable that the greatest concentration of the compound is located in the corm and therefore, it was tempting to assume that this organ represents the site of synthesis. However, PAGE (1984) and PAGE and VAN STADEN (1987) detected hypoxoside only in those *in vitro* tissues that displayed some form of root differentiation. This observation led these authors to propose that hypoxoside biosynthesis may occur in root tissue. Such a proposal implies that synthesis would take place in one organ, the roots, followed by subsequent transport of the molecule to the site of storage, the corm. Since the presence and level of hypoxoside is not known for either the roots or

leaves, it is possible that either of these organs could serve as the site of synthesis, with the corm fulfilling a storage role only.

This situation has been observed for the biosynthesis of other secondary metabolites, such as the quinolizidine alkaloids, sparteine and lupanine in *Lupinus polyphyllus* Lindl.(WINK and HARTMANN, 1982). In this plant, the highest level of the alkaloids was located in the roots but the synthesis of the compounds occurred in the leaves from where the molecules were transported to the rest of the plant, particularly the roots where they were stored.

There are, however, other reports in the literature for the biosynthesis of various alkaloids, that locate site of synthesis in the organ of storage. Caffeine has been reported to be synthesised and stored in the flowers and in the pericarp and seed coat of fruit of *Camellia sinensis* L. (SUZUKI, 1985; SUZUKI and WALLER, 1985). Evidence also exists that closely associates the formation of caffeine with leaf development in *Coffea arabica* L. and *Camellia sinensis* (FRISCHKNECHT, ELLER and BRAUMANN, 1982; TSUSHIDA and TAKEO, 1979; ASHIHARA and KUBOTA, 1986) and also with fruit development (SUZUKI and WALLER, 1984; 1985). The bisbenzylisoquinoline alkaloids of *Stephania cepharantha* Hayata are found in the tubers of the plant and since root cultures of this plant are capable of producing five of these alkaloids it seems likely that this organ synthesises the phytochemicals in the whole plant (SUGIMOTO, SUGIMURA and YAMADA, 1988).

Biosynthesis of the anthocyanins present in *Petunia hybrida* Hort. flowers occurs *in situ* in the epidermal layers of the petals (JOHNSSON, DONKER-KOOPMAN and SCHRAM, 1984). In contrast, the

flavonoids present in the epidermal layers of *Avena sativa* L. leaves, are apparently synthesised in the mesophyll tissue and transported to the epidermal layers (KNOGGE and WEISSENBÖCK, 1986).

The secondary metabolites that appear to be structurally related to hypoxoside, the diarylpentane-type compounds, the diarylheptanoids, [*n*]-gingerols, the *meta*,*meta*-bridged biphenyls and the 9-phenylphenalenones, have not been specifically investigated with respect to biosynthetic site.

Only reports of the tissue from which the diarylpentane type compounds were extracted, exist. These include the location of hinokeresinol, agatheresinol and sugiresinol in the heartwood and bark of various members of the Coniferae (ENZELL and THOMAS, 1965; 1966; HIROSE, OISHI, NAGAKI and NAKATSUKA, 1965; ENZELL, HIROSE and THOMAS, 1967; ENZELL, THOMAS and WAHLBERG, 1967; BERACIERTA and WHITING, 1978). No mention of the compounds in other parts of the plant were made. The diarylpentane type compound (Z,Z)-4,4'(1,4-pentadiene-1,5-dyl)diphenol has been located in the wood of *Ginkgo biloba* L. (PLIENINGER, SCHWARZ, JAGGY, HUBER-PATZ, RODEWALD, IRNGARTINGER and WEINGES, 1986) and the rhizomes of *Alpinia galanga* Willd.(BARIK, KUNDU and DEY, 1987).

While the biosyntheses of some of the diarylheptanoids have been investigated, the actual location of these processes has not received attention. ROUGHLEY and WHITING (1973) extracted radiolabelled curcumin from the rhizomes of *Curcuma longa* L. when radiolabelled precursors were applied. The radiolabelled precursors were, however, applied via the wick method to the stem just above the rhizome and thus,it is possible that biosynthesis of curcumin may have occurred in the shoot, followed by transport to the rhizome. In *Acer nikoense*

Maxim. the biosynthesis of acerogenin A occurred in young excised shoots (INOUE, KENMOCHI, FURUKAWA and FUJITA, 1987). However, several diarylheptanoids have been isolated from the stem bark of *Acer nikoense*, including acerogenin A (NAGAI, KUBO, FUJITA, INOUE and MATSUO, 1978; KUBO, NAGAI and INOUE, 1983; INOUE, KENMOCHI, FURUKAWA and FUJITA, 1987). Thus it is not clear whether synthesis of the acerogenin A occurs in the bark or whether the phytochemical is laid down in the stem tissue during development.

The diarylheptanoids have been reported to occur in a variety of organs. A number of diarylheptanoids have been isolated from the wood of *Alnus japonica* Sieb. & Zucc. of the Betulaceae (NOMURA, TOKOROYAMA and KUBOTA, 1981) while the centrolobine diarylheptanoids have been found in the heartwood of *Centrolobium robustrum* Mart. and *Centrolobium tomentosum* Benth. (ARAGAO CRAVEIRO, DA COSTA PRADO, GOTTLEIB and WELERSON DE ALBUQUEQUE, 1970). Garuganin III, a diarylheptanoid type compound, was found in the leaves and bark of *Garuga pinnata* Roxbg.(MISHRA, HARIBAL and SABATA, 1985). RAVINDRANATH and SATYANARAYANA (1980) isolated dihydroxycurcumin from the rhizome of *Curcuma longa* but did not report on its presence elsewhere in the plant. Five diarylheptanoids have been found in the rhizomes of *Curcuma zanthorrhiza* Roxbg.(UEHARA, YASUDA, AKIYAMA, MORITA, TAKEYA and ITOKAWA, 1987) and another five from the male flowers of *Alnus sieboldiana* Matsum. (HASHIMOTO, TORI and ASAKAWA, 1986). The diarylheptanoid type compound 1-(4'-hydroxy-3'-methoxyphenyl)-7-phenyl-3-heptanone was discovered in the fruits of *Alpinia oxyphylla* Miquel. (ITOKAWA, AIYAMA and IKUTA, 1981). MALTERUD

and ANTHONSEN (1980) located [7.0]-metacylophane in the root bark of *Myrica nagi* Thunb. ex Murray.

DENNIFF, MACLEOD and WHITING (1980) have shown that radiolabelled precursors are incorporated into 6-gingerol in excised rhizome slices of *Zingiber officinalis* Roscoe, undoubtably proving the ability of this organ to synthesise the phytochemical. However, the ability of other tissues was not examined.

The *meta*, *meta*-bridged biphenyls, myricanone and myricanol, have been found in the stembark of *Myria nagi* (CAMPBELL, CROMBIE, TUCK and WHITING, 1970) but to date no reports on the distribution of these compounds throughout the rest of the plant or of the tissues where synthesis occurs, have been made.

THOMAS (1971) obtained incorporation of radiolabel into the 9-phenylphenalenone, haemocorin, when he incubated labelled precursors with rhizome slices of *Haemodorum corymbosum* Vahl. This organ also apparently, contains high levels of the compound but the biosynthetic potential of the other organs has not been investigated. Lachnanthoside, the 9-phenylphenalenone found in the roots, became labelled in these organs when labelled precursors were fed via the roots to the whole plant of *Lachnanthes tinctoria* (EDWARDS, SCHMITT and WEISS, 1972). While it is not clear whether the roots were the biosynthetic site callus cultures derived from stolons of this plant are capable of synthesising lachnanthoside (BEECHER, SARG and EDWARDS, 1983).

In investigating the possible biosynthetic site for hypoxoside in *H. hemerocallidea* plants, the location and quantity of the phytochemical

in the different organs was investigated. On the basis of this data the biosynthetic potentials of excised organs and eventually, organs of whole plants, were ascertained by feeding [U-^{14}C]phenylalanine followed by extraction for radiolabelled hypoxoside.

Once the organ of synthesis was ascertained, an anatomical study at both the light microscope and electron microscope level was initiated. With the establishment of the tissues in which hypoxoside biosynthesis occurs, the question of precursor availability arose. From the data presented in Chapter Two it was reasonable to assume that, since both phenylalanine and particularly *t*-cinnamic acid are good precursors for the phytochemical, it is a shikimate derivative. Thus the locality of the shikimate pathway within the plant will be an important factor affecting hypoxoside biosynthesis, since this is the pathway from which the precursors for this biosynthetic pathway are derived. This is especially pertinent for precursor access, particularly if this pathway is confined to one set of organs and hypoxoside biosynthesis to another.

Despite the importance of the aromatic amino acids, the intracellular compartmentation of this pathway has not been clarified. The location of this pathway, within the cell, will clearly decide in which tissues and therefore in which organs, the pathway will be predominantly operative.

It has been proposed that the entire shikimate pathway, from carbon dioxide fixation to the end products, the aromatic amino acids, is located within plastids, particularly chloroplasts. This proposal is the result of investigations performed with isolated spinach chloroplasts (BICKEL and SCHULTZ, 1979; SCHULZE-SIEBERT, HEINEKE,

SCHARF and SCHULTZ, 1984, FIEDLER and SCHULTZ, 1985). While this pathway is irrefutably present in the chloroplasts (BICKEL, PALME and SCHULTZ, 1978; FIEDLER and SCHULTZ, 1985; MOUSDALE and COGGINS, 1985) it appears to be dependent on cytoplasmically derived phosphoenolpyruvate for its operation and it is unlikely that the chloroplasts are the only locality of the pathway within cells.

Other studies have shown that while chloroplast-derived aromatic amino acids are present in the cell, they do not account for the total amount of aromatic biosynthesis that occurs within the cell (BUCHHOLZ, REUPKE, BICKEL and SCHULTZ, 1979). Thus the suggestion has been made that isozymic enzymes of the skikimate pathway exist with different subcellular localisations (JENSEN, 1986) so that the pathway is present in its entirety in the cytosol as well as in the plastids. The indications are that the plastidial isozymes are subject to feedback inhibition by the end products of the pathway, L-phenylalanine, L-tyrosine and L-tryptophan (GOERS and JENSEN, 1984, RUBIN and JENSEN, 1985). The control of the cytosolic isozymes is not as rigid, however, as the enzymes of this pathway are apparently less sensitive to feedback inhibition by the end products (CARLSON and WIDHOLM, 1978; GOERS and JENSEN, 1984).

These observations have caused JENSEN (1986) to postulate that the less tightly regulated cytosolic shikimate enzymes supply aromatic precursors, in an overflow type mechanism, to secondary metabolism. Secondary metabolism, he argues further, would thus be dependent on the supply of erythrose-4-phosphate and phosphoenolpyruvate, from cytosolic carbohydrate metabolism, to the cytosolic shikimate pathway

and on the competition with protein synthesis for aromatic amino acid substrates.

The proposed existence of a plastidically localised shikimate pathway and separate cytosolic pathway, with the latter providing precursors for secondary metabolism, is a hypothesis which is not favoured by BAGGE and LARSSON, (1986). They suggest, on the basis of enzyme localisation work (FIEDLER and SCHULTZ, 1985), that the chloroplasts are the sole locality in which aromatic amino acid biosynthesis occurs in photosynthetic tissue. Furthermore, these authors claim that the rapid exportation of aromatic amino acids synthesised in isolated chloroplasts, to the experimental medium, in their studies and those of BICKEL, PALME and SCHULTZ (1978), lend support to their hypothesis. They reason that, in the experimental situation, this rapid transport of the aromatic amino acids out of the chloroplasts, is brought about by the relatively large volume of the incubation medium, which would be replaced in the *in vivo* situation by the sink effect of secondary metabolism. Since the aromatic amino acids can be rapidly transported out of the chloroplasts, BAGGE and LARSSON (1986) believe that they are the sole source of substrates for cytosolic based secondary metabolism. These authors do concede, however, that the evidence provided by JENSEN (1986) for plastidic and cytosolically separate pathways is convincing and that it is probable that both pathways exist.

The majority of the above studies have used species of plants such as spinach (*Spinica oleracea* L.) pea (*Pisum sativum* L.) tobacco (*Nicotiana silvestris* Spegaz. & Comes.) and *Vigna radiata* (L). Wilczek from which chloroplast isolation is relatively easy. JENSEN (1986) points out that while the details may be different, the basic

biosynthetic pathways of primary metabolisms are similar in all plants. Thus, it may well be possible that the plastidic and cytosolic pathways of aromatic acid biosynthesis, are present in *H. hemerocallidea*.

As mentioned earlier, the first committed step into secondary metabolism occurs when the aromatic amino acids are converted, by phenylalanine ammonia lyase, to cinnamic acid. The cinnamic acid hydroxylases are responsible for further conversions to the hydroxycinnamic acids. The subcellular localisation of these processes has not received much attention in the literature. STAFFORD and LEWIS (1977) showed that in spinach leaf extracts, phenylalanine was converted mainly to phenylacetic acids and some cinnamic acid. Subsequent to this, CZINCHI and KINDL (1977) located phenylalanine ammonia lyase and cinnamic acid hydroxylases on the microsomal membranes of cells in cucumber cotyledons. The cinnamic acid hydroxylases have thus far been isolated predominantly at membranes (CZINCHI and KINDL, 1975, 1977), while phenylalanine ammonia lyase activity has been attributed mainly to the cytosol, although reports have appeared which locate this enzyme in microsomes, chloroplasts and microbodies (ARMHEIN and ZENK, 1971, RUIS and KINDL, 1971, WEISSENBÖCK, 1975, GREGOR, 1976). BITSCH, TRIHBES and SCHULTZ (1984), from studies made with chloroplast isolates and spinach leaf extracts, claim that the subsequent conversions of phenylalanine to cinnamic acid and the hydroxycinnamic acids, are processes which are extraplastidic.

The evidence thus presented for the location of the shikimate pathway and the activities of phenylalanine ammonia lyase and cinnamic acid hydroxylase, make it apparent that the subcellular localisation of

these pathways is not completely elucidated and thus it is presumptuous to confine them to either the plastids or the cytosol until more evidence is available. The data from the literature suggests that the skikimate pathway is predominant in photosynthetic tissue, however.

It seems possible that most of the synthesis of the aromatic precursors for hypoxoside, may occur in the leaves of the plants. Thus two aspects of precursor availability had to be investigated. Firstly, the production of shikimate derivatives by the leaves and secondly the form in which these compounds were transported to the site of synthesis. These areas of precursor production and transport were examined in the following ways:

(i) The potential of the possible precursors to become radioactively labelled in different parts of the plant was examined when plants were exposed to ^{14}C-carbon dioxide.

(ii) The potential of possible precursors to be exported to different parts of the plant was studied when these compounds were applied via the aerial parts of the plant.

If the leaves are the primary source of precursor, then the dying back of these parts during a two month dormancy period in winter (WOOD, 1976, HEIDEMAN, 1979), would be expected to cause fluctuations in the levels of hypoxoside production. Thus a seasonal study was initiated where the organs containing hypoxoside, were collected at monthly intervals and assessed for hypoxoside content.

This was carried out for two reasons:

(i) to ascertain the best stage of growth at which corms should be harvested and

(ii) to discover the relationship between hypoxoside production and the pattern of growth of the plants.

3.2 MATERIALS AND METHODS

3.2.1. SITE OF SYNTHESIS

3.2.1.1. The detection and quantification of hypoxoside in the various organs of *H. hemerocallidea*

Plant material

Clonal plants of *H. hemerocallidea*, established according to the method of PAGE (1984) as described in 2.2.2.1 were used. The plants were nine months old and had been maintained under the environmental conditions of Pietermaritzburg prior to harvest at the end of March. Upon harvesting material was divided into leaves, roots and corms, flash frozen with liquid nitrogen, freeze dried for 24 hours, ground to homogeneous powders and stored at -20^{o}C until analysed.

Detection of hypoxoside

From each of the freeze dried and powdered leaves, roots and corms, 0.05 g of material was extracted for hypoxoside. This extraction and subsequent separation of the extracts on TLC and HPLC was performed as described in 2.2.2.1. From each 100 μl of extract prepared 10 μl was separated by HPLC.

Quantification of hypoxoside

For the quantification of hypoxoside, 0.05 g (dry mass) of the homogeneously powdered leaves, roots and corms, was extracted for hypoxoside and separated on TLC as described in 2.2.2.1. Material was taken from three plants in order to provide three replicates. The eluants from TLC were then used to quantify hypoxoside as described in

2.2.2.1 with the exception that, the corm eluants were diluted ten times in order that the levels should fall within the sensitivity range of the calibration curve. The percentage hypoxoside in each set of organs was derived as a mean of the three replicates and 95 percent confidence limits were determined.

The results from this quantification of hypoxoside, implicated the roots and/or corm as a possible site/s for hypoxoside biosynthesis and thus investigations with excised roots and corms were attempted, in order to determine the role of these organs in hypoxoside biosynthesis.

3.2.1.2. Application of ^{14}C-phenylalanine to excised roots and excised corms

Plant material

An 18 month old clonal plant, initiated and hardened off according to PAGE (1984) as described in 2.2.2.1, was used. Six months before use the plant was grown under the following constant conditions; temperature, 23 ± 2 ^{o}C, light intensity 55 μE m^{-2} s^{-1} and a photoperiod of 16 hours.

Application of radioactivity

The contractile roots were excised from the plant and sterilised under aseptic conditions as follows: 0.1 percent benzimidazole (benomyl), 2 minutes; 95 percent ethanol, 2 minutes; 0.1 percent mercuric chloride, 10 minutes. The sterilisation was followed with one 30 minute and two 10 minute washes in sterile distilled water. The roots were then cut into 1 cm lengths.

The excised corm was cut into slices, sterilised and washed as described in section 2.2.1.1 where corms were used as an explant source. After washing, dead and damaged tissue was removed and the corm slices were cut into 0.5 cm^3 pieces.

The prepared excised roots (4.28 g) and corm (5.68 g) were placed into 50 ml phosphate buffer (pH 5.5) containing 5.5 x 10^6 dpm and 13.8 x 10^6 dpm L(U-^{14}C)phenylalanine (spec. act. 18.98 GBq $mmol^{-1}$) respectively. The buffer, in each case, was contained in 100 ml conical flasks and was autoclaved at 121 ^oC and one bar pressure for 20 minutes before the filter sterilised radioactive precursor was added in a 1 ml volume of distilled water. Transferring of the excised organs to the treatment solution was done under aseptic conditions on a laminar flow bench.

The excised material was incubated on a shaker at 23 ± 2 ^oC in the dark for 7 days. At the end of this period the plant material was collected, washed with distilled water, flash frozen with liquid nitrogen, freeze dried for 24 hours, ground to a homogeneous powder and stored at -20 ^oC.

Extraction, HPLC separation and assay for incorporation of radioactivity into hypoxoside

From the freeze dried corm (2.10 g) and root material (0.53 g) , 0.5 g of material was extracted for hypoxoside as described in section 2.2.1.1. From the filtered extracts, 1 ml was withdrawn and assayed for radioactivity with 4 ml Beckman Ready-Solv scintillant and a Beckman LS 3200 scintillation counter. Extracts were scrutinised for peaks of radioactivity co-eluting with the retention time of authentic hypoxoside.

Extraction, HPLC separation and assay for radioactivity incorporated into cinnamic acid and the hydroxycinnamic acids

From the freeze dried root and corm material 0.5 g amounts were extracted for cinnamic acid and the hydroxycinnamic acids as described in 2.2.1.3. The extracts were purified with paper chromatography and analysed with HPLC as outlined in 2.2.1.3. Fractions of 1.5 ml were collected during the HPLC separations and these were assayed for radioactivity with 4 ml Beckman Ready-Solv liquid scintillant using a Beckman LS 3200 scintillation counter. The presence of radioactive peaks co-eluting with authentic *t*-cinnamic acid and the hydroxycinnamic acids was noted for each extract.

3.2.1.3. Investigations of biosynthetic site for hypoxoside using intact plants of *H. hemerocallidea*

As a result of the lack of success in attempting to use excised organs in order to determine the site of hypoxoside biosynthesis, investigations with intact plants were undertaken.

Plant material

Twelve-month old clonal plants were used. These were initiated and hardened off according to the method of PAGE (1984) as described in 2.2.2.1. Prior to use plants were maintained under the following conditions: 23 ± 2 ^{o}C, light intensity 55 μE m^{-2} s^{-1} and a photoperiod of 16 hours.

Application of ^{14}C-phenylalanine

The L(U-^{14}C)phenylalanine was applied to three plants by inserting the needle (0.45 mm) of a micro syringe containing the radioactivity into a 0.88 mm needle that had been cut to a 2 cm length and inserted into

the shoot apices of the plants (Figure 2.2.12). The plants were fed 1.11×10^6 dpm L($U^{14}C$)phenylalanine (spec. act. 18.98 GBq $mmol^{-1}$) in a 10 μl volume at weekly intervals. Plants were maintained under the following conditions for the duration of the experiment: temperature, 23 ± 2 ^oC; light intensity, 55 $\mu E\ m^{-2}\ s^{-1}$; 16 hour photoperiod. A plant was harvested at each of the following time intervals; 2, 4 and 8 weeks after the first application of radioactive precursor. Upon harvesting plants were divided into leaves, roots and corms, flash frozen with liquid nitrogen and freeze dried for 24 hours. Material was subsequently ground to a homogeneous powder and stored at -20 ^oC.

Extraction, HPLC separation and assay for radioactivity incorporated into hypoxoside in each organ

Half the dried mass of the leaves (0.32 g), roots (0.43 g) and corm (0.64 g) of plants from each time interval was extracted and 1 ml aliquots of the filtered extracts were assayed for radioactivity as described in section 3.2.1.2. The extracts were subsequently separated with TLC and analysed with HPLC. The procedures for extraction, TLC separation and HPLC analysis were performed according to the methods described in section 2.2.1.1. Fractions of 1.5 ml were collected from the HPLC separation and assayed for radioactivity. Radioactive peaks co-eluting with hypoxoside were noted.

Extraction, HPLC separation and assay for radioactivity incorporated into cinnamic acid and the hydroxycinnamic acids in each organ

The remaining halves of the dry mass of the leaves (0.32 g), roots (0.43 g) and corm (0.64 g) for plants from each time interval were extracted for cinnamic acid and the hydroxycinnamic acids. Extracts were separated by means of paper chromatography and then analysed with HPLC. The extraction, paper chromatography and HPLC separation were

performed as described in 2.2.1.3. Fractions of 1.5 ml were collected after separation by HPLC and these were assayed for radioactivity. Radioactive peaks co-eluting with the authentic standards were noted.

3.2.1.4. **Biosynthetic potential of various regions of the corm**

Since data obtained for experiment 3.2.1.3 indicated that the corm was the major site for hypoxoside biosynthesis, the biosynthetic potential of this organ was further investigated. Anatomical studies carried out by PAGE (1984) showed that transversely the corm consisted of two regions; an inner pith region surrounded by a cambium and an outer cortical region bound by a suberised epidermis. It was shown that hypoxoside was more abundant in the upper region of the corm than the lower region. Thus four regions of the corm were delineated, an upper inner pith region, an upper outer cortical region, a lower inner pith region and a lower outer cortical region (Figure 3.2.1). The biosynthetic potential of each of these regions was investigated.

Plant material

Eighteen-month old clonal plants initiated as described by PAGE (1984)(Section 2.2.2.1) were maintained under the following conditions until used: temperature 23 ± 2 ^{o}C, light intensity 55 μE m^{-2} s^{-1} and a photoperiod of 16 hours.

Application of ^{14}C-phenylalanine

The L(U-^{14}C)phenylalanine was administered to whole plants using the shoot apical method of application described in section 3.2.1.3 (Figure 2.2.12). The plants were fed 11.1 x 10^{6} dpm L(U-^{14}C)phenylalanine (spec. act. 18.98 GBq mmol^{-1}) in a 10 μl volume at weekly intervals for 8 weeks, during which time they were maintained

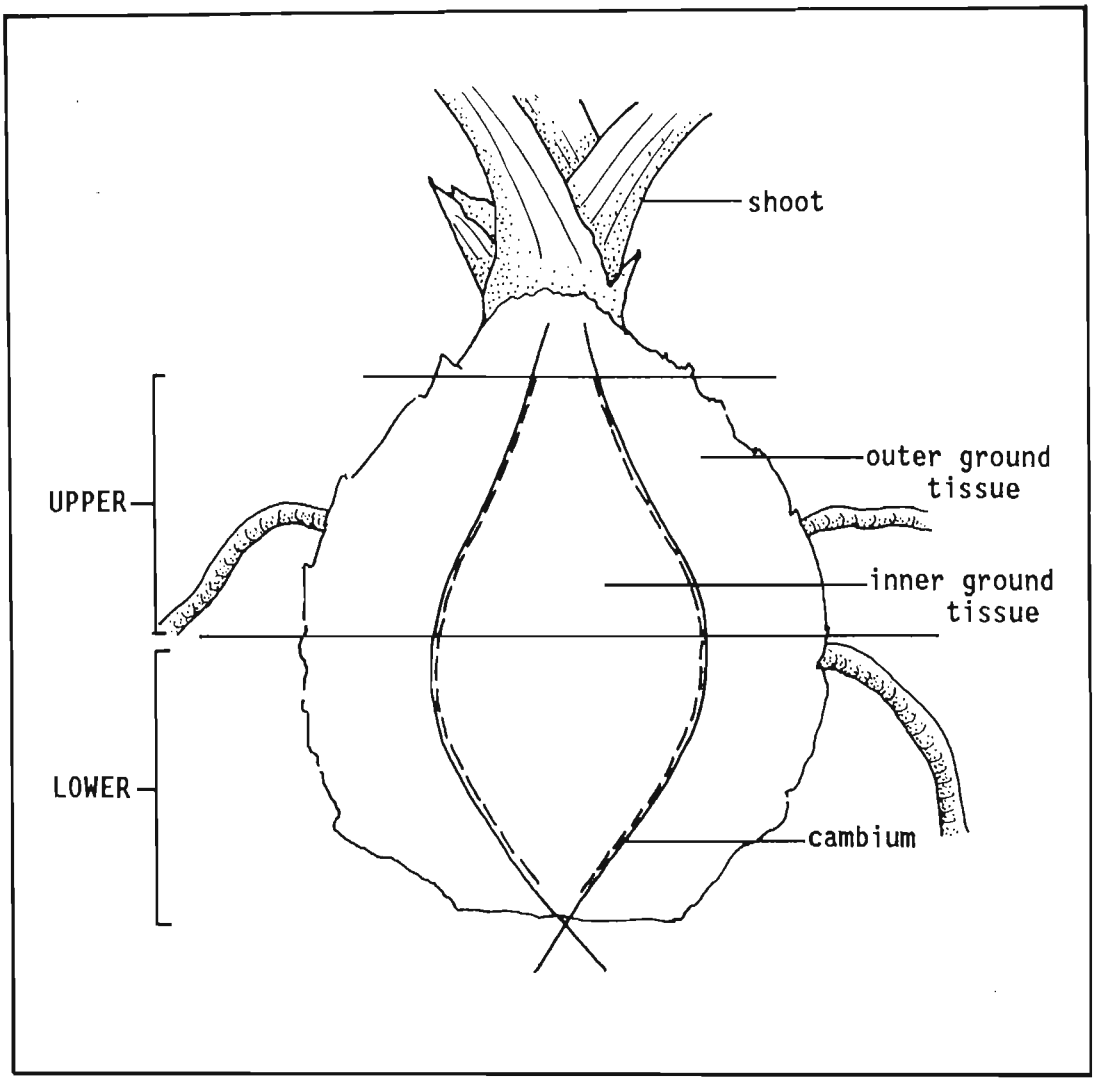


Figure 3.2.1: Diagrammatic representation of the delineation of corms of *H. hemerocallidea* into four regions; upper inner and outer regions, and lower inner and outer regions.

Figure 1.1: Morphology of *H. hemerocallidea* Fisch. & Mey.

under the following conditions: temperature 23 ± 2 ^{o}C, light intensity 55 μE m^{-2} s^{-1}, 16 hour photoperiod. Plants were harvested at 2,4 and 8 weeks after the first application. Three plants were used as replicates for each time interval. Upon harvesting, the corm of each plant was divided into upper and lower halves and then each half was divided into an inner and outer region using the cambium (Figure 3.2.2) as the dividing line. The four regions were then flash frozen with liquid nitrogen, freeze dried for 24 hours and stored at -20 ^{o}C until extraction.

Extraction, HPLC separation and assay for radioactivity incorporated into hypoxoside in the various corm regions

Half of the ground dried mass for each corm region at each time interval was extracted for hypoxoside. Aliquots of 1 ml from the filtered extracts were assayed for radioactivity. Extracts were separated with TLC and analysed with HPLC. The extraction procedure and methods for TLC and HPLC separation are described in 2.2.1.1. Fractions of 1.5 ml were collected from the HPLC separations and assayed for radioactivity as previously described (2.2.1.2). Peaks of radioactivity co-eluting with authentic hypoxoside were noted, and used to calculate the amount of radioactivity associated with hypoxoside in each region at each time interval. Values were obtained as a mean of three replicates.

Extraction, HPLC separation and assay for radioactivity incorporated into cinnamic acid and the hydroxycinnamic acids in various regions of the corm

The remaining half of the ground dry mass collected for each corm region at each time interval, was extracted, separated with paper chromatography and analysed by HPLC. The procedures for the

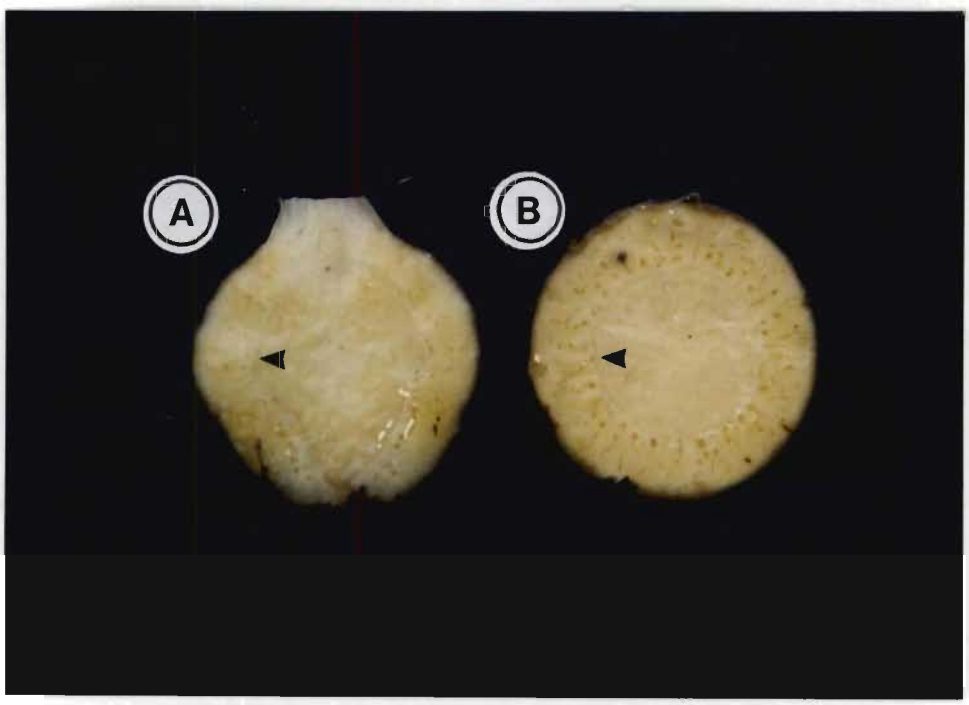


Figure 3.2.2: Longitudinal (**A**) and transverse (**B**) sections through an 18-month old corm of *H. hemerocallidea*, showing the position of the cambium used to divide the corm into quarters, for the study of the biosynthetic potential of various regions of the corm.

extractions, paper chromatographic and HPLC separations of the cinnamic acid and hydroxycinnamic acids are given in section 2.2.1.3. Fractions of 1.5 ml were collected from the HPLC separations and assayed for radioactivity as described in 2.2.1.2. Peaks of radioactivity co-eluting with the authentic standards, *t*-cinnamic acid, *p*-coumaric acid, caffeic, ferulic and sinapic acids were noted, and used to calculate the amount of radioactivity associated with the hydroxycinnamic acids at each time interval. Three replicates were used for each corm region.

3.2.1.5. Anatomical studies of the corm

Light Microscopy

Although PAGE (1984) examined the anatomy of the corm at the light microscope level, only transverse sections were made and it is not clear whether these were from the upper or lower regions of the corm. Thus a more extensive investigation of the anatomy of this organ was undertaken.

Plant material: Fifteen-week old clonal plants, propagated according to PAGE (1984)(Section 2.2.2.1), were the source of corm material. The corms were well washed after removal of roots and leaves.

Procedure: The young corms were quartered by division once longitudinally and once transversely (Figure 3.2.3). Corm specimens were immediately fixed in FAA (37 percent formaldelyde: acetic acid: 96 percent ethanol: water; 2: 1: 10: 7, volume for volume) at 23 ± 2 ^{o}C for a minimum time of 8 hours. Dehydration and wax embedding followed the procedure outlined in Table 3.2.1. Once material was wax infiltrated it was embedded into wax molds. Wax blocks were mounted onto wooden blocks and sections of a 10 μm thickness were produced with a Jung hand-microtome. The wax ribbons

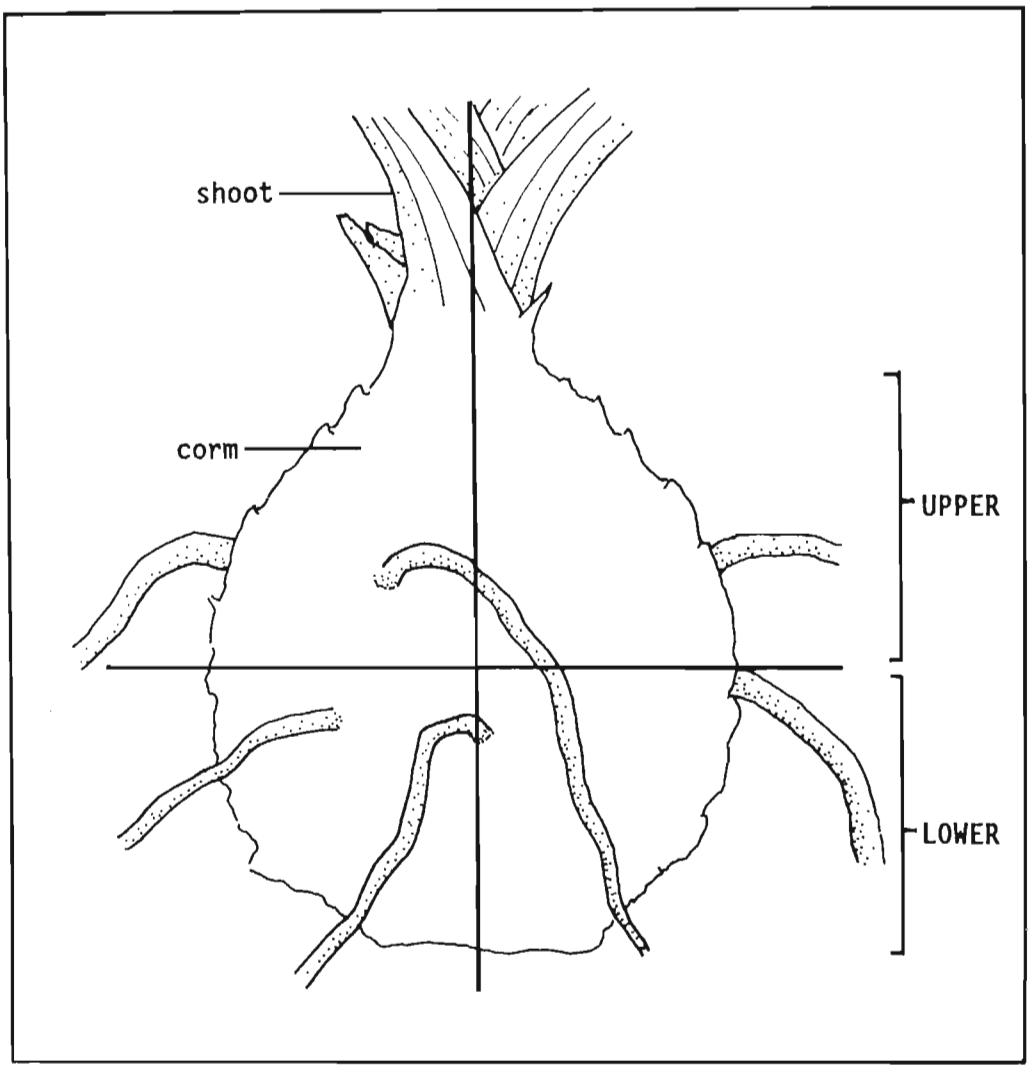


Figure 3.2.3: Diagrammatic representation of the division of 15-week old corms into four regions for light microscopic studies.

Table 3.2.1: The tertiary-butanol dehydration method and procedure used for wax infiltration of corm material of *H. hemerocallidea*

PROCEDURE	TREATMENT SOLUTION	COMPOSITION %	MINIMUM TIME h	TEMPERATURE °C
Dehydration				
I	*t*-butanol	20	24	23±2
	95% ethanol	50		
	water	30		
II	*t*-butanol	35	4	23±2
	95% ethanol	50		
	water	15		
III	*t*-butanol	55	4	23±2
	95% ethanol	40		
	water	15		
IV	*t*-butanol	75	4	23±2
	100% ethanol	25		
V	*t*-butanol	100	4	40
VI	*t*-butanol	100	4	40
Embedding				
VII	*t*-butanol	50	48h	40
	liquid paraffin	50		
VIII	liquid paraffin		24h	60
IX	liquid paraffin * + wax pellets		24h	60
	* CAPS OF VIALS REMOVED			
X	Pure molten wax		48h	60

produced were mounted onto glass slides with HAUPT'S (1930) adhesive prior to staining with safranin and fast green (JENSEN, 1962), and clearing of the wax with xylene. Sections were then mounted in Depex Mounting medium with coverslips and viewed with an Olympus BH2 light microscope.

Electron Microscopy

These studies were attempted in order to elucidate intracellular distribution of the phenolic compounds within the corm tissue. Since hypoxoside appears to be the major phenolic in the corm, it was assumed that most of the phenolic material detected at the electron microscope (EM) level would be representative of hypoxoside distribution. Because leaching of phenolics from vacuole into the cytoplasm during fixation is a common problem (MUELLER and BECKMAN, 1974), fixation of material in caffeinated glutaraldehyde (MUELLER and GREENWOOD, 1978) was attempted. However, this did not prevent leaching of hypoxoside, thus, the following procedure was devised:

Plant material: Corms of 18-month old clonal plants (obtained according to PAGE (1984)) were excised and dissected under 2 percent osmium tetroxide into 2 mm^3 blocks.

Procedure: The corm blocks were fixed in 2 percent osmium tetroxide, buffered with 0,05 M sodium cacodylate buffer (pH 7.2), for 2 hours. Two 30 minute washes in 0.05 M sodium cacodylate buffer followed. Material was then dehydrated by passage through an ethanol series where material remained for 15 minutes sequentially in the following solutions: 10 percent ethanol, 25 percent ethanol, 50 percent ethanol, 75 percent ethanol and absolute ethanol. During the 75 percent ethanol dehydration, material was simultaneously stained with 1 percent uranyl acetate. After dehydration the material was impregnated with propylene oxide, followed by gradual impregnation

with Spurrs resin over a 72 hour period. The resin was polymerised at 70 ^{o}C for 12 hours.

Specimens were cut out of the resin wafer, fixed to resin stubs and trimmed to the required size. Glass knives made on a LKB knifemaker (Type 7801B) were used to cut initially semi-thin sections (2 μm) and finally ultrathin sections (0.05 μm). Sectioning was carried out with a Reichert Jung Ultracut E microtome. The ultrathin sections were mounted on 300 mesh Gilder copper grids, restained with uranyl acetate (20 minutes in the dark), rinsed and stained with lead citrate (15 minutes) in the presence of sodium hydroxide pellets (REYNOLDS, 1963).

Sections were viewed with the aid of a JEOL 100CX electron microscope at an accelerating voltage of 80 kV.

3.2.2. TRANSPORT AND PRODUCTION OF POTENTIAL PRECURSORS

3.2.2.1. The detection and quantification of the hydroxycinnamic acids in various organs of *H. hemerocallidea*

Plant Material

Nine-month old clonal plants established as described earlier (section 2.2.2.1) were potted out and maintained under natural conditions until harvesting at the end of March. Plants were divided into leaves, roots and corms. Each portion was flash frozen with liquid nitrogen and freeze dried for 24 hours. Prior to storage at -20 ^{o}C material was milled to a homogeneous powder. Three plants were used as replicates.

Detection of cinnamic acid and the hydroxycinnamic acids

From the freeze dried leaves, roots and corms, 0,25 g of material was extracted and in each case was separated with paper chromatography and analysed by HPLC as described in section 2.2.1.3. UV absorbent peaks at 265 nm that co-eluted with the authentic standards *t*-cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid were noted.

Quantification of the hydroxycinnamic acids

A second set of extractions from the leaves, roots and corms of the three plants, using 0,25 g of material, was carried out. After separation with paper chromatography, quantification of the hydroxycinnamic acids was made according to the method described in section 2.2.1.3. A mean percentage for each hydroxycinnamic acid, for each organ, was calculated from three replicates at a 95 percent confidence level.

3.2.2.2. Fixation and incorporation of ^{14}C-carbon dioxide into hydroxycinnamic acids by intact *H. hemerocallidea* plants

Plant Material

Twelve-month old plants, which were initiated from shoot-producing callus (PAGE, 1984), were used. Prior to use the plants were maintained under the following conditions for 2 months: temperature, 23 ± 2 ^{o}C; light intensity, 55 μE m^{-2} s^{-1}; 16 hour photoperiod.

Application of $^{14}CO_2$ to plants

Three plants were placed into four bell jars as illustrated in (Figure 3.2.4). The bell jars were sealed firmly onto glass plates with petroleum jelly. The radioactivity was introduced when 11.1 x 10^{6} dpm

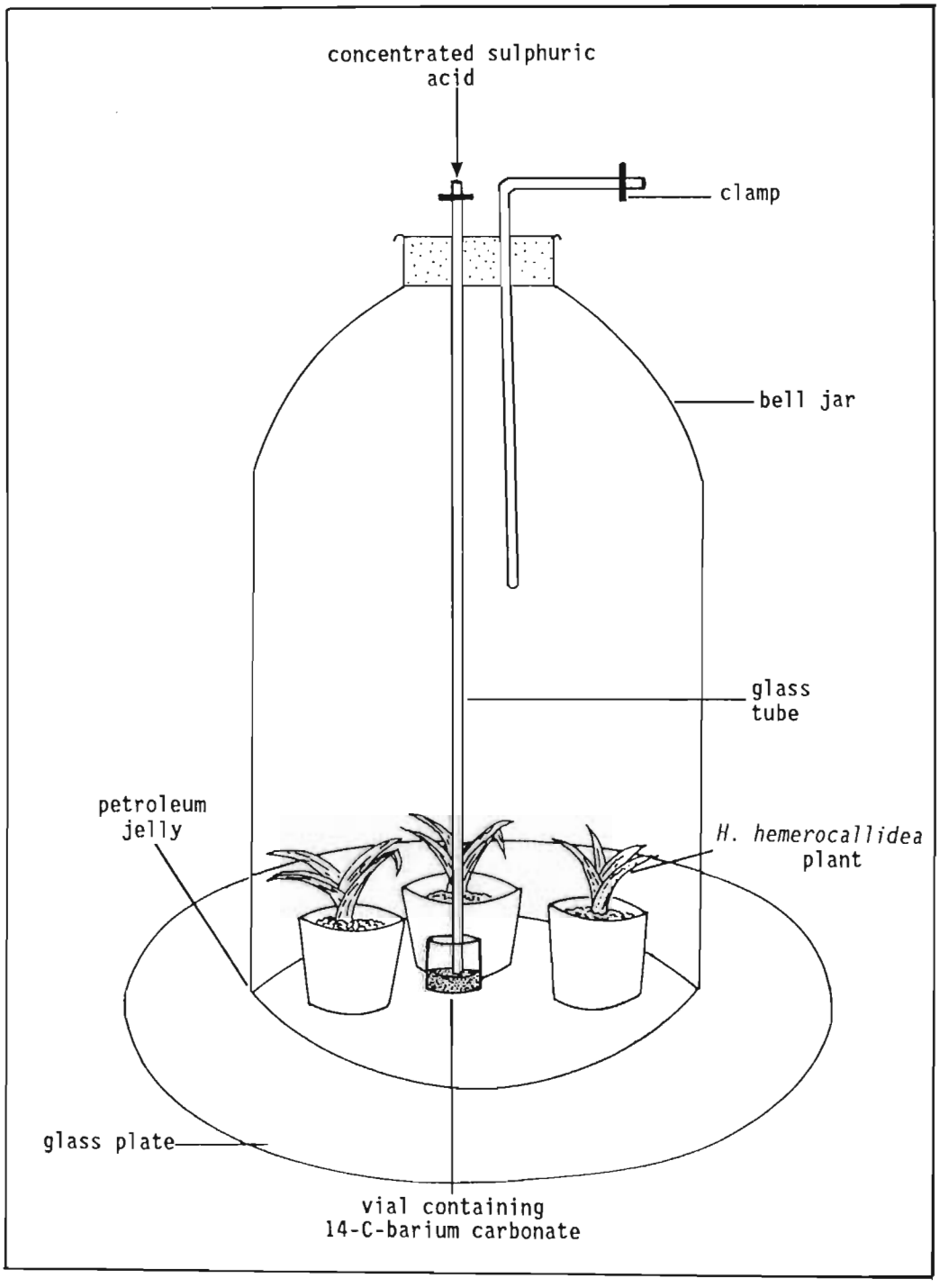


Figure 3.2.4: The apparatus used to treat 12-month old plants of *H. hemerocallidea* with ^{14}C-carbon dioxide.

^{14}C-barium carbonate (spec. act. 2.15 GBq $mmol^{-1}$)mixed with 1 mg of unlabelled barium carbonate was exposed to concentrated sulphuric acid, releasing $^{14}CO_2$ into each jar. Plants were fed the $^{14}CO_2$ 4 hours after the beginning of the light period. Plants were maintained in the bell jars under the conditions already described. After 6, 24, 96 and 192 hours the plants were harvested, for each time period, from one bell jar by dividing the plants into leaves, roots and corms, flash freezing each component with liquid nitrogen and freeze drying for 24 hours . After milling, the material was stored at -20 ^{o}C.

Extraction, HPLC separation and assay for radioactivity incorporated into cinnamic acids and the hydroxycinnamic acids

The freeze dried and milled organs of the plants from each time interval (0.1g) were extracted for the hydroxycinnamic acids. One milliliter of each extract was assayed for radioactivity. The extracts were separated on paper and analysed by HPLC as described in section 2.2.1.3. Fractions of 1.5 ml were collected from the HPLC separation and assayed for radioactivity as described in 2.2.1.2. Peaks of radioactivity co-eluting with the authentic standards, *t*-cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid were quantified as a percentage of the total radioactivity recovered from each plant. Three replicates were used for each treatment.

3.2.2.3. The application of ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid to intact plants of *H. hemerocallidea*

Plant Material

Eighteen-month old plants, obtained from shoot-producing callus according to the method described by PAGE (1984) (section 2.2.2.1), were used. Plants were maintained under the following conditions: temperature, 23 ± 2 ^{o}C, light intensity 55 μE m^{-2} s^{-1}; photoperiod 16 hours.

Application of radioactive compounds

The radiolabelled compounds were applied to the shoot apices of the intact plants using the method described in section 3.2.1.3 (Figure 2.2.12). The following quantities of radioactivity were applied: 1.11 x 10^{6} dpm L(U-^{14}C)phenylalanine (spec. act. 18.98 GBq mmol^{-1}), 1.11 x 10^{6} dpm (3-^{14}C)*t*-cinnamic acid (spec. act. 1.77 GBq mmol^{-1}), 1.11 x 10^{6} dpm (2-^{14}C)*p*-coumaric acid (spec. act. 3.84 MBq mmol^{-1}) and 0.47 x 10^{6} dpm (1-^{14}C)caffeic acid (spec. act. 1.49 MBq mmol^{-1}). In each case the radioactivity administered was contained in a 10 μl volume. Three plants were used per treatment.

The above quantities of radioactivity were applied to plants at weekly intervals for 8 weeks. The experiment was terminated with the harvesting of the plants by dividing these into leaves, root and corms and flash freezing the components with liquid nitrogen. Plant material was freeze dried for 24 h, milled and stored at -20 ^{o}C.

Extraction, HPLC separation and assay for radioactivity incorporated into cinnamic acid the hydroxycinnamic acids

Half of the freeze dried material collected from the leaves (±1.0 g), roots (±1.0 g) and corms (±1.25 g) of the plants for each treatment was extracted for the hydroxycinnamic acids and separated using paper chromatography followed by HPLC as described in section 2.2.1.3. Fractions of 1.5 ml were collected from the HPLC separation and were assayed for radioactivity as described in 2.2.1.2.

Peaks of radioactivity co-eluting with the authentic standards were noted.

3.2.3. SEASONAL FLUCTUATIONS IN HYPOXOSIDE

The seasonal fluctuations in the hypoxoside concentration in the upper and lower halves of the corms over a 12 month period were monitored.

Plant Material

Six-month old plants, propagated from shoot-producing callus (PAGE, 1984) as described in section 2.2.2.1, were transplanted into sand in plastic pots and transferred to environmental conditions at the end of October. Since plants dehydrate rapidly in plastic pots compared to those planted in the ground, a weekly watering was given to supplement moisture derived from rainfall. Watering was stopped from the end of May to mid-August when plants were dormant. From the end of February (4 months after the plants were potted out), three randomly selected plants, were harvested at the end of each month for 12 months. At harvesting plants were divided into leaves, roots and corms, divided into upper and lower halves, the material flash frozen with liquid nitrogen and freeze dried for 24 h. Dry masses of plants were

recorded. Plant material was then milled and stored at -20 ^{o}C until further analysis.

Extraction and quantification of hypoxoside

Freeze dried material (0.05 g) from the upper and lower halves of corms from plants for each month was extracted for hypoxoside and separated on TLC as described in section 2.2.2.1. The eluants from TLC were then used to quantify hypoxoside as described in 2.2.2.1 with the exception that the corm eluants,in this case, were diluted by a factor of ten, in order that the levels should fall within the sensitivity range of the calibration curve. Material from three plants was analysed at each monthly interval to provide replicates for the derivation of the mean percentage hypoxoside for the roots, upper and lower corms. Ninety-five percent confidence limits were calculated.

3.3. RESULTS

3.3.1. SITE OF SYNTHESIS

3.3.1.1. The detection and quantification of hypoxoside in various organs of *H. hemerocallidea*

Upon HPLC separation of the extracts from all of the organs, leaves, roots and corm, peaks of UV absorbance at 257 nm were detected at the retention time of hypoxoside (Figure 3.3.1). However, in the case of the leaves this peak was small and indistinct (Figure 3.3.1 B), becoming better defined and pronounced in the extract from the root (Figure 3.3.1 C). The UV absorbent peak coinciding with the retention time of hypoxoside in the corm, was large and well defined (Figure 3.3.1 D). When the levels of these compounds present in the leaves, roots and corms, with similar retention times to hypoxoside, were determined it was found that the corm contained approximately 20 times more hypoxoside than the roots (Table 3.3.1). The level in the leaves, however, was too low to be quantified with the *p*-nitroaniline reagent.

Thus both the roots and corms contained hypoxoside, with the latter organ being very much richer in the compound than the former. Hence, both were investigated for biosynthetic potential.

3.3.1.2. Application of ^{14}C-phenylalanine to excised roots and corm

The roots and corm took up the applied radioactivity to approximately the same extent. In both cases the uptake was relatively poor (Table 3.3.2). When the hypoxoside extracts were separated with HPLC (Figure

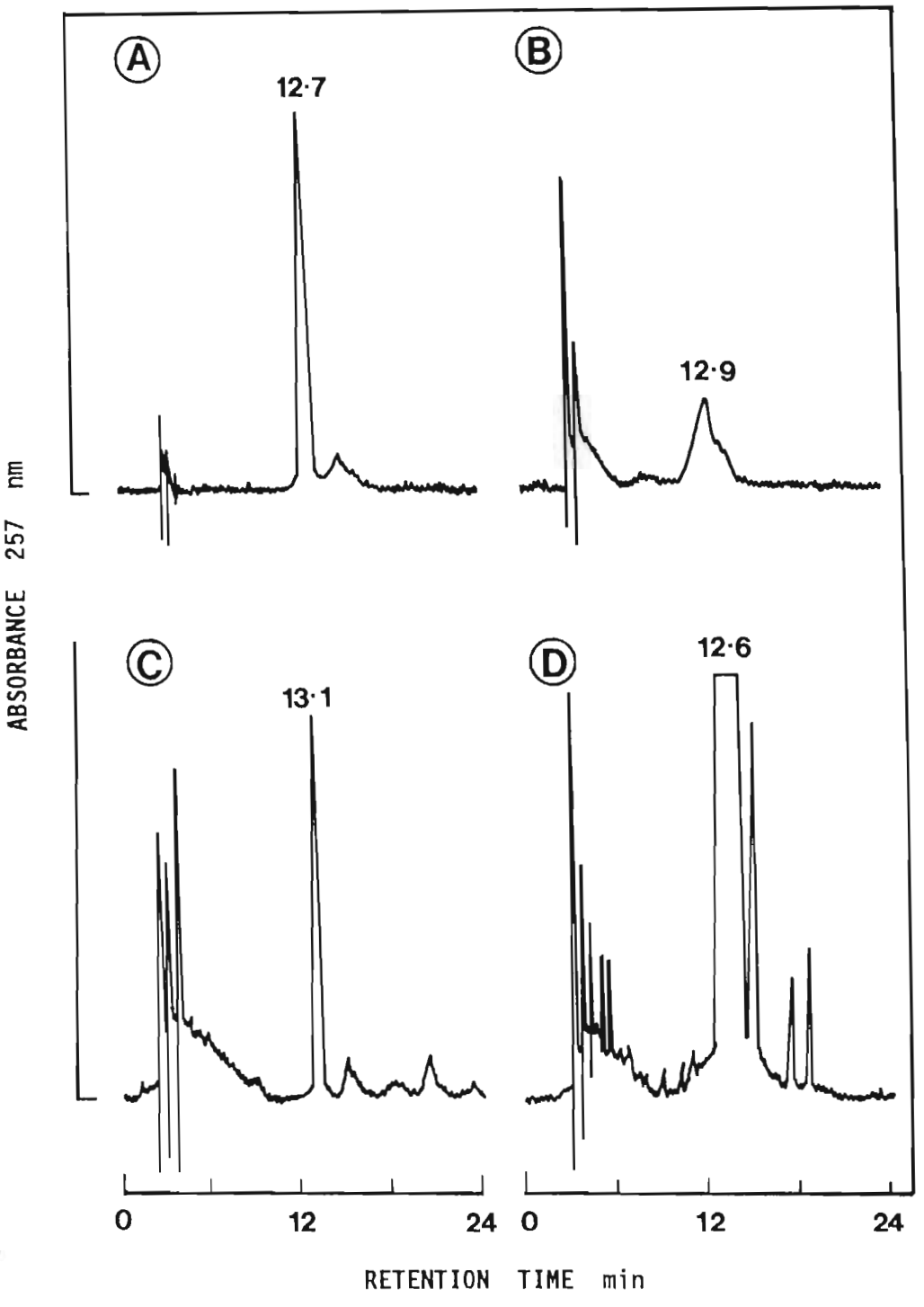


Figure 3.3.1: The UV absorbance traces obtained for the HPLC separation of authentic hypoxoside (A), leaf extracts (B), root extracts (C) and corm extracts (D) from 9-month old plants of *H. hemerocallidea*.

Table 3.3.1: The percentage hypoxoside (dry mass) recovered from the leaves, roots and corms of 9-month old plants of *H. hemerocallidea*. Three replicates were used and 95 percent confidence limits were calculated.

ORGAN ANALYSED	HYPOXOSIDE %
Leaves	undetectable
Roots	0.109 ± 0.019 *
Corms	2.403 ± 0.472 *

* indicates values that are not significantly different calculated with analysis of variance and a multiple range test at $p < 0.05$.

Table 3.3.2: The radioactivity, expressed as a percentage of the total radioactivity applied, detected in the excised roots and corm of *H. hemerocallidea* 7 days after the application of ^{14}C-phenylalanine.

EXCISED ORGAN	RADIOACTIVITY RECOVERED %
Roots	10.56
Corm	7.78

3.3.2 A and B), the only peaks of radioactivity detected in both extracts, occurred at the retention time of 3 minutes.

Thus, in neither the excised roots nor the excised corm did, hypoxoside become isotopically labelled. When the incorporation of radioactivity into cinnamic acid and the hydroxycinnamic acids was examined by HPLC separation of these extracts (Figure 3.3.3 A and B), both extracts yielded a large peak of radioactivity at the retention time of *t*-cinnamic acid. None of the other peaks of radioactivity detected co-eluted with the other standards. Thus the ^{14}C-phenylalanine was channelled into *t*-cinnamic acid but not any of the other hydroxycinnamic acids, unless incorporation of radioactivity into these acids was transient.

3.3.1.3. Investigations into the biosynthetic site for hypoxoside in intact plants of *H. hemerocallidea*.

Uptake of radioactivity

When ^{14}C-phenylalanine was applied to the shoot apical region of the plants at weekly intervals for 8 weeks, a considerable amount of the total radioactivity recovered was recovered from the leaves at all of the harvests 2, 4, and 8 weeks (Table 3.3.3). While very much less radioactivity was recovered from the roots, the amounts recovered were still slightly better than those recovered from the corms at each time interval.

Incorporation of radioactivity into hypoxoside

Peaks of radioactivity co-eluting with hypoxoside, were not detected upon HPLC separation of the extracts obtained from both the leaves and roots, at any of the time intervals (Figures 3.3.4 and 3.3.5). The

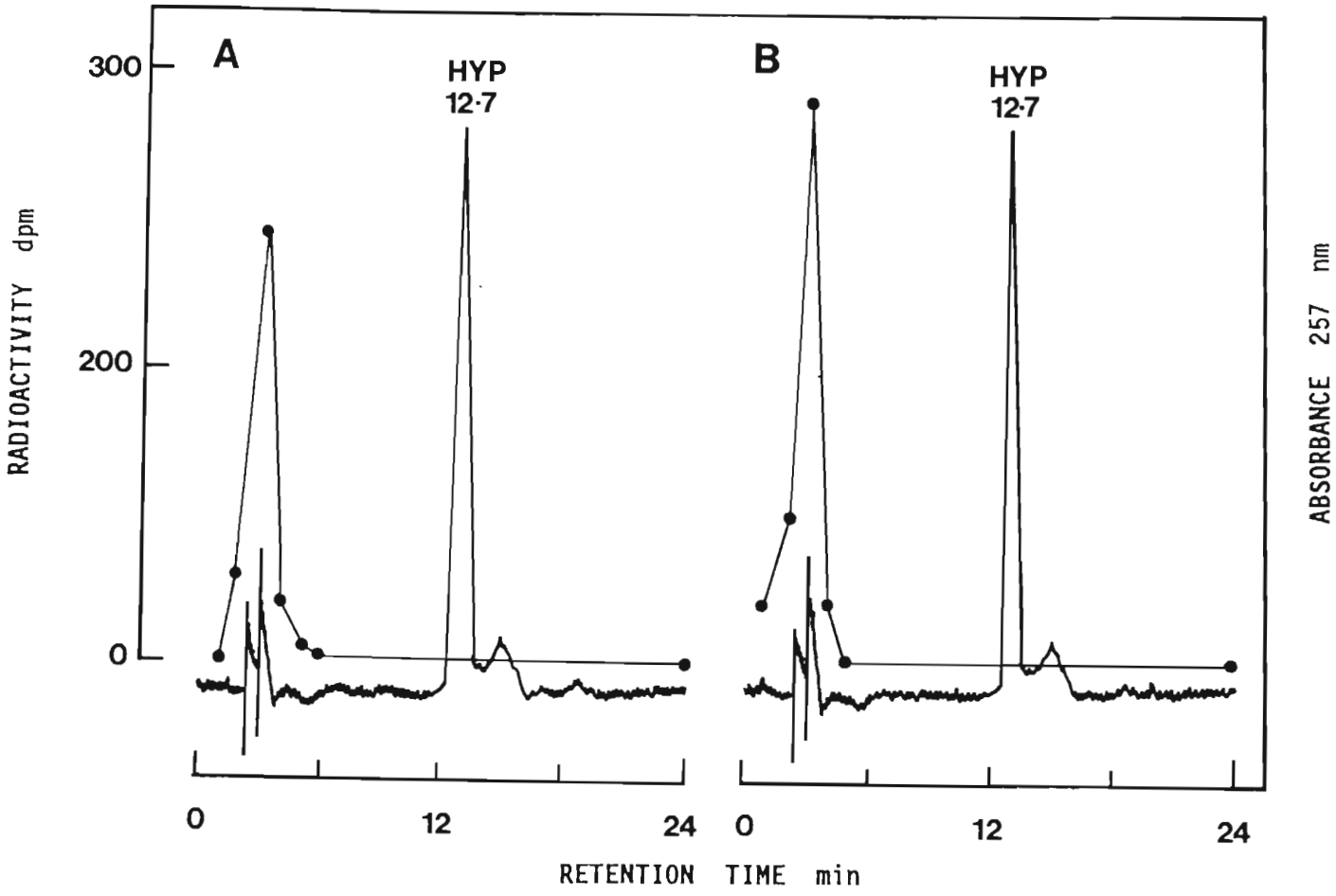


Figure 3.3.2: The peaks of radioactivity detected (●—●) upon HPLC separation of hypoxoside containing extracts from excised root (A) and corm tissue (B) of *H. hemerocallidea* treated with ^{14}C-phenylalanine for 7 days. HYP= hypoxoside.

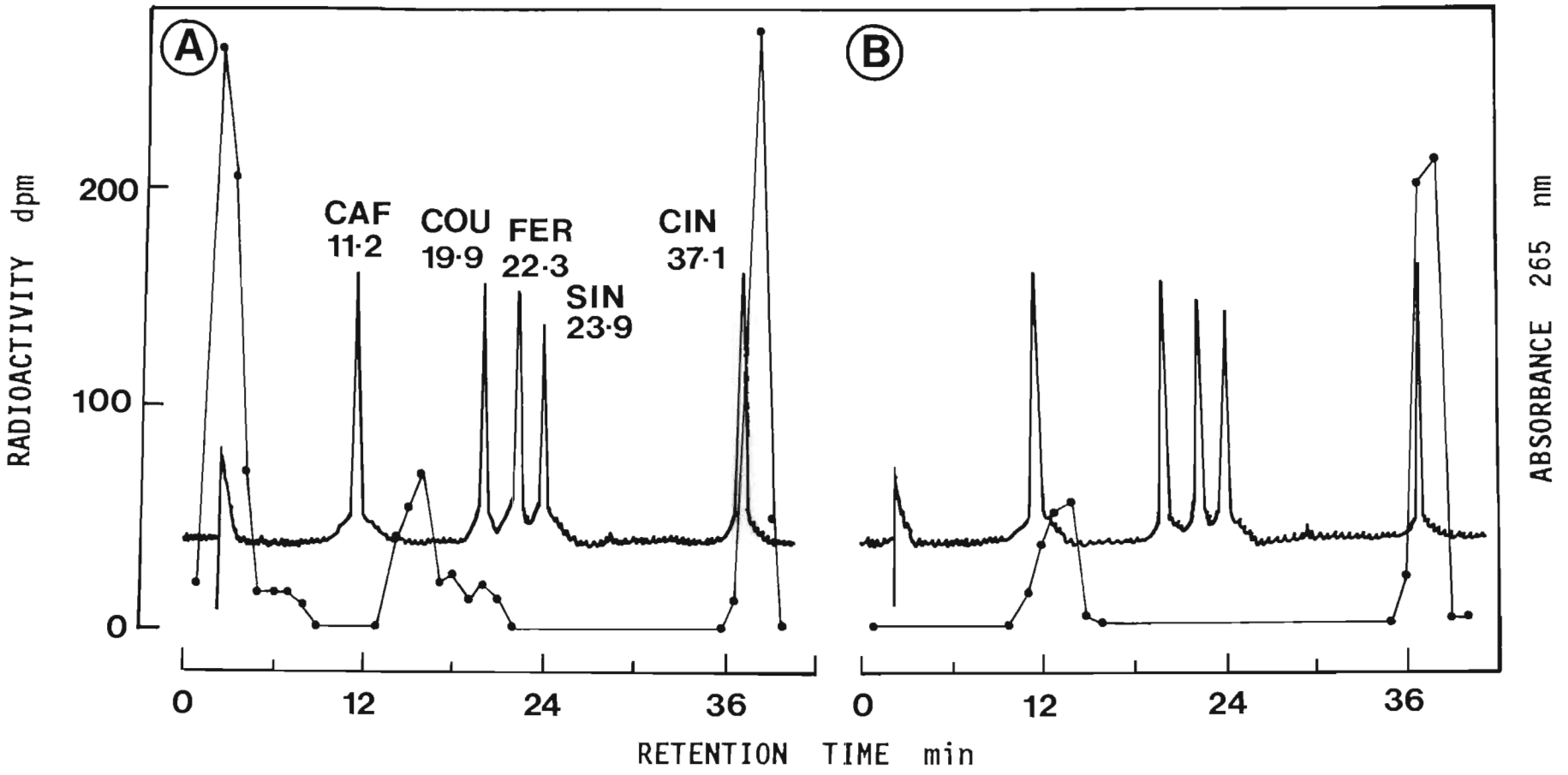

A
B
CAF
11·2
COU
19·9
FER
22·3
SIN
23·9
CIN
37·1
RADIOACTIVITY dpm
ABSORBANCE 265 nm
200
100
0
0
12
24
36
0
12
24
36
RETENTION TIME min

Table 3.3.3: The radioactivity recovered (expressed as a percentage of the total radioactivity applied) from the leaves, roots and corms of ^{14}C-phenylalanine treated *H. hemerocallidea* plants harvested at 2,4 and 8 weeks.

ORGAN	RADIOACTIVITY RECOVERED %		
Time of Harvest (weeks)	2	4	8
Leaves	17.26	14.32	14.77
Roots	2.93	0.88	0.97
Corm	1.09	0.59	0.77

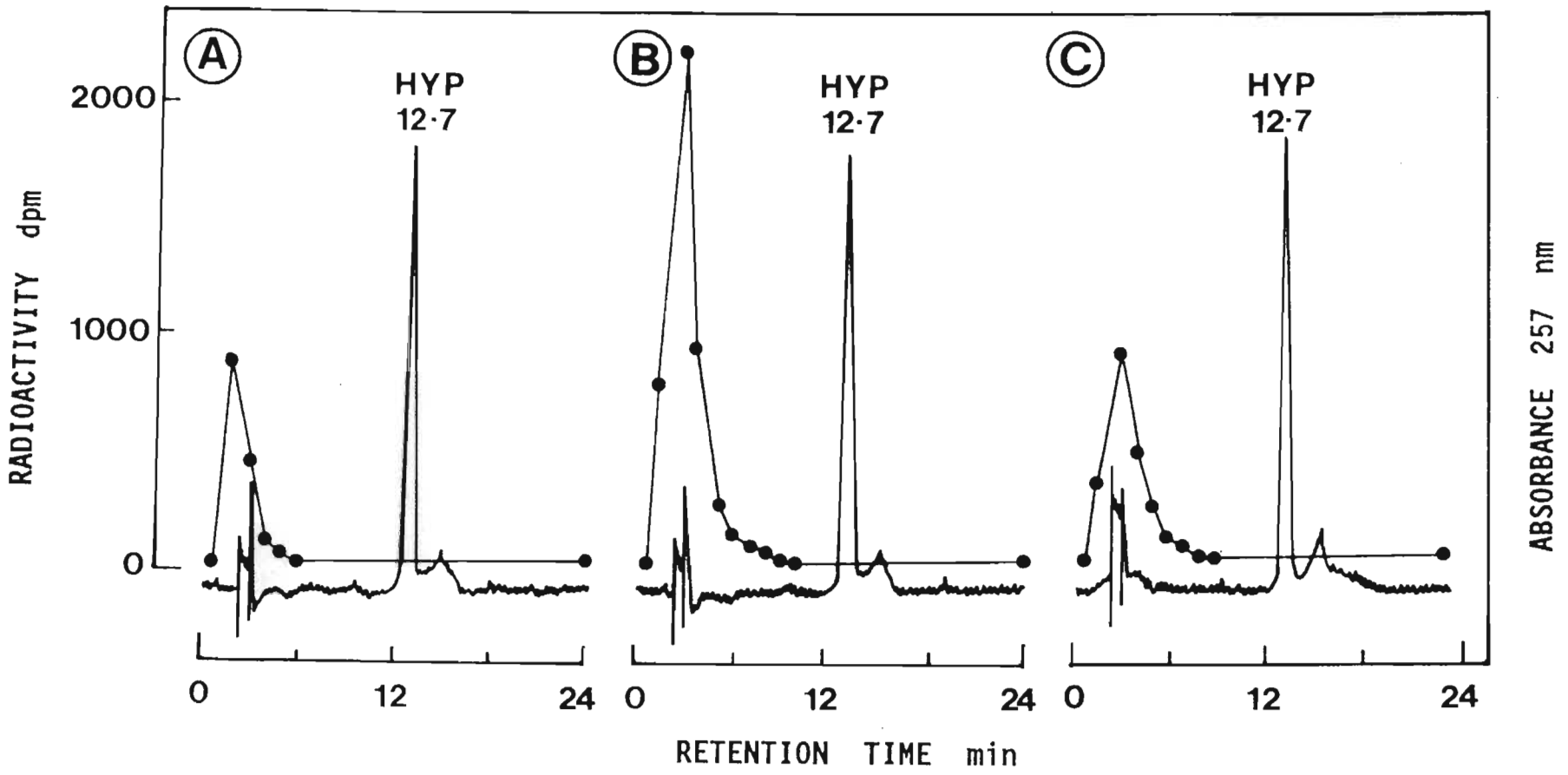


Figure 3.3.4: The peaks of radioactivity detected (●—●) upon HPLC separation of hypoxoside containing extracts of leaves from ^{14}C-phenylalanine treated, intact plants of *H. hemerocallidea* harvested after 2 (A), 4 (B) and 8 weeks (C), respectively. HYP= hypoxoside.

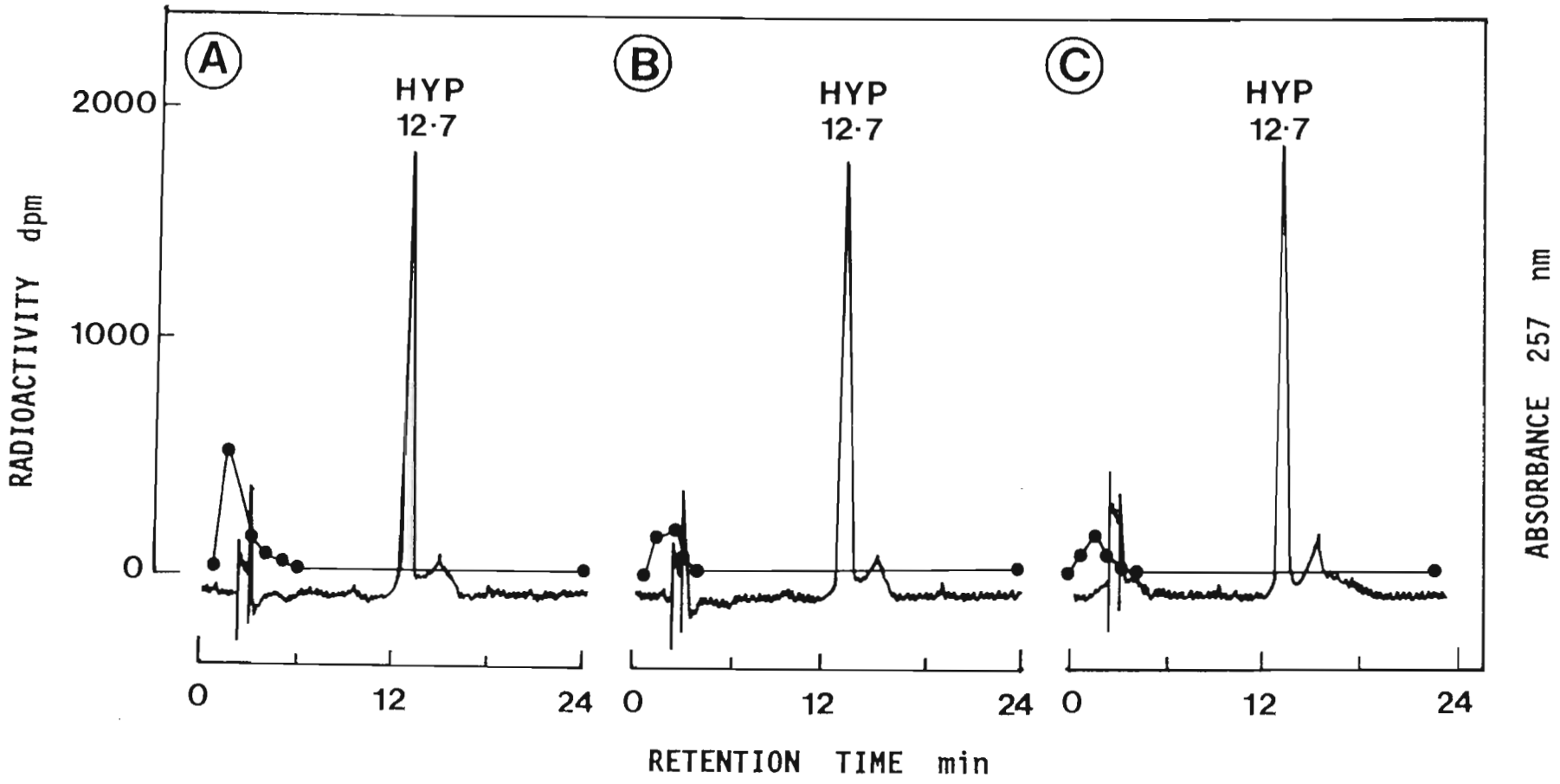


Figure 3.3.5: The peaks of radioactivity detected (●—●) upon HPLC separation of hypoxoside containing extracts of roots from ^{14}C-phenylalanine treated, intact plants of *H. hemerocallidea* harvested after 2 (**A**), 4 (**B**) and 8 weeks (**C**), respectively. HYP= hypoxoside.

HPLC separation of extracts from the corm, produced results that differed to those obtained for the other organs. After 2 weeks a small peak of radioactivity was detected coincident with the retention time of authentic hypoxoside (Figure 3.3.6 A). At subsequent time intervals (Figure 3.3.6 B and C) this peak of radioactivity increased considerably in size. When the radioactivity incorporated into hypoxoside in the corm was calculated as a percentage of that applied to the plant (Table 3.3.4), there was an increase of 0.013 percent between the values obtained for the two and eight week harvests. This data strongly suggests that the corm was the organ in which hypoxoside biosynthesis was occurring in this experiment, since no radioactive hypoxoside was detected in the roots or leaves, both of which contained more of the applied radioactivity than the corm.

Incorporation of radioactivity into cinnamic acid and the hydroxycinnamic acids

HPLC separation of the leaf, root and corm extracts, yielded in all cases, at all time intervals, major peaks of radioactivity which co-eluted with the retention times of caffeic and *p*-coumaric acid (Figure 3.3.7). In comparison, the peaks that co-eluted with sinapic, ferulic and *t*-cinnamic acids were small and indistinct. When the levels of radioactivity incorporated into *t*-cinnamic, *p*-coumaric and caffeic acid in the leaves, roots and corm were calculated as a percentage of the radioactivity present in each organ, at each time, a distinct trend was observed. In the leaves and roots (Figure 3.3.8 A and B) there was a tendency for the levels associated with *p*-coumaric and caffeic acids to increase during the 8 weeks. The incorporation of radioactivity into the latter acid showed the greatest increase. In the corm extracts the opposite trend was observed (Figure 3.3.8 C). The levels of incorporation of radioactivity into *p*-coumaric acid

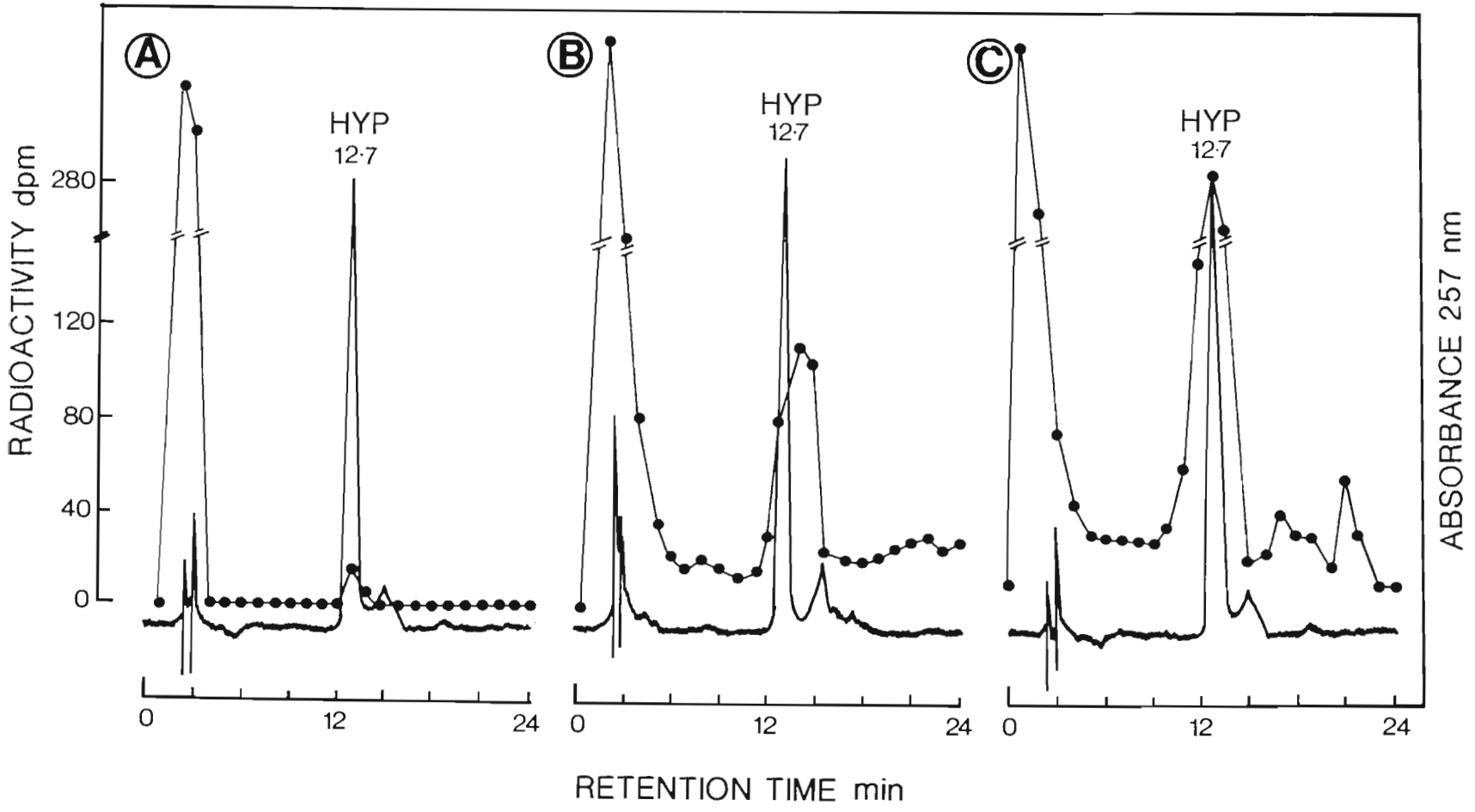


Figure 3.3.6: The peaks of radioactivity detected (●—●) upon HPLC separation of hypoxoside containing extracts of corms from ^{14}C-phenylalanine treated, intact plants of *H. hemerocallidea* harvested after 2 (**A**), 4 (**B**) and 8 weeks (**C**), respectively. HYP= hypoxoside.

Table 3.3.4: The incorporation of radioactivity (expressed as a percentage of the total radioactivity applied) into hypoxoside in the corms harvested at 2,4 and 8 weeks from plants of *H. hemerocallidea* treated with ^{14}C-phenylalanine.

TIME OF HARVEST weeks	INCORPORATION OF RADIOACTIVITY %
2	0.001
4	0.008
8	0.013

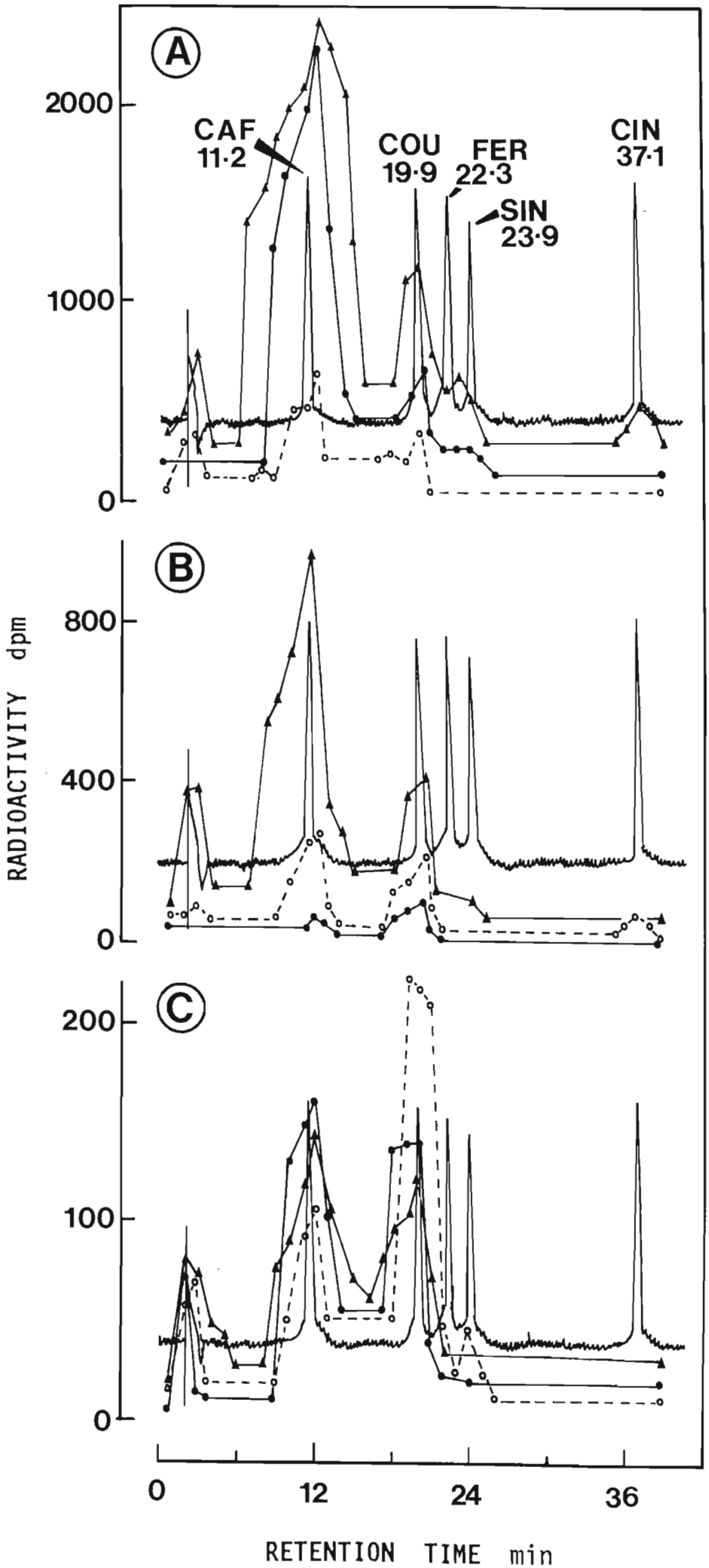
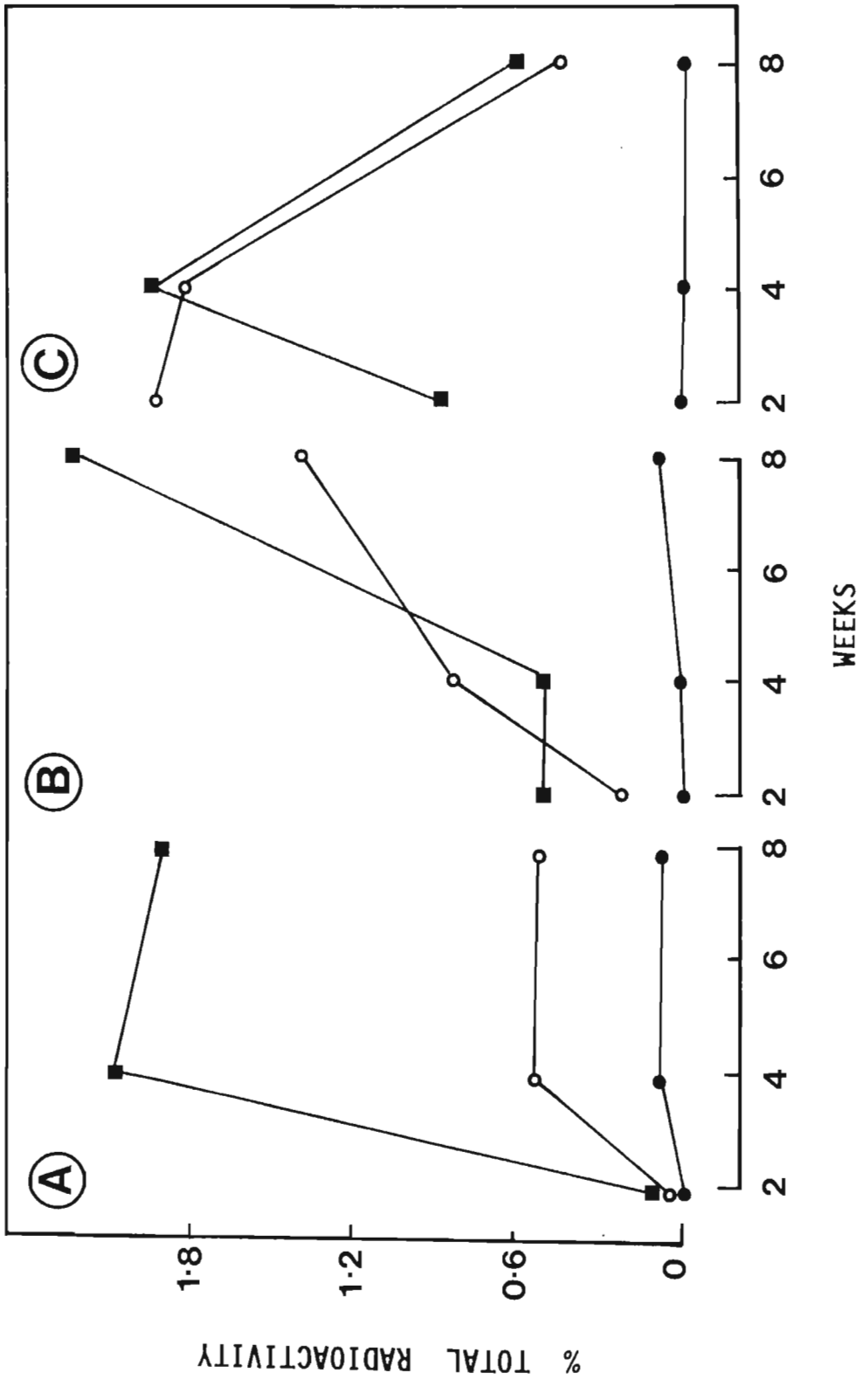

A
2000
1000
0
CAF
11·2
COU
19·9
FER
22·3
SIN
23·9
CIN
37·1
B
800
400
0
C
200
100
0
0
12
24
36
RETENTION TIME min
RADIOACTIVITY dpm
ABSORBANCE 265 nm

Figure 3.3.8: The changes in radioactivity incorporated (expressed as a percentage of the total radioactivity applied) into *t*-cinnamic acid (●—●), *p*-coumaric acid (○—○) and caffeic acid (■—■) in the leaves (**A**), roots (**B**) and corms (**C**) of ^{14}C-phenylalanine treated, intact plants of *H. hemerocallidea* over an 8 week experimental period.


A
B
C
1·8
1·2
0·6
0
2
4
6
8
WEEKS
% TOTAL RADIOACTIVITY

decreased, while after an initial sharp increase, those associated with caffeic acid, also decreased sharply. In all of the organs, the levels of radioactivity associated with *t*-cinnamic acid, remained at a more or less constant level.

3.3.1.3. **Biosynthetic potential of various regions of the corm**

Uptake of radioactivity

The radioactivity recovered from the corms, as whole organs, increased slightly over the 8 week experimental period but this increase was not significant (Table 3.3.5). If the radioactivity taken up by the corms was calculated as a percentage for each region of the corm (upper inner and outer and lower inner and outer), the distribution of the radioactivity changed over the 8 week period. Initially (2 weeks) most of the radioactivity was present in the inner region of the corm, both in the upper and lower portions, with the difference being the greatest in the lower portion (Figure 3.3.9 A). After 4 weeks (Figure 3.3.9 B) the inner region had accumulated most of the radioactivity in the upper portion, while the outer region in the lower portion had accumulated most of the radioactivity in this half. The difference in accumulated radioactivity between the lower portions, was not significant however. The pattern of distribution of radioactivity after 8 weeks was almost identical (Figure 3.3.9 C).

Distribution of radioactivity associated with hypoxoside following the application of ^{14}C-phenylalanine

At the 2 week harvest of the experiment most of the radioactivity associated with hypoxoside was located in the inner regions, both in the upper and lower halves (Figure 3.3.10 A) of the corms. These differences were significant. This pattern changed at 4 weeks (Figure

Table 3.3.5: The radioactivity recovered (expressed as a percentage of the total radioactivity applied) from corms harvested from ^{14}C-phenylalanine treated plants of *H. hemerocallidea* at 2, 4 and 8 weeks. Three replicates were used and 95 percent confidence limits were calculated.

TIME OF HARVEST weeks	RADIOACTIVITY RECOVERED %
2	3.31 ± 3.01
4	4.69 ± 3.22
8	5.49 ± 4.18

Figure 3.3.9: The distribution of radioactivity (measured as a percentage of the total radioactivity recovered from the whole corm) between the upper inner (**UI**), upper outer (**UO**), lower inner (**LI**) and lower outer (**LO**) regions of corms harvested from ^{14}C-phenylalanine treated, intact plants of *H. hemerocallidea* after 2 (**A**), 4 (**B**) and 8 weeks (**C**),respectively. The solid bar represents the least significant difference values calculated in each case at $p < 0.05$.

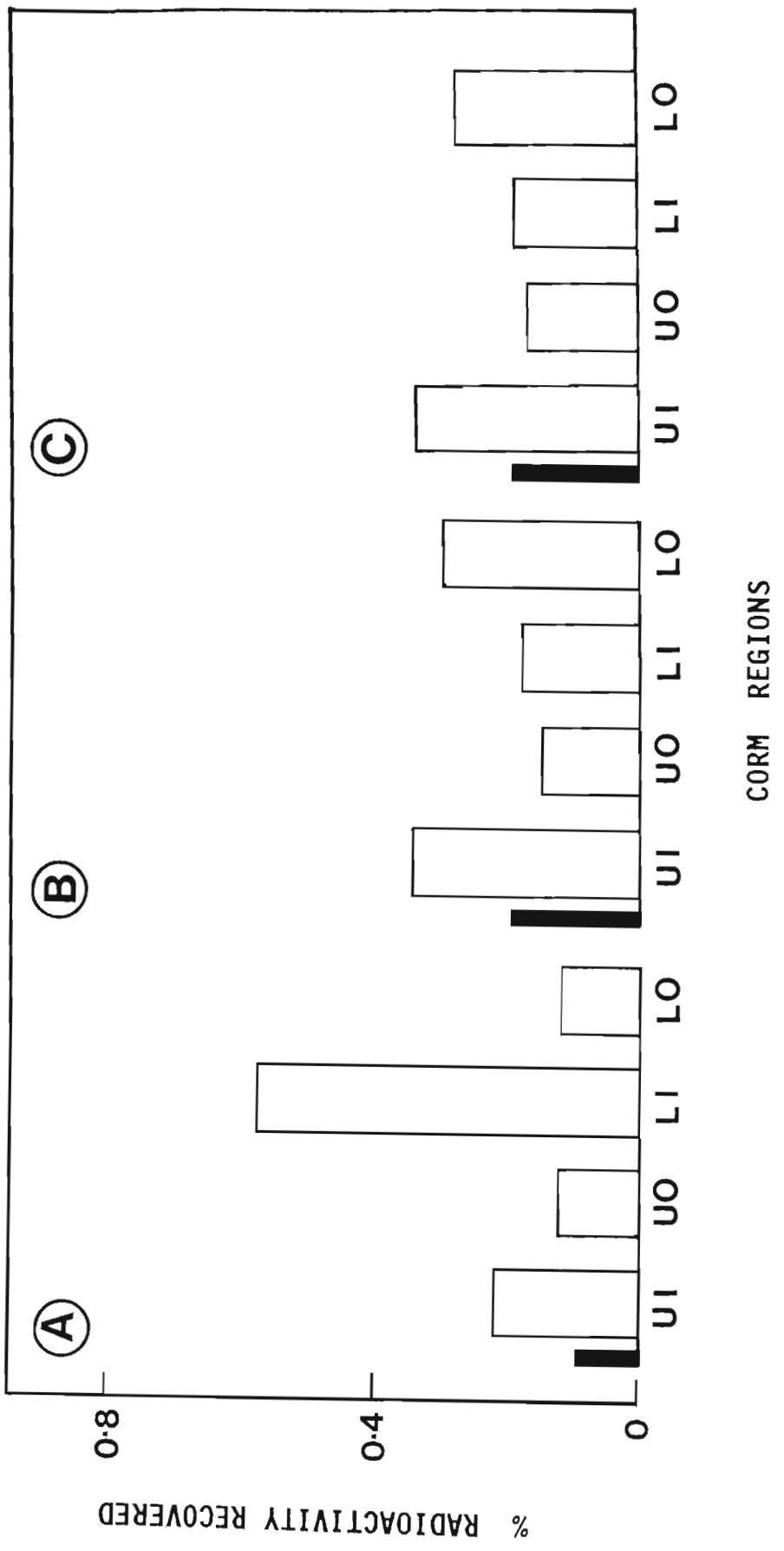
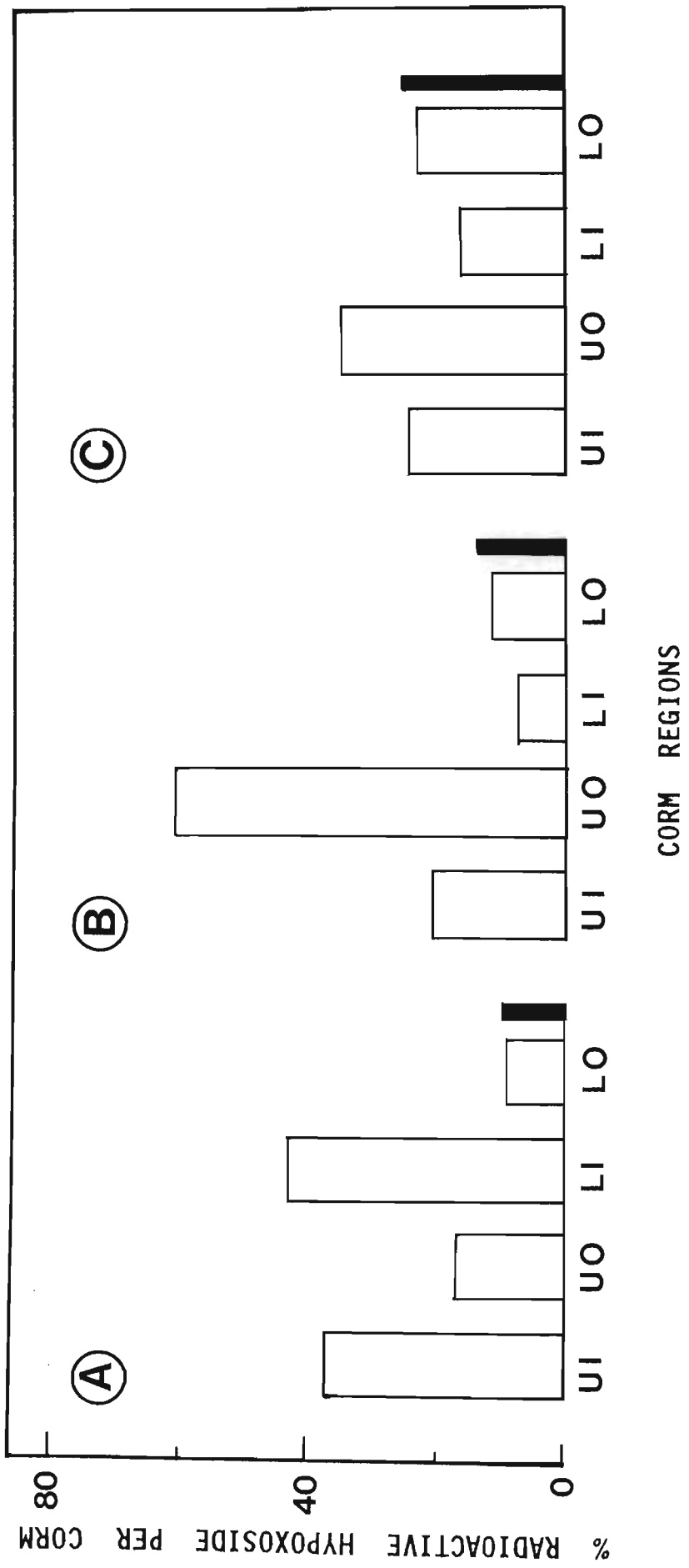

% RADIOACTIVITY RECOVERED
0·8
0·4
0
A
B
C
UI
UO
LI
LO
UI
UO
LI
LO
UI
UO
LI
LO
CORM REGIONS

Figure 3.3.10: The distribution of radioactivity incorporated into hypoxoside (measured as a percentage the total radioactivity recovered from the whole corm) between the upper inner (**UI**), upper outer (**UO**), lower inner (**LI**) and lower outer (**LO**) regions of corms from ^{14}C-phenylalanine treated, intact plants of *H. hemerocallidea* harvested after 2 (**A**), 4 (**B**) and 8 weeks (**C**) respectively. The solid bar represents the least significant difference values calculated in each case at $p < 0.05$.


% RADIOACTIVE HYPOXOSIDE PER CORM
80
40
0
A
B
C
UI
UO
LI
LO
CORM REGIONS

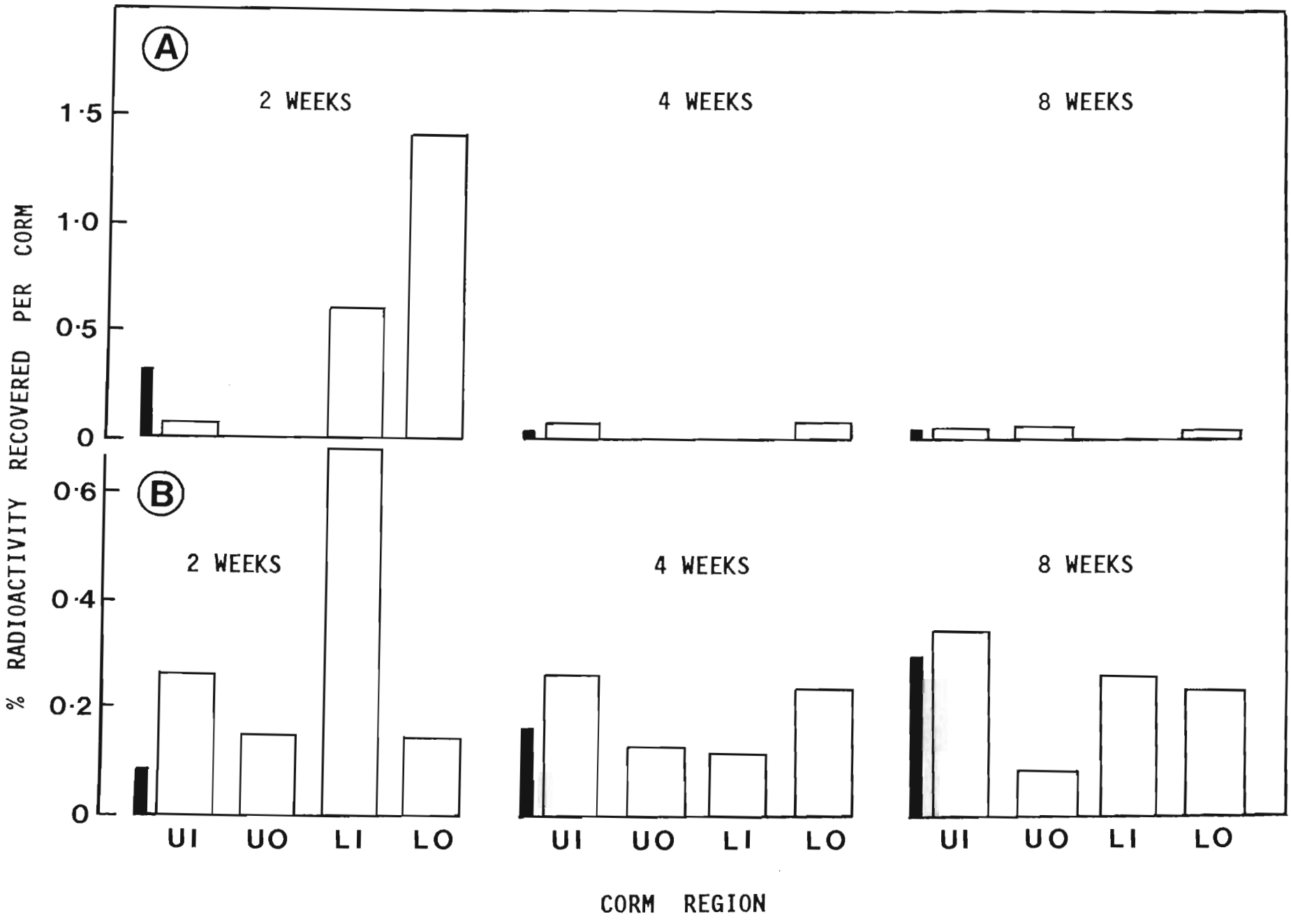
3.3.10 B) with a substantially greater amount of radioactivity being associated with the outer region in the upper half of the corm. The lower half of the corm contained lower levels of radioactivity in association with hypoxoside with the outer region containing slightly more than the inner. The pattern after 8 weeks (Figure 3.3.10 C) was similar, with the outer regions in both the upper and lower halves of the corm containing more radioactivity associated with hypoxoside than the inner regions. The difference between the upper regions was not as marked in comparison with the corms from the plants harvested at 4 weeks.

Distribution of radioactivity associated with the hydroxycinnamic acids following the application of ^{14}C-phenylalanine

Since *p*-coumaric and caffeic acids were the only hydroxycinnamic acids to become substantially labelled in the corm upon treatment with ^{14}C-phenylalanine, only the results for these will be discussed. Thus ferulic, sinapic and cinnamic acids with which very little radioactivity was associated, will be excluded. The radioactivity associated with *p*-coumaric acid (Figure 3.3.11 A) was initially high in the lower region of the corm with the greatest concentration of radioactivity being detected in the outer region and very little in the upper region. At the 4 and 8 week intervals (Figure 3.3.11 A) the levels of radioactivity associated with *p*-coumaric acid were negligible.

The radioactivity associated with caffeic acid at 2 weeks (Figure 3.3.11 B) mirrors that associated with hypoxoside at the same time (Figure 3.3.10 A) with most of the radioactivity being present in the inner region of the corm. At 4 and 8 weeks no distinct pattern

Figure 3.3.11: The incorporation of radioactivity (expressed as a percentage of the total radioactivity recovered from the corm) into *p*-coumaric acid (**A**) and caffeic acid (**B**) in the upper inner (**UI**), upper outer (**UO**), lower inner (**LI**) and lower outer (**LO**) regions of corms harvested from ^{14}C-phenylalanine treated, intact plants of *H. hemerocallidea* after 2, 4 and 8 weeks, respectively. The solid bar represents the least significant difference values calculated in each case at $p < 0.05$.


A
2 WEEKS
4 WEEKS
8 WEEKS
1·5
1·0
0·5
0
B
0·6
0·4
0·2
0
UI UO LI LO
% RADIOACTIVITY RECOVERED PER CORM
CORM REGION

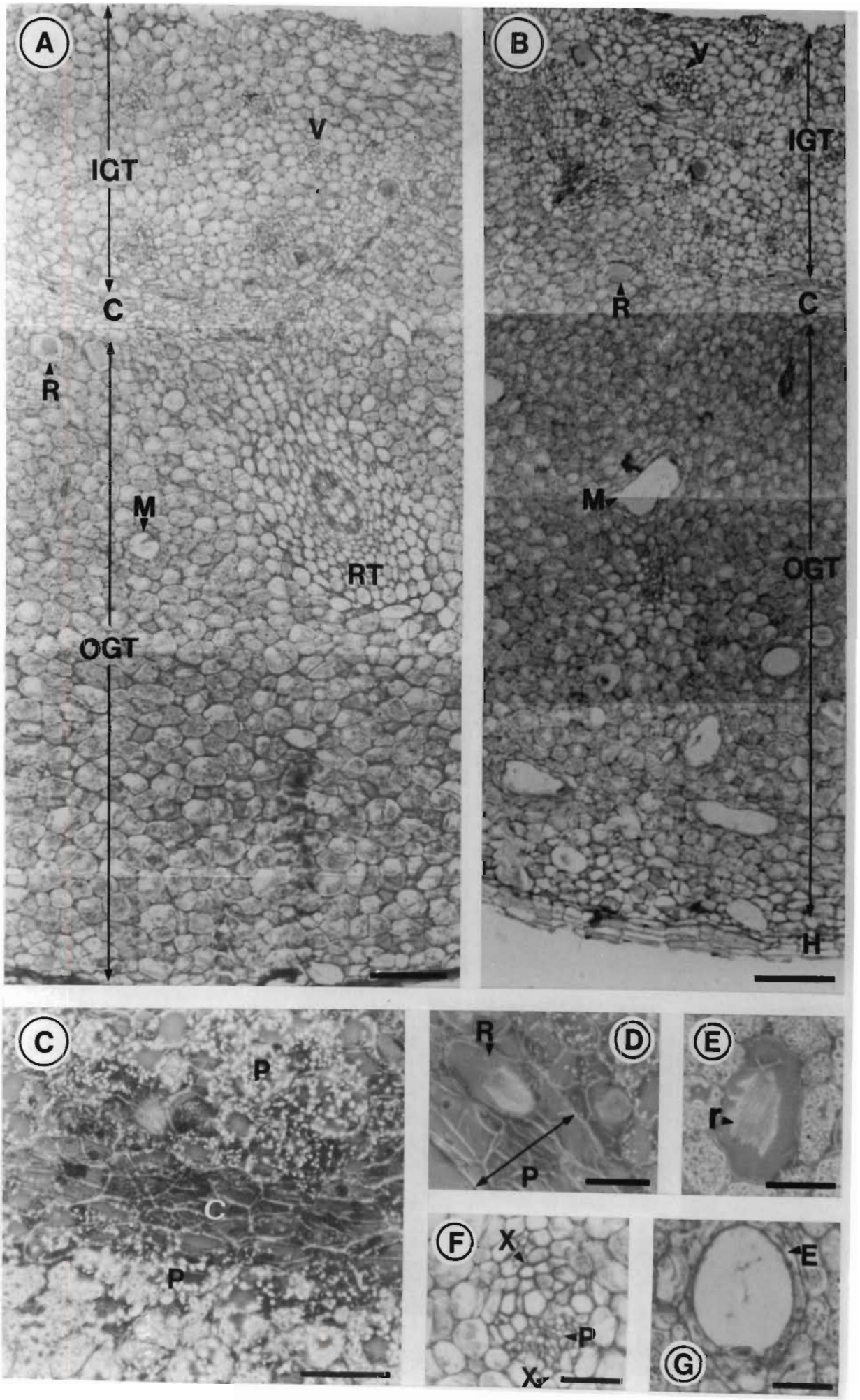
emerged except that the levels of radioactivity associated with the upper outer regions were low at both harvests (Figure 3.3.11 B).

3.3.1.4. **Anatomical studies of the corm.**

Light microscopy

The transverse sections through the upper half of the 15-week old corms revealed three defined tissue regions (Plate 3.3.1 A). At the centre of the corm a ground tissue comprised of vascular bundles and starch storing parenchyma cells was observed. Staining with safranin and fast green showed that the vascular bundles had an arrangement of phloem and xylem which is characteristic of concentric amphivasal bundles (Plate 3.3.1 F). Surrounding this inner ground tissue area was a cambium of densely packed, actively dividing cells (Plate 3.3.1 C) amongst which xylem elements were observed. The ground tissue that lay outside this area made up most of the corm and contained parenchyma cells densely packed with starch. Root traces were also observed in this tissue and proved to be of cambial origin when serial sections of the corm were studied.

The lower region of the corm (Plate 3.3.1 B) was arranged similarly to the upper region, consisting of an inner ground tissue area surrounded by a cambium and an extensive outer ground tissue area. An epidermis with an underlying immature storied hypodermis, was also evident (Plate 3.3.1 D). Only the phelloid cells of the phellem were observed as cork cells had not been formed at this stage. In hand sections made with one year old corms this storied hypodermis was also present in the upper corm.


A
V
IGT
C
R
M
RT
OGT
B
V
IGT
R
C
M
OGT
H
C
P
C
P
R
D
P
E
r
F
X
P
X
E
G

In the outer ground tissue regions of both the upper and lower parts of the corm, mucilage cavities were present (Plate 3.3.1 G). These consisted of a mucilage filled cavity surrounded by a layer of small epithelial cells. Also present in the upper and lower corms, in both the outer and inner ground tissue regions were cells containing raphides (Plate 3.3.1 E). The raphide cells were also observed in the hypodermal layers (Plate 3.3.1 D). In the longitudinal section through a 15-week old corm (Plate 3.3.2 A) a number of regions could be discerned. At the apex of the corm the leaf bases and the shoot apex were found. The shoot apex consisted of a two celled layer of tunica underlying which was the corpus (Plate 3.3.2 B). At the bases of the developing leaf primordia a primary thickening meristem (Plate 3.3.2 C) was evident. The primary thickening meristem joined the cambium (Plate 3.3.2 D) which separated the inner ground tissue area, in which vascular traces (Plate 3.3.2 E) were prevalent, from the outer ground tissue region. The outer ground tissue region contributed to the major part of the corm with the parenchymatous tissues in the upper and lower regions showing no differences (Plate 3.3.2 F & G) bar the presence of a larger number of mucilage cavities in the lower half of the corm.

Electron microscopy

Tissue taken from all regions of the corm, upper and lower, inner and outer regions (Plate 3.3.3 A) when examined at the electron microscope level contained cells with osmiophilic bodies present in the vacuoles (Plate 3.3.3 B). Since these osmiophilic bodies (Plate 3.3.3 C) were present only in cells densely packed with amyloplasts (Plate 3.3.3 B and D) it is assumed that these cells represent the starch rich parenchyma tissue that was observed at the light microscope level in the ground tissue in both the inner and outer regions of the corm.

Plate 3.3.2: Light micrographs of longitudinal sections through the corms of 15-week old plants of *H. hemerocallidea*.

Figure A: Diagrammatic representation of a longitudinal section through the corm constructed from light micrographs. At the apex of the corm the shoot apex (**SA**) and the leaf primordia (**LP**) from the bases of which a primary thickening meristem (**PM**) extends into a cambium (**C**). The cambium separates the inner ground tissue (**IGT**) from the outer ground tissue (**OGT**). Vascular bundles (**V**) are more numerous in the inner region while mucilage cavities (**M**) which are present in the outer ground tissue are larger and more numerous in the lower region. The solid bar represents 250 μm.

Figure B: The shoot apical meristem showing the two cell layered tunica (T) underlied by the corpus (**C**). The solid bar represents 100 μm.

Figure C: The primary thickening meristem (**PM**) at the base of a leaf primordium (**LP**), the anticlinally dividing meristematic cells of which were observed. The solid bar represents 100 μm.

Figure D: The cambium (**C**) bordered on either side by the starch storing parenchyma cells (**P**) which make up most of the inner and outer ground tissue. The solid bar represents 100 μm.

Figure E: The vascular traces (**V**) that were observed transversing the in the inner region. The solid bar represents 100 μm.

Figure F: The outer ground tissue found in the upper region of the corm. This tissue consisted of closely packed starch storing parenchyma cells (**P**). The solid bar represents 100 μm.

Figure G: The outer ground tissue found in the lower region of the corm. This tissue consisted of closely packed starch storing parenchyma cells (**P**) and frequent mucilage cavities (**M**). The solid bar represents 100 μm.

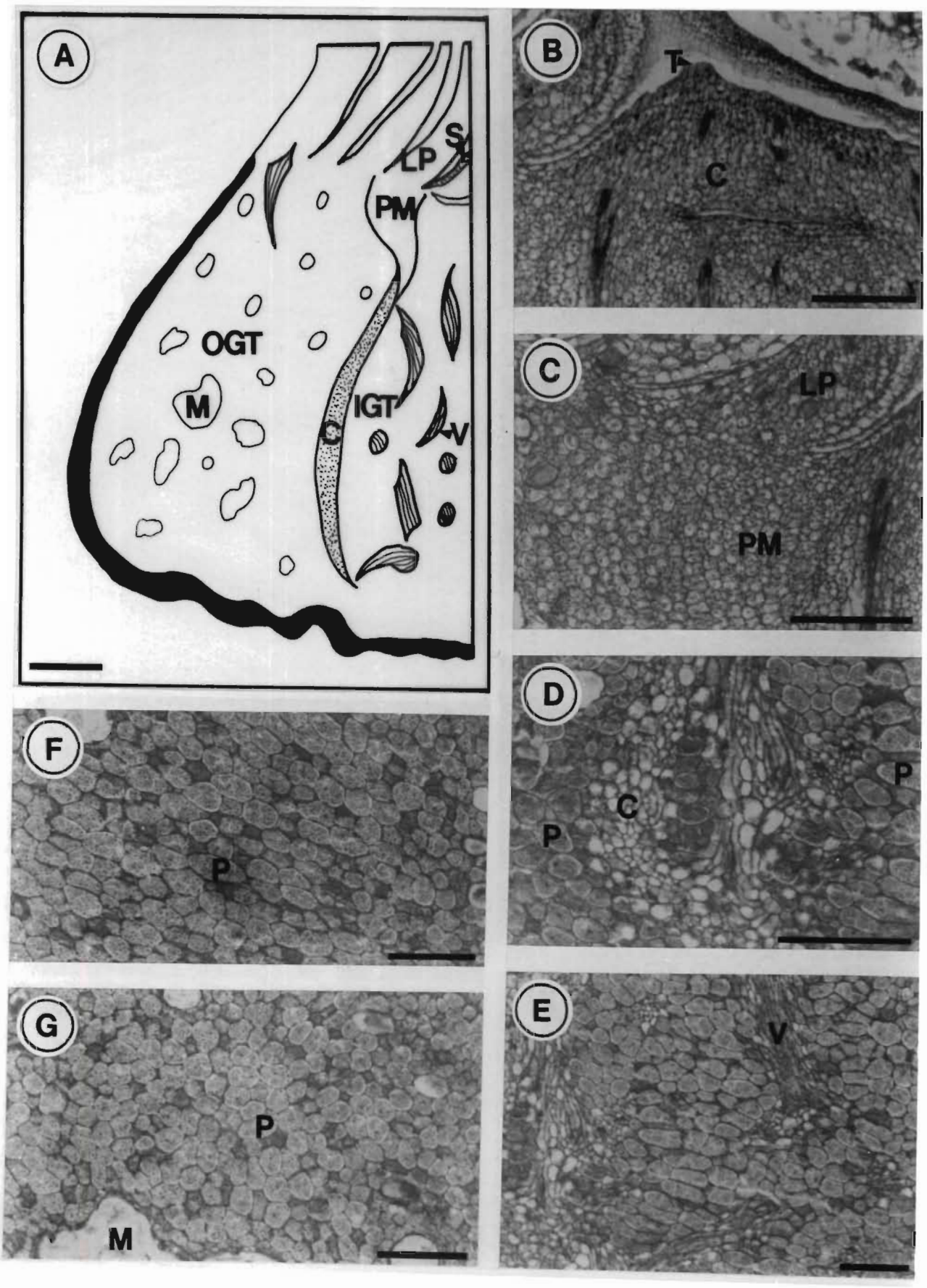

A
S
LP
PM
OGT
M
IGT
C
V
B
T
C
C
LP
PM
D
P
C
P
F
P
G
P
M
E
V

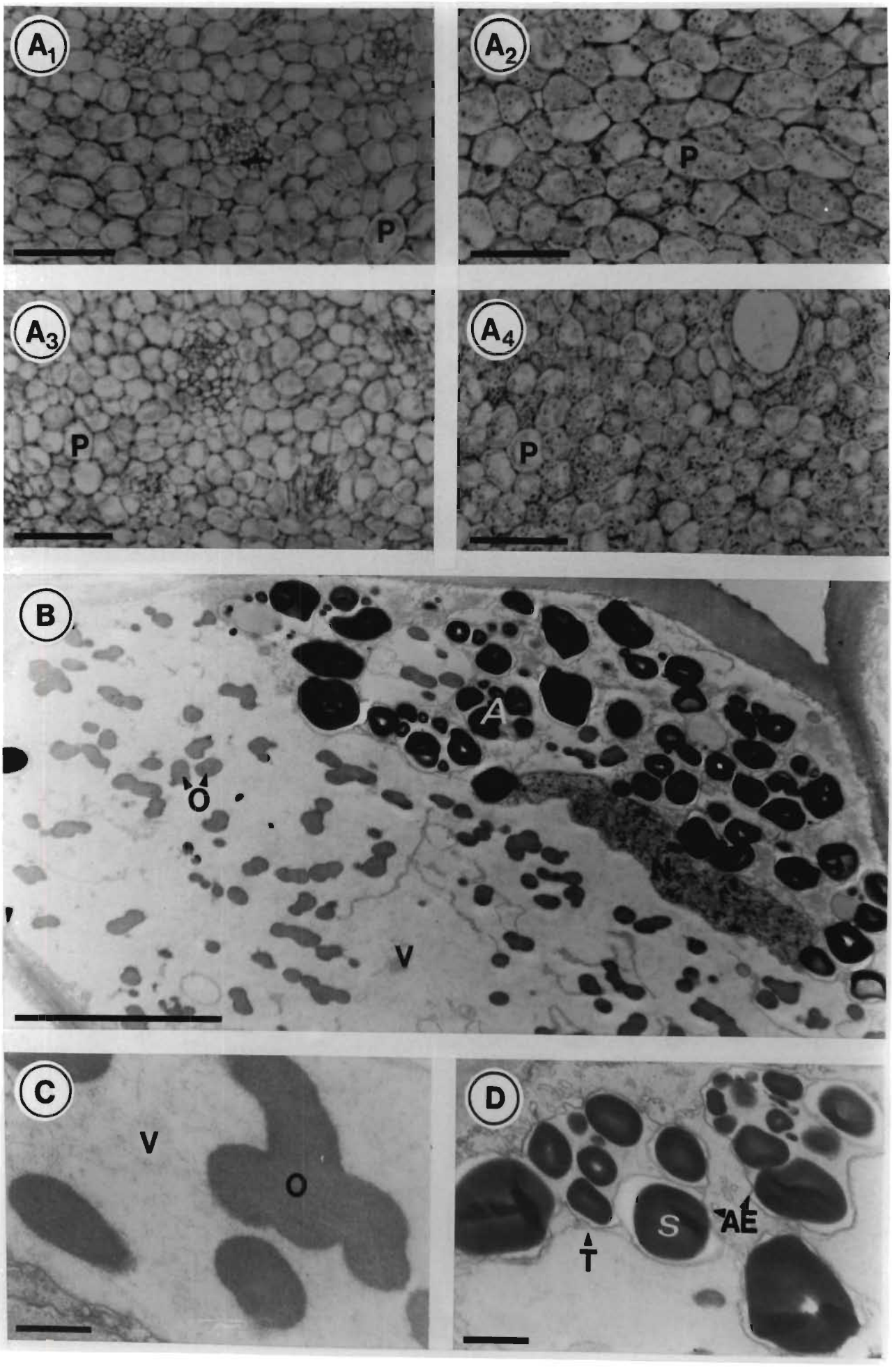
Plate 3.3.3: Light and electron micrographs of the ground tissue from the corms of 18-month old plants of *H. hemerocallidea*.

Figure A: Light micrographs of transverse sections through the corm tissue showing the starch storing parenchyma cells (**P**) found in the upper inner (A_1) and outer (A_2) regions and the lower inner (A_3) and outer (A_4) regions. The solid bar represents 100 μm.

Figure B: Electron micrograph of a transverse section through the starch storage parenchyma cells found in the ground tissue of the corm. Osmiophilic bodies (**O**) were evident in the vacuole (**V**) while numerous amyloplasts (**A**) were observed in the cytoplasm. Also visible was the nucleus (**N**). The solid bar represents 5 μm.

Figure C: Electron micrograph of the osmiophilic bodies (**O**) observed in the vacuoles (**V**) of the starch rich parenchyma cells of the ground tissue of the corm. The granular nature of these aggregates was clearly visible. The solid bar represents 1 μm.

Figure D: Electron micrograph of the tonoplast (**T**) and amyloplast envelopes (**AE**) of a starch storing parenchyma cell of the ground tissue of the corm showing that the membrane integrity was preserved. The solid bar represents 1 μm.


A1
P
A2
P
A3
P
A4
P
B
A
O
V
C
V
O
D
S
AE
T

At the electron microscope level these cells were characterised by being highly vacuolate, rich in amyloplasts with many osmiophilic bodies present in the vacuoles. Closer examination of the cytoplasm of these cells yielded interesting details. However, it must be mentioned that the cytoplasm did have a slightly disturbed appearance which was due to poor fixation, making the interpretation of any cytoplasmic detail difficult and tentative. This disturbance was caused by omitting fixation in glutaraldehyde since this caused leaching of the phenolics from the tissue. The obvious membrane integrity of the plasmalemma and tonoplast observed in (Plate 3.3.3 B, C and D) suggests, however, that some confidence can be placed on structures observed in the cytoplasm.

Present in the cytoplasm of these cells, were areas consisting of convoluted membranes and vesicles (Plate 3.3.4 A) in association with osmiophilic bodies. Closer examination of these structures (Plate 3.3.4 B) suggest that they consist of tubular endoplasmic reticulum. Also observed were areas in the cytoplasm that were densely packed with vesicles containing osmiophilic bodies and multivesicular bodies (Plate 3.3.4 C & D). Generally the cytoplasm was rich in endoplasmic reticulum (ER) suggesting either high synthetic or transport activity.

3.3.2. TRANSPORT AND PRODUCTION OF POTENTIAL PRECURSORS

3.3.2.1. The detection and quantification of the hydroxycinnamic acids in various organs of *H. hemerocallidea*

HPLC analysis

Upon separation by HPLC, peaks of UV absorbance measured at 265 nm were detected at the retention times of caffeic, *p*-coumaric, ferulic,

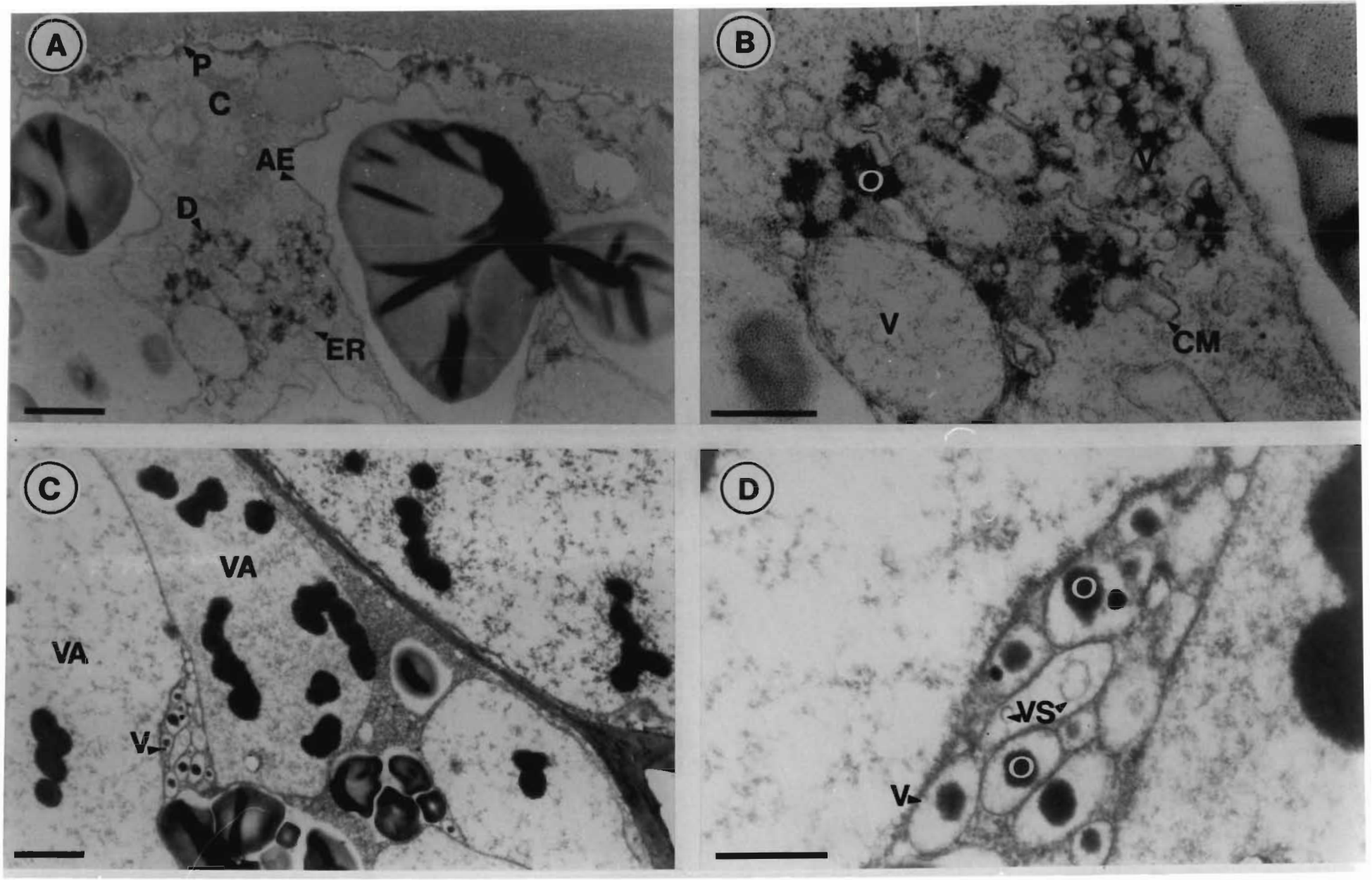
Plate **3.3.4:** Electron micrographs of transverse sections through the starch storing parenchyma cells, present in the ground tissue of the corms from 18-month old plants of *H. hemerocallidea*, showing cytoplasmic detail.

Figure A: The convoluted membranes of tubular endoplasmic reticulum (**ER**) associated with densely staining material (**D**) which were visible in the cytoplasm (**C**). The preservation of the membrane integrity of the plasmalemma (**P**) and the amyloplast envelopes (**AE**) can be observed. The solid bar represents 1 μm.

Figure B: The convoluted membranes (**CM**) and vesicles (**V**) with closely associated osmiophilic material (**O**) which were observed in the cytoplasm of a starch storing parenchyma cell. The solid bar represents 0.5 μm.

Figure C: A dense cluster of vesicles (**V**) which were observed adjacent to the vacuole (**VA**). The solid bar represents 1 μm.

Figure D: Detail of the vesicles showing the inclusion of osmiophilic material (**O**) which was seen within some of these structures whilst others contain smaller vesicles (**VS**). The solid bar represents 0.5 μm.


A
P
C
AE
D
ER
B
V
V
CM
C
VA
VA
V
D
VS
V

sinapic and *t*-cinnamic acid (Figure 3.3.12 A) in extracts from the leaves, roots and corm (Figure 3.3.12 B, C & D) of *H. hemerocallidea* plants. Although the peaks coinciding with caffeic and *p*-coumaric acids were well defined in all of the extracts, those associated with ferulic, sinapic and *t*-cinnamic acids tended to be small.

Quantification of the hydroxycinnamic acids

When quantified in the leaves, roots and corms caffeic acid was the most abundant hydroxycinnamic acid detected, particularly in the roots (Figure 3.3.13). Sinapic acid, surprisingly, was the second most abundant hydroxycinnamic acid in all of the organs followed by *p*-coumaric and ferulic acids. The levels of the latter two compounds did not differ significantly from one another. Of all the organs, the roots were the richest in caffeic acid. The corm did not contain as high a level of these acids as did the leaves and the roots.

3.2.2.2. Fixation and incorporation of ^{14}C-carbon dioxide into the hydroxycinnamic acids by intact *H. hemerocallidea* plants.

Distribution of radioactivity

At all of the harvest times the level of radioactivity present in the corm was lower compared to that detected in the leaves and roots. However, this difference was not significant in all cases (Table 3.3.6). The level of radioactivity detected in the roots tended to be higher than that found in the leaves but once again this difference was not always significant. On average most of the radioactivity was present in the leaves and roots. Twenty-four hours after pulsing with $^{14}CO_2$ the levels of radioactivity present in each organ reached a constant value.

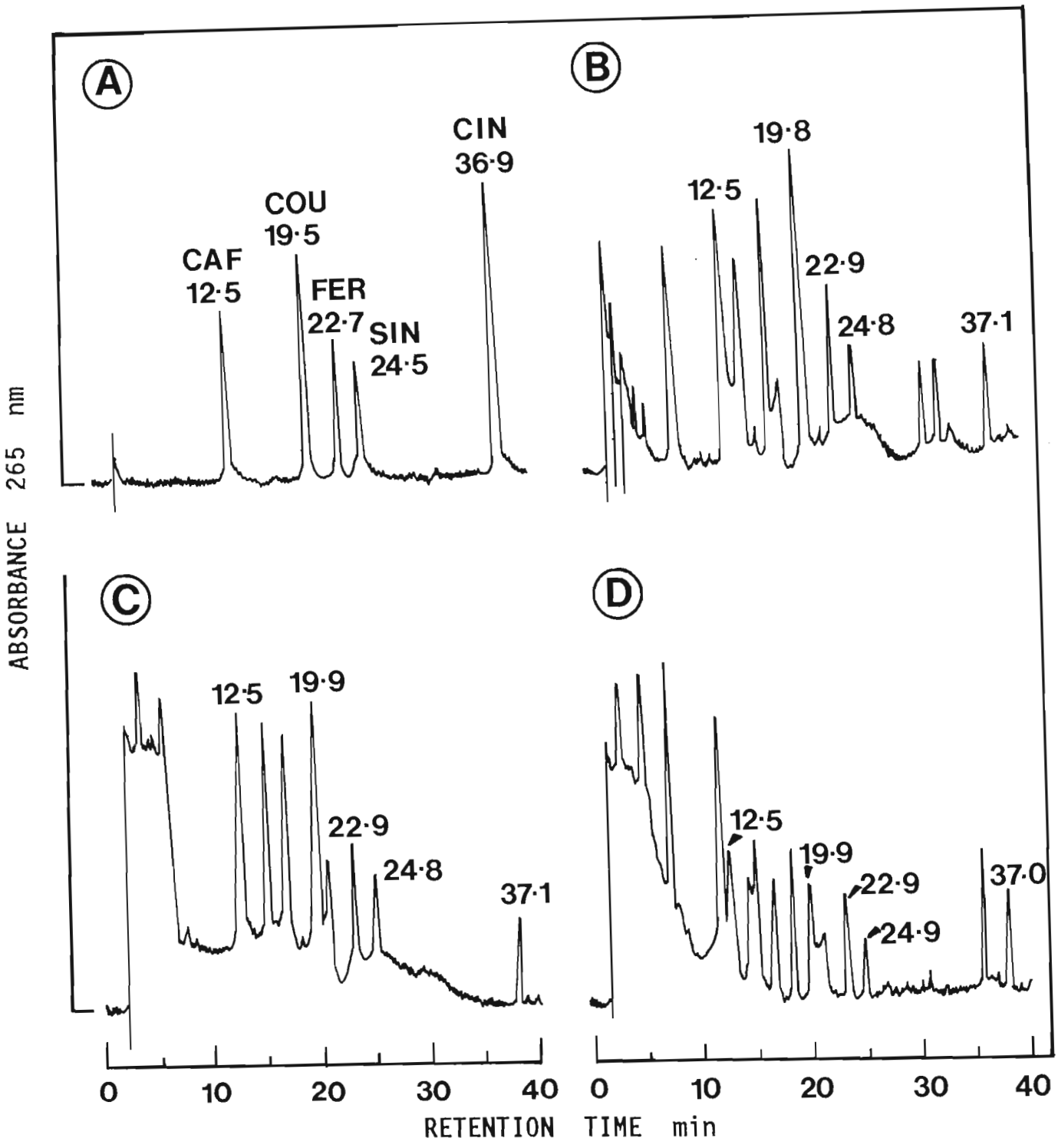


Figure 3.3.12: The UV absorbance traces obtained upon the HPLC separation of authentic *t*-cinnamic acid and the hydroxycinnamic acids (**A**), leaf (**B**), root (**C**) and corm extracts (**D**) of 9-month old plants of *H. hemerocallidea*. CAF= caffeic acid, COU= *p*-coumaric acid, FER= ferulic acid, SIN= sinapic acid and CIN= *t*-cinnamic acid.

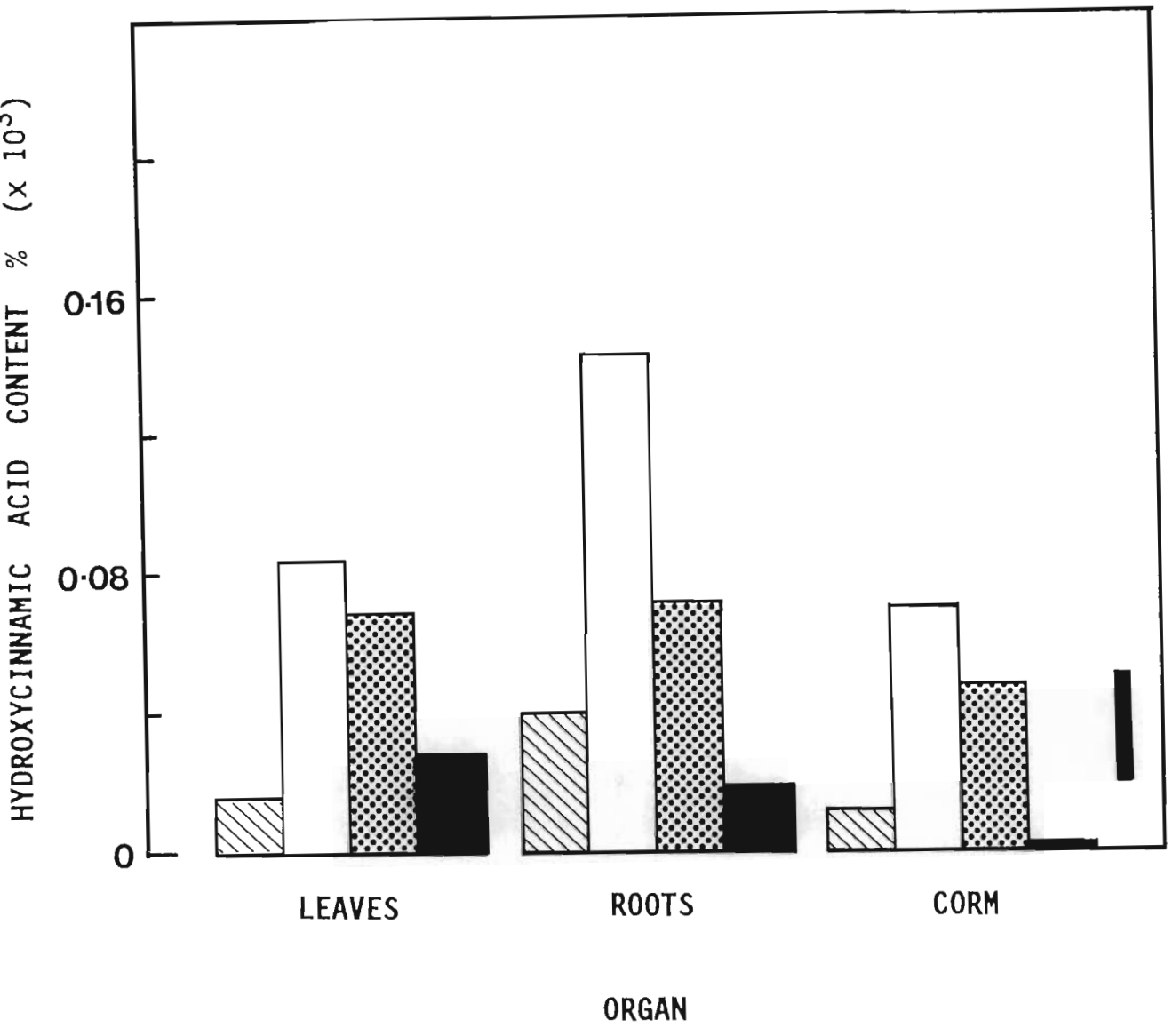


Figure 3.3.13: The levels of the hydroxycinnamic acids (calculated as a percentage of the dry mass) detected in the leaves, roots and corms of 9 month old *H. hemerocallidea* plants. ▨= *p*-coumaric acid, □= caffeic acid, ⊡= sinapic acid and ■= ferulic acid. The solid bars represent the least significant difference values calculated at $p < 0.05$.

Table 3.3.6: The distribution of radioactivity (expressed as a percentage of the total radioactivity recovered) between the leaves, roots and corms of plants of *H. hemerocallidea* 6, 24, 96 and 192 hours after pulsing with ^{14}C-carbon dioxide. Three replicates used and 95 percent confidence level were calculated.

PLANT ORGAN	RADIOACTIVITY RECOVERED %			
Time of Harvest h	6	24	96	192
Leaves	29.5 ± 7.2 **	65.3 ± 21.1*	34.9 ± 7.5*	40.9 ± 18.3*
Roots	52.7 ± 12.8*	23.3 ± 17.5 *	53.3 ± 6.2 *	47.3 ± 17.2*
Corm	17.8 ± 13.9 *	11.4 ± 4.0 *	11.8 ± 1.4 *	11.7 ± 2.4*

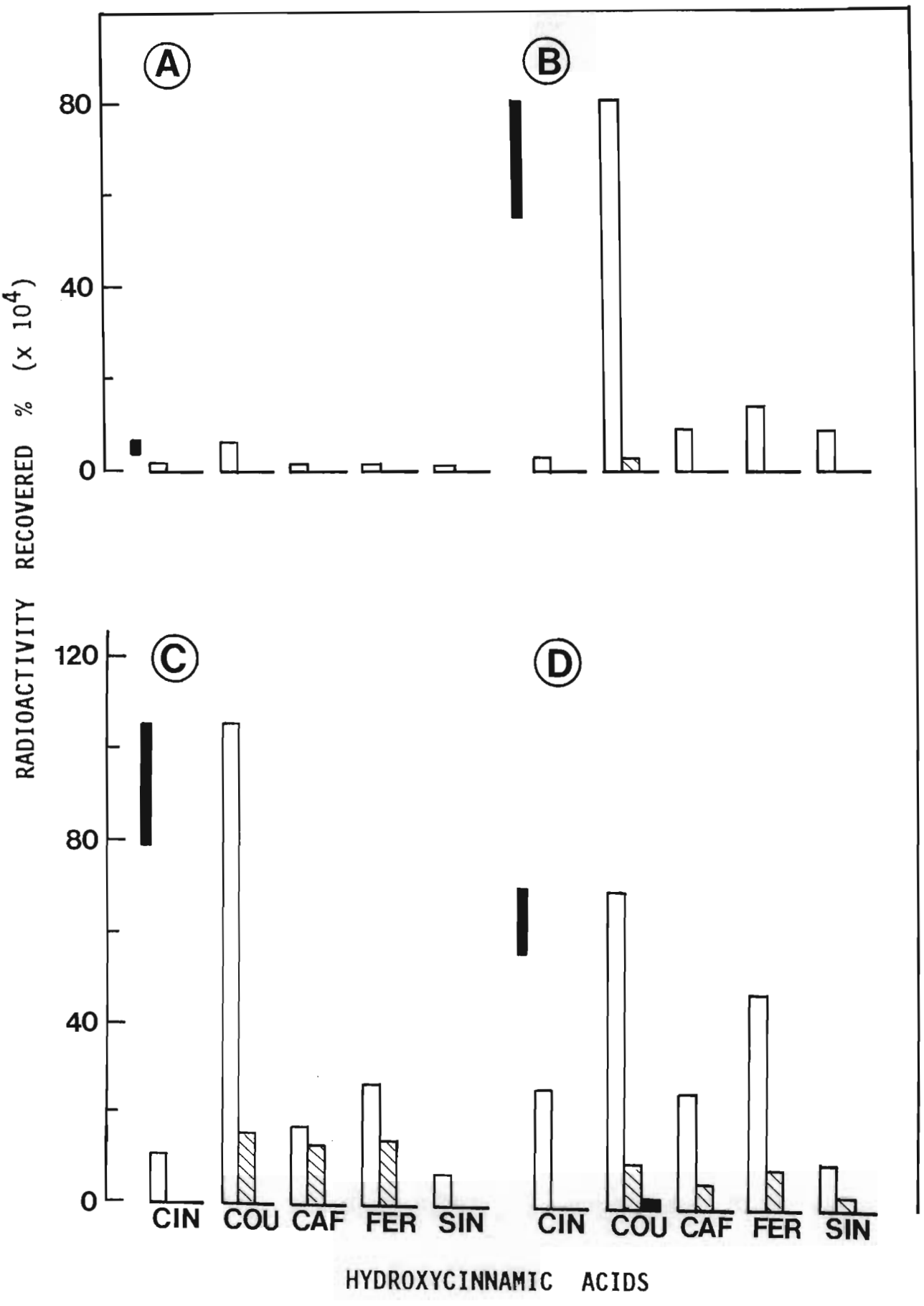
* indicates, within each time interval, values that are not significantly different calculated using analysis of variance and a multiple range test at $p < 0.05$.

Incorporation of radioactivity into cinnamic acid and the hydroxycinnamic acids

Six hours after application, very little radioactivity was associated with the hydroxycinnamic acids (Figure 3.3.14 A). The leaves were the only organs in which incorporation into these acids had occurred; the greatest level of radioactivity being associated with the retention time of *p*-coumaric acid. After 24 hours, the situation in the leaf extracts remained the same, with the exception that the radioactivity associated with *p*-coumaric acid, was much greater than that detected at the 6 hour harvest (Figure 3.3.14 B). After 24 hours the roots were the only other organ in which incorporation of radioactivity into these acids occurred; most of the incorporation being into *p*-coumaric acid.

The incorporation of radioactivity into cinnamic acid and the hydroxycinnamic acids in the leaf extracts after 96 hours (Figure 3.3.14 C), remained similar to that obtained after 24 hours, with the exception that the levels were higher. In the roots similar levels of radioactivity were incorporated into *p*-coumaric, caffeic and ferulic acids. However, these levels of incorporation were much lower than those detected in the leaves.

After 192 hours the level of radioactivity associated with *p*-coumaric acid appeared to decrease in the leaves, while the incorporation into the other acids increased slightly (Figure 3.3.14 D). Radioactivity recovered from roots harvested from plants after 192 hours was detected in association with *p*-coumaric, caffeic, ferulic and sinapic acids. The levels of incorporation were still much lower than those detected in the leaves. Only at this time interval was radioactivity


A
B
C
D
80
40
0
120
80
40
0
RADIOACTIVITY RECOVERED % (x 10⁴)
CIN
COU
CAF
FER
SIN
CIN
COU
CAF
FER
SIN
HYDROXYCINNAMIC ACIDS

in association with the hydroxycinnamic acids detected in the corm and then only with *p*-coumaric acid.

Thus, in all of the organs, *p*-coumaric acid seemed to be the first hydroxycinnamic acid into which radioactivity was incorporated or accumulated. This accumulation became more pronounced with time.

3.3.3.3. The application of ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid to intact plants of *H. hemerocallidea*.

Recovery of radioactivity

The recovery of applied radioactivity in all treatments was low (Table 3.3.7). The applied radioactivity was recovered most efficiently from plants treated with ^{14}C-*t*-cinnamic acid, followed by those treated with ^{14}C-phenylalanine, while the radioactivity recovered from the ^{14}C-*p*-coumaric and ^{14}C-caffeic acid treatments, was poor. The recovery of the radioactivity from the ^{14}C-*t*-cinnamic acid treatment was significantly better than that obtained for the ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid treatments, while the recovery obtained for ^{14}C-phenylalanine was significantly better than the ^{14}C-caffeic acid treatment. The method of extraction used would recover radioactivity present in the soluble phase of the cell. Any cell wall bound radioactivity would not be accounted for.

The distribution of recovered radioactivity between organs was very similar for all treatments (Table 3.3.7). In each case the leaves and corms contained similar levels of radioactivity while much lower levels of radioactivity were found in the roots.

Table 3.3.7: The percentage radioactivity recovered per plant and the distribution of this recovered radioactivity between the various organs when ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid, ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid were applied exogenously to intact plants of *H. hemerocallidea*. Three replicates were used and 95 percent confidence limits calculated.

TREATMENT	RADIOACTIVITY RECOVERED %	DISTRIBUTION OF RADIOACTIVITY %		
		Leaves	Roots	Corm
L(U-^{14}C)phenyl-alanine	15.1±8.8**	45.3±21.3^{a}	13.2±9.9^{b}	41.4±13.6^{a}
(3-^{14}C)*t*-cinnamic acid	26.8±4.5*	50.2±13.9^{a}	3.3±1.1^{b}	46.5±46.8^{a}
(2-^{14}C)*p*-coumaric acid	5.1±2.2 **	55.9± 9.4^{a}	1.3±0.9^{b}	43.1± 8.2^{a}
(2-^{14}C)-caffeic acid	3.1±0.9 *	45.8± 6.9^{a}	1.3±0.6^{a}	52.8± 7.3^{a}

* indicates values that are not significantly different calculated using analysis of variance and a multiple range test at $p < 0.05$.

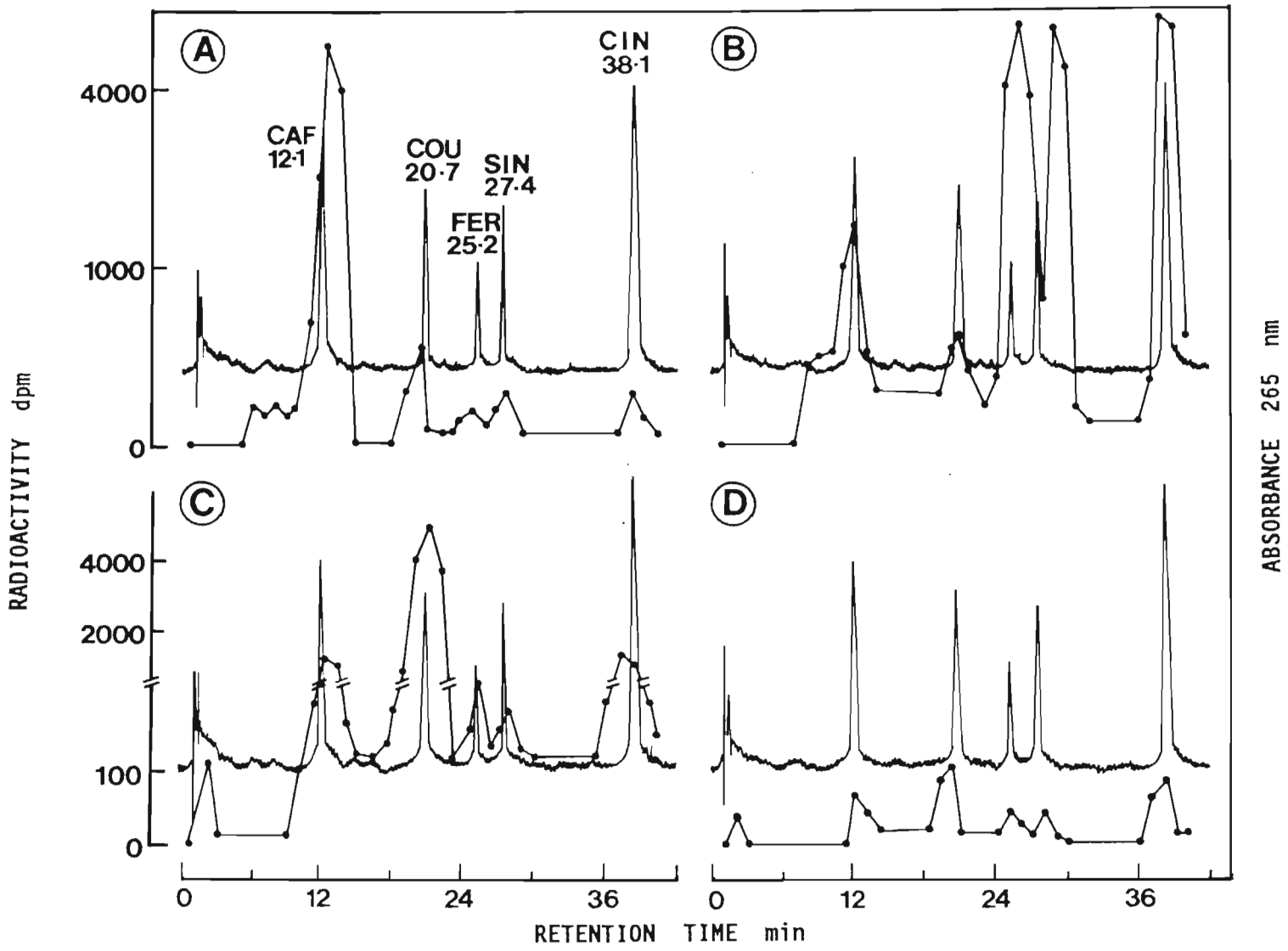
^{a,b} indicates values that are not significantly different calculated using analysis of variance and a multiple range test at $p < 0.05$.

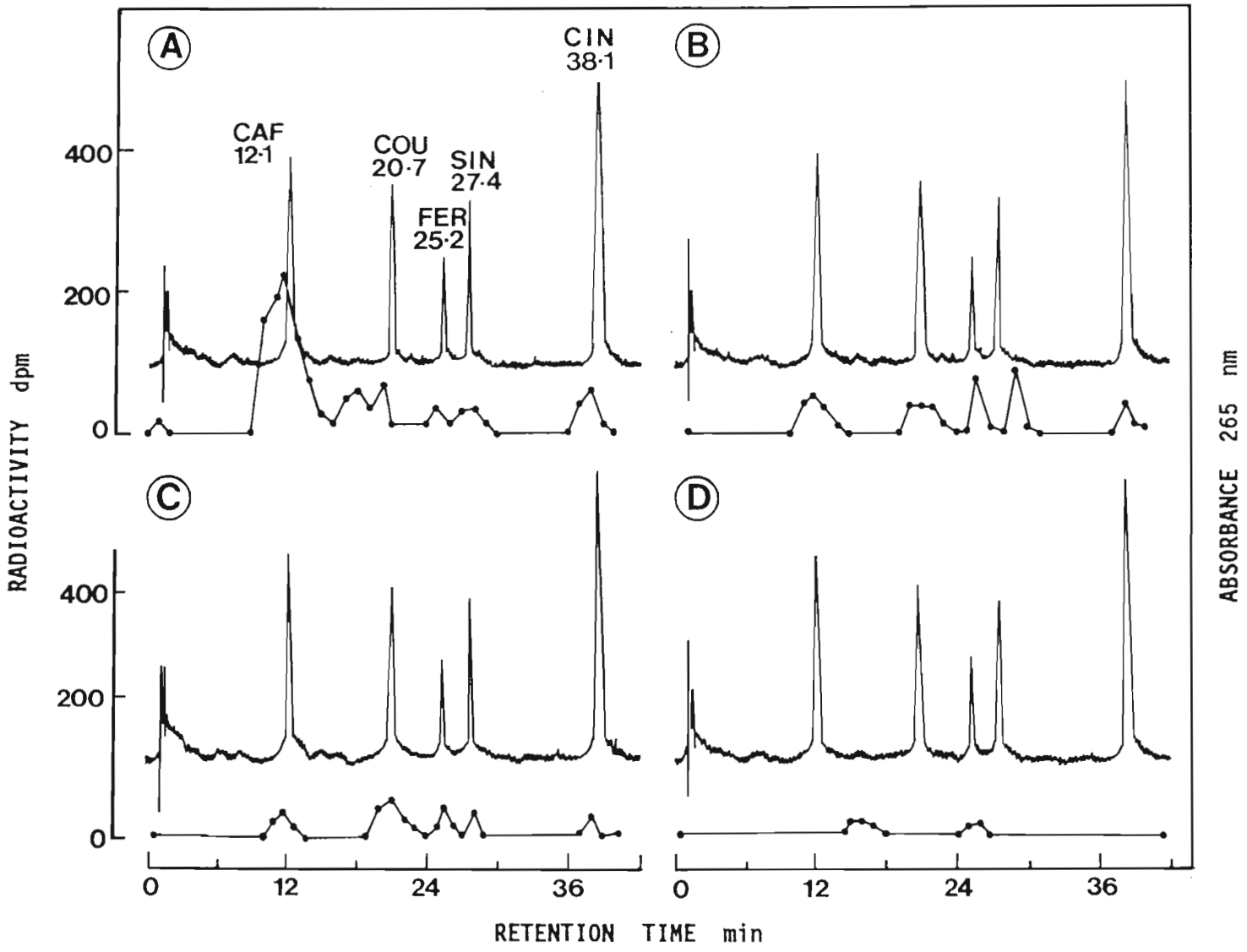
Incorporation of radioactivity into cinnamic acid and the hydroxycinnamic acids

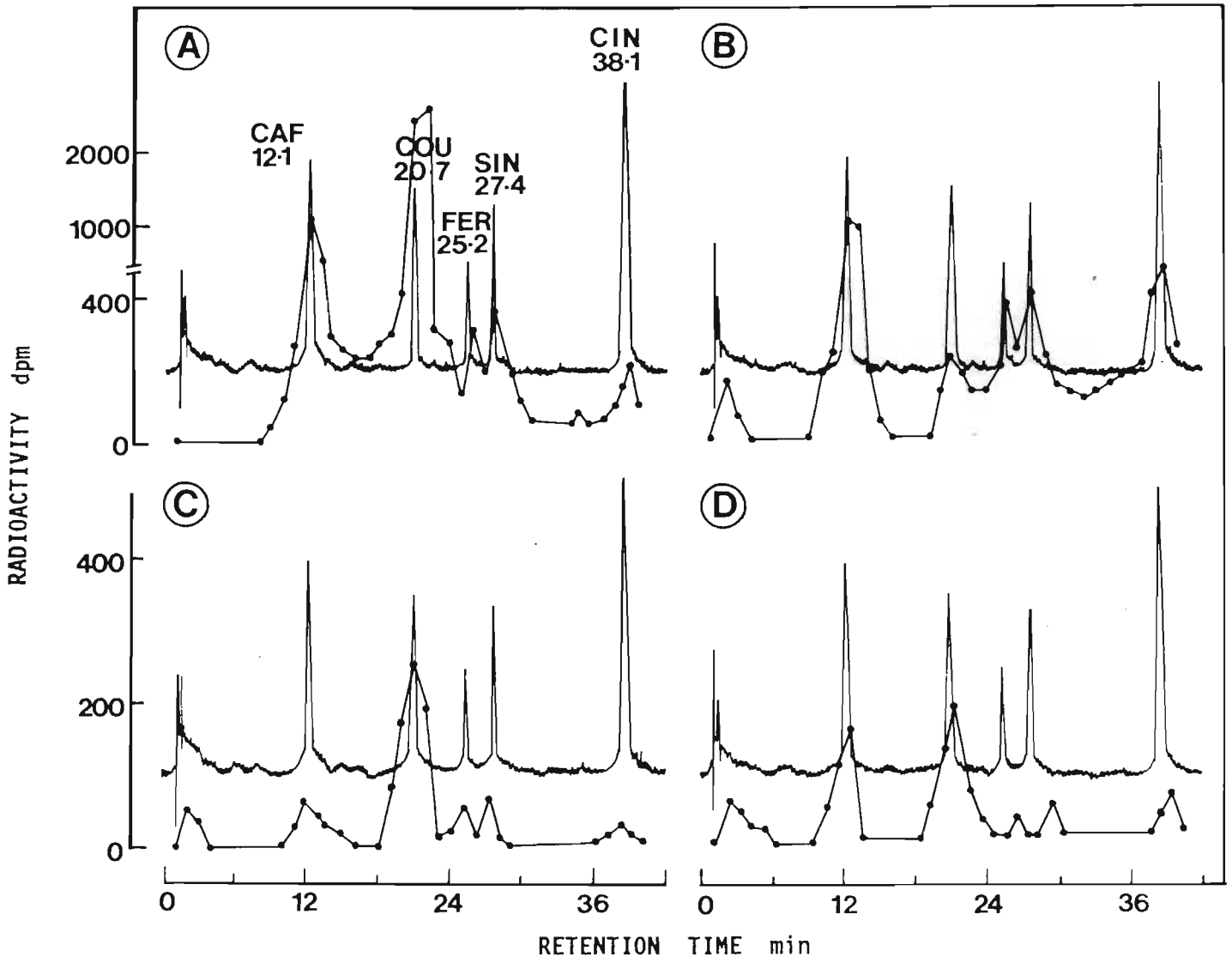
Upon separation with HPLC, the leaf extracts from all of the treatments yielded peaks of radioactivity which co-eluted with the retention times of all the authentic standards used (Figure 3.3.15). In the root extracts, such peaks of radioactivity were detected in the treatments only where ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid and ^{14}C-*p*-coumaric acid were applied (Figure 3.3.16). The corm extracts, upon separation with HPLC yielded peaks of radioactivity that co-eluted with all the standards (Figure 3.3.17).

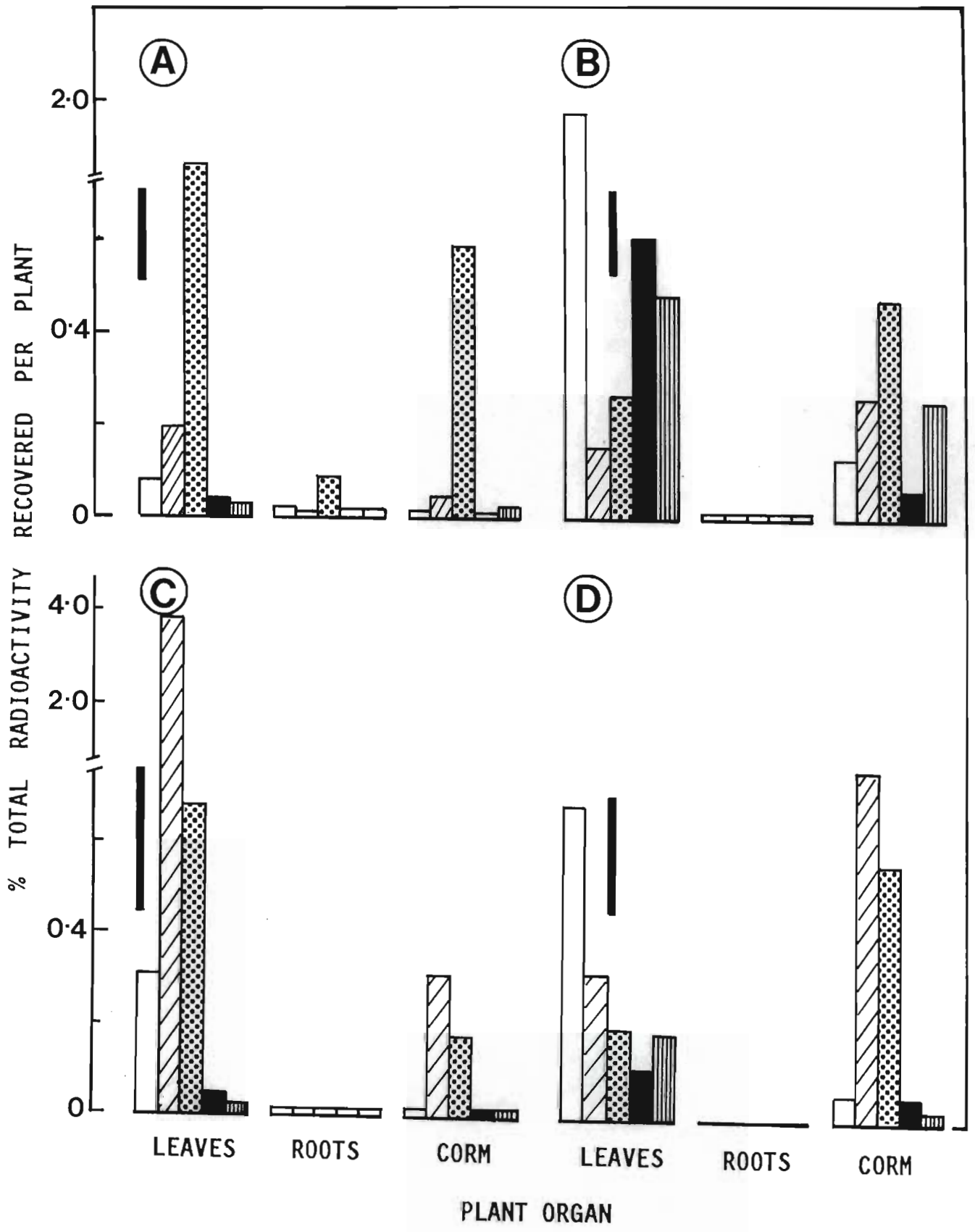
The quantification of the peaks of radioactivity recovered at the retention times of cinnamic acid and the hydroxycinnamic acids in the extracts from the leaves, roots and corms for all of the treatments is presented in Figure 3.3.18. When ^{14}C-phenylalanine was applied (Figure 3.3.18 A) most of the radioactivity recovered from the leaves was associated with caffeic acid and to a lesser extent with *p*-coumaric acid. In the roots very little radioactivity was associated with these acids. In the corm extracts almost all of the radioactivity recovered was associated with caffeic acid.

Application of ^{14}C-*t*-cinnamic acid (Figure 3.3.18 B) produced a different distribution of the recovered radioactivity associated with the hydroxycinnamic acids. Most of the radioactivity recovered from the leaves in this treatment was associated with *t*-cinnamic, ferulic and sinapic acids. Much less radioactivity was incorporated into *p*-coumaric and caffeic acids. Once again very little radioactivity was recovered in association with cinnamic acid and the hydroxycinnamic acids in the roots. In the corm extracts, more radioactivity was associated with *p*-coumaric and caffeic acids than in the leaf extracts


A
B
C
D
CAF
12·1
COU
20·7
SIN
27·4
FER
25·2
CIN
38·1
4000
1000
0
4000
2000
100
0
0
12
24
36
RETENTION TIME min
RADIOACTIVITY dpm
ABSORBANCE 265 nm


A
B
C
D
CAF
12·1
COU
20·7
SIN
27·4
FER
25·2
CIN
38·1
400
200
0
RADIOACTIVITY dpm
ABSORBANCE 265 nm
0
12
24
36
RETENTION TIME min


A
B
C
D
CAF
12·1
COU
20·7
FER
25·2
SIN
27·4
CIN
38·1
2000
1000
400
0
400
200
0
0
12
24
36
RETENTION TIME min
RADIOACTIVITY dpm
ABSORBANCE 265 nm


A
B
C
D
2·0
0·4
0
4·0
2·0
0·4
0
% TOTAL RADIOACTIVITY RECOVERED PER PLANT
LEAVES
ROOTS
CORM
LEAVES
ROOTS
CORM
PLANT ORGAN

while less radioactivity was detected at the retention times of ferulic and sinapic acids.

In leaf extracts of plants treated with ^{14}C-*p*-coumaric acid (Figure 3.3.18 C), most of the radioactivity recovered was present in *p*-coumaric and caffeic acids with some incorporation into *t*-cinnamic acid. The root extracts contained very little radioactivity in association with these acids. In the corm extracts most of the radioactivity was detected at the retention times of *p*-coumaric and caffeic acids.

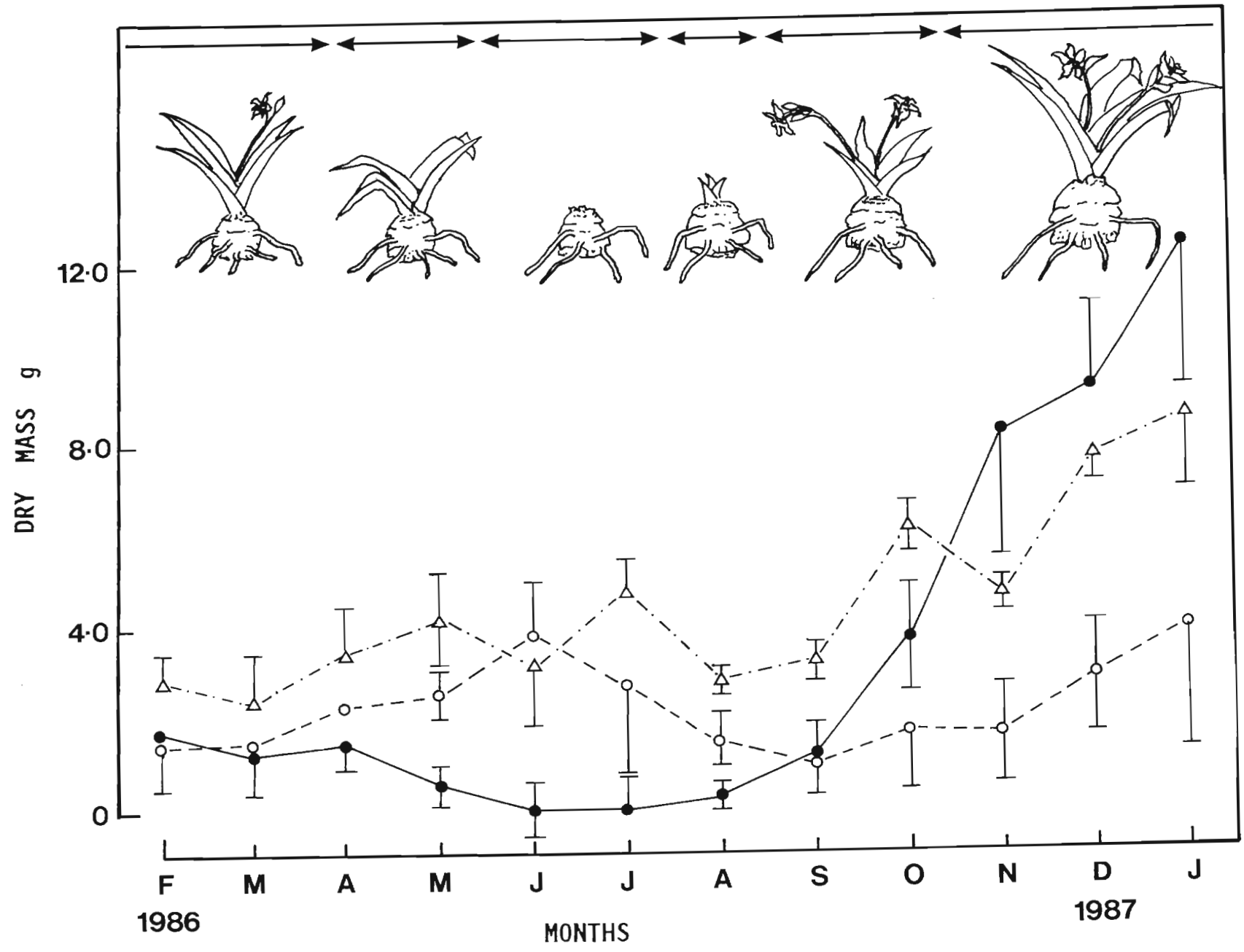
In extracts from the leaves of plants treated with ^{14}C-caffeic acid (Figure 3.3.18 D), a large quantity of the recovered radioactivity was associated with *t*-cinnamic acid, followed by lower incorporation into the hydroxycinnamic acids. In the roots radioactivity was not associated with these acids. In the corm extracts most of the radioactivity was associated with *p*-coumaric and caffeic acids.

While the distribution of the applied radioactivity differed in the leaf extracts, the results from the corm extracts follow a similar trend in all of the treatments, with most of the radioactivity being associated with *p*-coumaric and caffeic acids.

3.3.3. SEASONAL FLUCTUATIONS IN HYPOXOSIDE

Plant growth

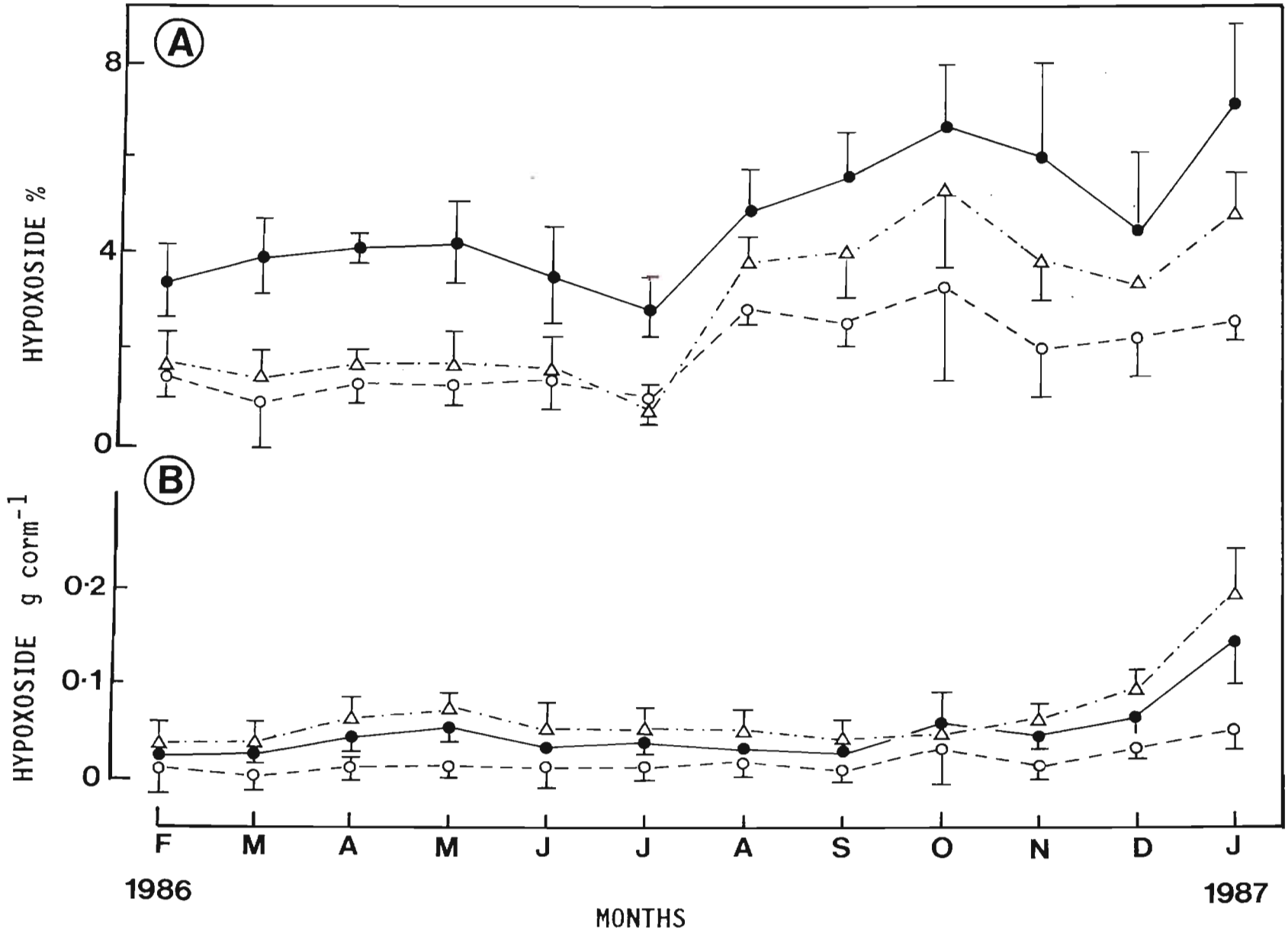
In the early part of the study (February 1986 to May 1986) the general growth trend was for the corm and roots of the plants to increase in mass, while the leaf mass began to lag (Figure 3.3.19). During this period the corms contributed greatest part to the dry mass of the


DRY MASS g
12·0
8·0
4·0
0
F
M
A
M
J
J
A
S
O
N
D
J
1986
1987
MONTHS

plants, when compared to the leaves and roots. By June the plants were dormant and had lost their leaves. From June through to September the roots decreased in mass. From June to July corm mass remained approximately constant. During August, however, a decrease in corm mass was observed. August was the time at which corms broke dormancy and produced shoots. From August through to January the leaves increased substantially in mass with a flush of growth occurring between October and November. From September through to October the corm mass increased but decreased in November at the time when the flush of shoot occurred. From November to January the corm increased in mass with growth decreasing slightly in January. From September to the end of January the mass associated with the roots increased.

Quantification of hypoxoside

In most cases the percentage of hypoxoside, calculated on a dry mass basis, was significantly higher in the upper corm than in the lower corm (Figure 3.3.20 A). However, the fluctuations in the percentage hypoxoside contained in the two halves of the corms, showed a similar trend. Thus only the level in the whole corm will be considered further. From February through to the end of June the percentage of hypoxoside remained constant. During July this level decreased, but not significantly so. From August to October, however, the level increased significantly and then decreased again through to the end of December, but not significantly so. In quantifying hypoxoside as a percentage of the dry mass of the corm, the values obtained, become dependent on the growth of the corm and do not actually reflect the times at which hypoxoside biosynthesis may be occurring. Thus the content of hypoxoside per corm was calculated (Figure 3.3.20 B). While the upper corm contained more hypoxoside than the lower corm,


A
8
4
0
HYPOXOSIDE %
B
0·2
0·1
0
HYPOXOSIDE g corm⁻¹
F
M
A
M
J
J
A
S
O
N
D
J
1986
1987
MONTHS

the trend in fluctuations was the same. Towards the end of summer (March, April, May) the level of hypoxoside increased slightly, but not significantly so. During winter (June, July and August) the level decreased slightly and then remained constant, decreasing again in spring (September). From October through to December, when the shoot was growing rapidly, the amount of hypoxoside present in the corms increased very slightly. Between December and January, when the growth of the leaves and corm slowed down, the level of hypoxoside increased significantly.

Thus, hypoxoside biosynthesis appeared to occur when growth of the shoot slowed down (December to January) and stopped altogether (January to May). During these times growth of the corm did occur as the mass of these organs increased.

3.4. DISCUSSION

3.4.1. SITE OF SYNTHESIS

The level of hypoxoside detected in the 9-month old corms (2.4 percent) was lower than those of 3.5 to 4.5 percent quoted by DREWES, HALL, LEARMONTH and UPFOLD (1984). These authors did not report the age of the corms used, nor at what time of year the plants, from which they obtained corms, were harvested. Thus, both age and time of collection, could explain the difference in levels obtained in the current work and those of the authors mentioned. PAGE (1984) analysed the hypoxoside content of various regions of corms of *H. hemerocallidea* and reported that the upper halves of the corms were richer in hypoxoside than the lower halves. PAGE (1984) also analysed corms at quarterly intervals throughout a year (February, May, July and October) and observed a seasonal fluctuation in the hypoxoside levels in the corms, with the observed levels reaching the lowest value in winter.

The level of 0.109 percent hypoxoside, detected in the root material of *H. hemerocallidea* was twenty times less than that found in the corms. This level was, however, directly comparable with that found in the root producing callus (Chapter 2.3.1.1.).

The data obtained from the quantification of hypoxoside in the various organs of *H. hemerocallidea* suggests that the corm is the site for biosynthesis of this phytochemical, since it contains by far the greatest concentration of hypoxoside. However, the detection of the compound in all organs and the fact that PAGE and VAN STADEN (1987) detected hypoxoside *in vitro* in tissue displaying root

differentiation, meant that the possible role of all the organs in hypoxoside biosynthesis, had to be considered.

Results obtained from the excised root and corm investigations were disappointing, with extensive browning of the tissue occurring. In both of these organs the applied ^{14}C-phenylalanine appeared to be converted to *t*-cinnamic acid which was shown in Chapter 2 to be a precursor for hypoxoside, yet no incorporation into hypoxoside occurred in either excised organ. A number of reasons could provide an explanation for this situation.

(i) Poor uptake by, and/or transport of the applied radioactivity to the biosynthetic tissues.

(ii) The extensive wounding, resultant from the excision of the corm and roots, may have elicited a wound response, the consequence of which was that the precursors for hypoxoside were channelled into the phenolics associated with the browning that occurs with such a wound response.

(iii) The corm and/or roots may not be the site of hypoxoside biosynthesis. The role of these organs, in relation to this phytochemical may just be one of storage .

The values recorded for the uptake of the ^{14}C-phenylalanine by the root and corm tissue, are comparable for those obtained for ^{14}C-phenylalanine and *t*-cinnamic acid uptake by the corm tissue of whole plants in the experiment in section 2.3.1.6, where incorporation into hypoxoside occurred.

The considerable browning which occurred in the excised organ tissue, was indicative of wounding, since this response is brought about by the metabolism of phenolics during wounding (LUH and PHITHAKPOL, 1972;

HERRMANN, 1976; MAYER and HAREL, 1979). The phenolic acids play an important role in the browning of tissue through their conversion to polyphenolic compounds (HERRMANN, 1976). In the presence of oxygen, the enzyme polyphenol oxidase (PPO) oxidises these phenolic compounds to quinones, which polymerise into brown pigments (MATHEW and PARPIA, 1971; LUH and PHITHAKPOL, 1972; RIBÉREAU-GAYON, 1972; RAMIREZ-MARTINEZ and LUH, 1973 ; HANSON and HAVIR, 1979; ROBINSON, 1980; RHODES, WOOLTORTON and HILL, 1981).

Upon wounding , there is often an increase in the enzymes responsible for conversions in the phenolic metabolic pathways, including phenylalanine ammonia lyase, cinnamic acid-4-hydroxylase and hydroxycinnamate (RHODES and WOOLTORTON, 1978). Increased levels of phenylalanine ammonia lyase activity have been reported in pea seedlings (HYODO and YANG, 1971), citrus fruit peel (RIOV, MONSELISE and KAHAN, 1968), excised bean axes (WALTON and SONDHEIMER, 1968), sweet potato (MINAMIKAWA and URITANI, 1965a, 1965b), swedes (RHODES and WOOLTORTON, 1971), buckwheat (ARMHEIN and ZENK, 1971) and gherkin (ENGELSMA, 1968). This increased activity of phenylalanine ammonia lyase can be linked to the ethylene produced during the wounding (HYODO, KURODA and YANG, 1978; CAMM and TOWERS, 1973).

The increased levels of the enzymes of phenolic metabolism and the channelling of the phenolic acids into the compounds responsible for tissue browning, suggests that this browning effect is important in the wound response. It is thought that this production of brown pigments occurs as a defense mechanism against microbial infection (RHODES and WOOLTORTON, 1978).

Since the browning observed, is caused by the oxidation of phenolic compounds, it can be postulated that much of the phenylalanine that was taken up and converted to phenolic acids, was channelled into the polyphenolic compounds associated with the wounding, at the expense of hypoxoside biosynthesis.

It became obvious from this experiment that a system, where the wounding was kept to a minimum and one which examined the biosynthetic potential of all of the organs, was required. Thus, whole plants treated and harvested over time, were used.

In the experiment where ^{14}C-phenylalanine was applied to whole plants, radioactivity was incorporated into hypoxoside in the corms. This was observed throughout the experiment, making it obvious that this organ was the major site for hypoxoside biosynthesis. This is further supported by the finding that the other organs, particularly the leaves, contained most of the radioactivity recovered. Nevertheless, while the corm may be the predominant site for biosynthesis, the possibility that the roots are capable of synthesising this biochemical, cannot be disregarded, firstly because a quantifiable level of hypoxoside was detected in these organs and secondly, this compound is synthesised by root producing callus, as shown in this study (section 2.3.1.2) and by PAGE (1984). The likelihood that hypoxoside could be synthesised in the roots and transported to the corm tissue or *vice versa*, would be dependent on the ease with which hypoxoside transport could occur in these tissues.

VAN STADEN, PAGE and FORSYTH (1986) applied hypoxoside to soybean callus (*Glycine max* (L) Merr. var. Acme) and found that while the compound was present in the cytoplasm, a fact determined with

electron microscopic studies, cell division ceased. Once sequestered in the vacuole, cell division commenced. Furthermore, PAGE (1984) and VAN STADEN, PAGE and FORSYTH (1986) reported that in the corm material, electron microscopic studies revealed that, the majority of the phenolic compounds appeared to be localised in the vacuole. However, an association of osmiophilic bodies with the ER was also reported (VAN STADEN, PAGE and FORSYTH, 1986). Whether this association of osmiophilic bodies with the ER of corm cells is indicative purely of synthesis or transport within the cell, is unknown. It seems likely, however, that in view of the inhibitory effect of hypoxoside on cell division, that the synthesis and sequestering of this phytochemical would be intracellularly confined. Thus it seems unlikely that transport of hypoxoside would occur to any extent in the tissues of *H. hemerocallidea*, suggesting that hypoxoside biosynthesis occurs in the tissues where it is found to have accumulated.

The possibility that the leaves synthesise hypoxoside, appears to be unlikely, firstly because such low levels of it have been detected in the leaves and shoot producing callus (section 2.3.1.1) and secondly, because no incorporation of radioactivity into hypoxoside occurred in these organs, which accumulate most of the radioactivity when ^{14}C-phenylalanine is applied. It is very probable that the small quantity of hypoxoside detected in these organs came from the basal part of leaves at the junction with the corm.

For the compounds structurally related to hypoxoside, the diarylpentane type compounds, the diarylheptanoids, the [*n*]-gingerols, the *meta,meta*-bridged biphenyls and the 9-phenylphenalenones, only a few definite reports exist on sites of synthesis.

The diarylheptanoid, acerogenin A is synthesised in the shoots of *Acer nikoense* (INOUE, KENMOCHI, FURUKAWA and FUJITA, 1987), the [*n*]-gingerol, 6-gingerol, is synthesised by excised rhizome slices of *Zingiber officinalis* and the 9-phenylphenalenone, haemocorin, is synthesised by rhizome slices of *Haemodorum corymbosum* (DENNIFF, MACLEOD and WHITING, 1980; THOMAS, 1971). These organs were also the locality at which most of the major accumulation of these compounds occurred within the respective plants.

From the examination of the levels of radioactivity incorporated into cinnamic acid and the hydroxycinnamic acids in the treatment of whole plants with ^{14}C-phenylalanine, it is interesting to find that, in all of the organs at all of the time intervals, most of the radioactivity associated with these acids was associated with *p*-coumaric and caffeic acids. Additionally, the levels of incorporation in the leaves and roots increased while those in corm decreased over the eight week period. The decrease in the levels of radioactivity associated with *p*-coumaric and caffeic acids, tend to suggest utilisation of these hydroxycinnamic acids. This is interesting in view of the fact that the corm appeared to be the organ in which most hypoxoside synthesis was located.

When the different corm regions were examined for their biosynthetic potential, the data obtained after 2 weeks indicated that the localisation of biosynthesis was dependent on precursor availability. Since at this time most of the radioactivity was present in the inner part of the corm, this is where most biosynthesis occurred. This initial localisation of radioactivity in the inner part of the corm, can be explained by the observation that this region contained a large number of vascular bundles, which meant that any radioactive compound

entering the corm from the shoot apical region, would reach this area of the corm first. After 4 weeks, when the radioactive distribution equalised between the regions, it appeared that the upper outer region of the corm was more proficient at this process. After 8 weeks this effect was not so pronounced.

It seems that it can not be conclusively stated that hypoxoside biosynthesis occurs predominantly in the the outer region of the corm. However, it is certain that the upper region is more efficient at this process than the lower. This latter conclusion agrees with PAGE'S (1984) report that more hypoxoside was detected in the upper halves of the corms than the lower halves of this organ. Since corms of the type found in *H. hemerocallidea* die back from the bottom (THOMPSON, 1972), the older more senescent tissue is located in the lower region whilst the younger tissue is found in the upper half. Secondary metabolism is a process which is usually associated with mature tissues. It was hoped that the electron microscope studies would help to elucidate this situation. Because the upper regions of the corm have been observed to contain a higher concentration of hypoxoside than the lower halves, hypoxoside must be recovered or broken down in the corm tissues as they senesce. At present how such a process would occur is unknown.

A pattern correlating the levels of radioactivity incorporated into *p*-coumaric acid and caffeic acid, with corm region, as function of time, was not found. *p*-Coumaric acid appeared in the corms at 2 weeks and then disappeared suggesting rapid utilisation while caffeic acid was abundant in the corm at 2 weeks, particularly in those regions where hypoxoside biosynthesis occurred. After this, the levels in the

various regions equalised, giving no clue as to the possible involvement of these acids in hypoxoside biosynthesis.

PAGE (1984) drew the conclusion that the osmiophilic bodies detected in the cells of corm origin, when this tissue was examined at the electron microscope level, were due to phenolic compounds. However, PAGE does point out that at the electron microscope level, definite phenolic identification is not an easy task. The phenolics are stainable (BRISSON, ROBB and PETERSON, 1976; McCLURE, 1979; HAYAT, 1981) but identifying which osmiophilic material is a result of their presence is difficult. Because phenolics have a more granular appearance than lipids (BRISSON, ROBB and PETERSON, 1976), PAGE (1984) considered the osmiophilic bodies in the vacuoles of corm cells to be of phenolic origin. Since hypoxoside is present in the corm in high concentrations, PAGE (1984) consequently assumed that the osmiophilic bodies would be representative of hypoxoside distribution. This assumption is adhered to throughout the current study.

In PAGE's (1984) electron microscopic studies of corm material, she detected osmiophilic globules in the vacuoles of the cell, sometimes in conjunction with the tonoplast. The cytoplasm in these cells was also densely packed with osmiophilic material. PAGE (1984) reported that MUELLER and BECKMAN (1974) consider this type of phenolic distribution to be an artifact due to poor fixation and leaching of phenolics, but she came to the conclusion that, in the study with *H. hemerocallidea*, corms fixation was adequate. The current study shows that as far as the distribution of phenolics within the corm cells is concerned, the observations by made PAGE (1984) were artifacts.

In the current study, osmiophilic bodies, similar to those observed by PAGE (1984), were detected in the vacuoles of the corm cells but never in contact with the tonoplast and very little osmiophilic material was observed in the cytoplasm. Thus, it is clear that fixation in glutaraldehyde caused leaching of the phenolics from the vacuole, while fixation with osmium tetroxide, only preserved the phenolics in the vacuole as aggregates. This was, however, at the expense of good fixation of the cytoplasm. Although this effect is recognised in this study, it is not thought to have occurred to an extent where it allays interpretation of structures observed in the cytoplasm of these cells.

There are many reports that the vacuole provides the site for localisation of secondary metabolites (MATILE, 1984; YAMAKI, 1984) including alkaloids (MATILE, 1978; SAUNDERS, 1979; DEUS and ZENK, 1982; NEUMANN, KRAUSS, HIEKE and GRÖGER, 1983; HOMEYER and ROBERTS, 1984) and phenolics (GROB and MATILE, 1979; LEIGH, AP REES, FULLER and BANFIELD, 1979; WAGNER, 1979; MOSKOWITZ and HRAZDINA, 1981; YAMAKI, 1984).

Whether these cells with vacuoles rich in what is presumed to be phenolic material, are the site for hypoxoside biosynthesis is unknown. Cells containing granular osmiophilic material in their vacuoles, were always observed to have many amyloplasts within the cytoplasm. The density of the rough ER and the tubular nature of this ER, suggests that these cells were highly synthetic or that there was a considerable degree of transport occurring (ROBARDS, 1970; GUNNING and STEER, 1975). As the occurrence of plasmodesmata, observed in these cells, was very low the possibility that the ER was serving only as transport organelle, is low. The discovery of tubular ER in

association with vesicles and densely staining osmiophilic bodies, suggests that the ER in these cells was synthetically active.

The subcellular localisation of hypoxoside biosynthesis is a matter that can only be speculated upon. The biosynthesis of secondary metabolites appears, in many cases to be a process, which is invariably localised in cells. This is not surprising considering their often toxic effect on cellular metabolism.

There are reports that ubiquinone biosynthesis occurs in mitochondria of cells of *Solanum tuberosum* L., *Narcissus pseudonarcissus* L. and *Spinacia oleracea* (LÜTKE-BRINKHAUS, LIEDVOGEL and KLEINIG, 1984). Tocopherol biosynthesis has been reported to occur in the chloroplasts and microsomes of cells of *Calendula officinalis* L. leaves (JANISZOWSKA, 1987) while casbene, a diterpene, appears to be synthesised in proplastids (DUDLEY, DUEBER and WEST, 1986). The enzymes responsible for the synthesis of monoterpenes in *Narcissus pseudonarcissus*. flowers are located in the stroma of the chromoplasts of the cells (METTAL, BOLAND, BEYER and KLEINIG, 1988). Esculetin, a dihydroxycoumarin, is reported to be synthesised in the chloroplasts of *Saxifraga stolonifera* W.(SATO, 1967). The synthesis of alkaloids has been reported to occur in the cytoplasm (DEUS-NEUMANN and ZENK, 1984; ZENK, 1985),in plastids (WINK and HARTMANN, 1981) and in vesicles (AMANN, WANNER and ZENK, 1986). It is thought that anthocyanin biosynthesis may be located in ER-bound multi-enzyme complexes (HOPP and SEITZ, 1987). While the hydroxycinnamic acids are sequestered in the vacuoles of cells of the leaves of *Raphanus sativus* L., their synthesis appears to occur in the cytoplasm (STRACK and SHARMA, 1985).

As mentioned earlier, the subcellular localisation of phenylalanine ammonia lyase and the cinnamic acid hydroxylases has not been elucidated; phenylalanine ammonia lyase activity being found mainly in the cytosol although there are reports which locate the activity of this enzyme in microsomes, chloroplasts and microbodies (ARMHEIN and ZENK, 1971; RUIS and KINDL, 1971; WEISSENBÖCK, 1975; GREGOR, 1976). BITSCH, TRIHBES and SCHULTZ (1984) maintain that the cinnamic acid hydroxylases are confined to the cytosol. CZINCHI and KINDL (1977) reported that in cucumber cotyledon cells, phenylalanine ammonia lyase and the cinnamic acid hydroxylases co-occur in the ER at a density which makes these organelles suitable as a compartment in which phenylalanine ammonia lyase and the cinnamic acid hydroxylases are controlled, most likely as a membrane bound enzyme complex.

The tentative hypothesis that hypoxoside is formed within the tubular ER and the observed vesicles, associated with it and the tonoplast, prior to transport into the vacuole where it is sequestered, is put forward. Further electron microscope studies are required but will be difficult to perform as the corm tissue is difficult to fix and section.

While the upper region of the corm was younger than the lower half of this organ, cells, in which the vacuolar osmiophilic material and amyloplasts were observed, were present in all parts of the corm. Thus it was not surprising to find that all regions of the corm were capable of synthesising hypoxoside. Furthermore, from the light microscope studies it was evident that the upper outer region of the corm contained the greatest concentration of these cells; the inner region containing many vascular bundles and the lower outer region being bisected by mucilage cavities. Thus the discovery by PAGE

(1984) that the upper outer region of the corm contained the highest percentage of hypoxoside is explained by this observation , as well as the present finding that the outer region of the upper half of the corm was apparently the corm region with the greatest potential for hypoxoside biosynthesis.

PAGE (1984) refers to the cambial region between the inner and outer ground tissue as a primary thickening meristem. According to CHEADLE (1937), ZIMMERMAN and TOMLINSON (1970) and ESAU (1977) this is in fact a cambium. A primary thickening meristem is found but at the apex of the corm at the leaf bases and it is continuous with the cambium.

3.4.2. TRANSPORT AND PRODUCTION OF CINNAMIC ACID AND THE HYDROXYCINNAMIC ACIDS

Cinnamic acid and the hydroxycinnamic acids accumulate in many plant tissues in the form of glycosides, esters or amides and are usually sequestered in the vacuole (STRACK, 1977; HASLAM, 1985; STRACK and SHARMA, 1985). Despite their being sequestered in the vacuoles of cells, they are considered to be essential to anabolic metabolism, being components of primary cell walls (HARTLEY, JONES and WOOD, 1976; FRY, 1982) and lignin (BROWN and NEISH, 1955; GROSS, 1979; HASLAM, 1985). Nevertheless, these shikimate derivatives, which are also precursors for many more complex phenolics, have been repeatedly reported to inhibit plant growth. There are many accounts of the phytotoxicity of ferulic acid (WANG, YANG and CHUANG, 1967; DEL MORAL and MULLER, 1970; McPHERSON, 1971; RASMUSSEN and EINHELLIG, 1977; BLUM and DALTON, 1985) and *p*-coumaric and caffeic acids have also been reported to act as allelopathic agents and to inhibit growth (WANG, YANG and CHUANG, 1967; DEL MORAL and MULLER, 1970; McPHERSON, 1971;

RASMUSSEN and EINHELLIG, 1977; BLUM, DALTON and RAWLINGS, 1984; BLUM, DALTON and SHANN, 1985a, 1985b). These are facts that have to be considered when the transport and availability of cinnamic acid, *p*-coumaric acid and caffeic acid as precursors for hypoxoside, are examined.

Quantification of these acids in the organs of *H. hemerocallidea* revealed that in all the tissues caffeic acid and sinapic acid accumulated to the greatest extent and this may indicate that these acids are the most stable of the hydroxycinnamic acids in this plant.

The observation that when 14-C carbon dioxide was administered to plants, the first organs to accumulate radioactivity in association with the hydroxycinnamic acids, were the leaves, suggests that in intact plants of *H. hemerocallidea* the shikimate pathway operates predominantly in the leaves. Throughout the duration of the experiment, the continual increase of radioactivity incorporated into these acids in the leaves compared to the roots, which even at 6 hours contained similar levels of radioactivity to the leaves, further substantiates the theory that the leaves are the main source of cinnamic acid and hydroxycinnamic acids for the rest of the plant.

The form in which these acids are transported from the leaves to the rest of the plant, is unknown. Although initially radioactivity accumulated predominantly in association with *p*-coumaric acid in all of the organs when ^{14}C-carbon dioxide was administered to plants, the transport of the acid within the plant was not efficient. When ^{14}C-*p*-coumaric acid was applied to the shoots of intact plants, compared to similar treatments with ^{14}C-*t*-cinnamic acid and ^{14}C-caffeic acids, the radioactivity associated with this acid was less effectively

transported to the roots and corm. However, since it was the first hydroxycinnamic acid to become radioactively labelled in all organs when 14-C carbon dioxide was applied, it does not appear that the hydroxycinnamic acids for which it forms a precursor, namely caffeic, sinapic and ferulic acids, can be predominant transport forms either. This apparently leaves cinnamic acid and phenylalanine as the most readily transported molecules amongst these shikimate derivatives.

Cinnamic acid was detected in all experiments usually at a low level except when ^{14}C-*p*-coumaric or caffeic acids were applied to intact plants. Cinnamic acid has been reported to be a strong inhibitor of phenylalanine ammonia lyase activity (MINAMIKAWA and URITANI, 1965b; SATO, KIUCHI and SANKAWA, 1982) and thus it is likely that, within the cell, rapid conversion of this shikimate derivative would be necessary to ensure that phenylpropanoid synthesis is kept operable. In the treatments where ^{14}C-*p*-coumaric acid and ^{14}C-caffeic acid were applied, substantial levels of ^{14}C-cinnamic acid were detected in the leaves in both treatments, but more so in the caffeic acid treatment which also resulted in a greater level of radioactivity, in association with the hydroxycinnamic acids, being transported to the corm. Thus the questions arise as to whether cinnamic acid is the transport form and whether the transport of *p*-coumaric acid and caffeic acid was dependent on the ease with which they were converted to cinnamic acid.

The present data does not answer this. It does show, however, that when these acids are applied to the whole plant they are transported, but in what form this occurs is not known at present. The situation is further exacerbated by the ready conversion of these acids to one another when they are applied exogenously. Nevertheless, it seems

probable that transport occurs in a form which occurs before hydroxylation and that accumulation occurs in the form of *p*-coumaric acid initially. The accumulation of the radioactivity in association with *p*-coumaric and caffeic acids to more or less the same extent in the corm does not elucidate their role, if any, as precursors for hypoxoside.

3.4.3. SEASONAL FLUCTUATIONS IN HYPOXOSIDE

While the percentage hypoxoside contained in the corms changes markedly with the seasons, it does not truly indicate when hypoxoside biosynthesis is occurring. These figures imply that the levels of hypoxoside decrease in winter (July) and that synthesis of this phytochemical occurs in early spring and throughout the early growing season. The conclusion drawn from the seasonal variation in the percentage hypoxoside present in the corms is that these variations are more indicative of fluctuations in corm dry mass than hypoxoside biosynthesis.

When the actual amount of hypoxoside per corm was used to indicate seasonal fluctuations instead, the conclusions reached were that hypoxoside biosynthesis occurred towards the end of the growing season when most of the growth of the leaves had occurred and that when the leaves were lost during dormancy, synthesis of the phytochemical appeared to stop. Corm growth did occur concurrently with increased hypoxoside production. Thus after the period of dormancy it seems that energy is expended in establishing the leaves, after which energy and precursors are available for corm growth and hypoxoside production.

It also appears that the corm's capacity, in terms of percentage hypoxoside stored, increases with age. This could be explained by the possibility that the ratio of ground tissue, which is envisaged to consist mostly of the storage tissue, increases in proportion to the other types of tissue, such as vascular and meristematic tissue, as the corm increases in size. Thus, this increase in percentage, would rather be a reflection of a greater proportion of storage cells being present, rather than an increase in the capacity of these storage cells, to synthesise the compound.

The sapogenin concentration, expressed as a percentage, is reported to increase with age in the seedlings of *Dioscorea tokoro* Makino (AKAHORI, YASUDA, OKUNO, TOGAMI, OKANISHI and IWAO, 1969) and in the tubers of a number of *Dioscorea* species (CRUZADO, DELPIN and ROARK, 1965; KARNICK, 1968; BLUNDEN, HARDMAN and HIND, 1970). KARNICK (1968) observed that maximum diosgenin content of *Dioscorea deltoidea* Wall. and *Dioscorea prazeri* Prain & Burk. occurred when the leaves of the plant were wilting and dying, just prior to dormancy. The diosgenin content, measured as a percentage, of tubers of *Dioscorea hondurensis* Knuth. decreased during the dormancy breaking period whilst in *Dioscorea sylvatica* Ecklon. this parameter increased with the appearance of the leaves (BLUNDEN, HARDMAN and HIND, 1970).

Diosgenin production in the tubers of *Dioscorea deltoidea* has been reported to reach its maximum at the end of the growing season, before dormancy occurs (BINDROO, BHAT and KACHROO, 1984) and the authors comment that the natural growth pattern of the plant appears to be influencing the sapogenin content of the tubers. This is obvious since synthesis of any phytochemical, will be dependent on precursor availability, a factor that is related to growth. BINDROO, BHAT and

KACHROO (1984) draw attention to the fact that the production of a secondary metabolite is closely related to precursor availability from primary metabolism, a process that is dependent on the ontogenetic development of the plant. The time at which synthesis of a secondary metabolite occurs, may be dependent on where it is synthesised in the plant and what purpose, if any, it fulfils.

Caffeine accumulation in *Camellia sinensis* L. appears to occur in all parts of the plant from the leaves, stems and roots to the flowers and seeds (SUZUKI, 1985; ASHIHARA and KUBOTA, 1986). Synthesis of this alkaloid has been reported to occur in the younger leaves but the authors do not specify whether these leaves were fully expanded or not (ASHIHARA and KUBOTA, 1986). SUZUKI (1985) reported that the most caffeine biosynthesis occurs in the flowers, while these organs are still in bud. Further studies have determined that caffeine biosynthesis also occurs in the fruit of *Camellia sinensis* and *Coffea arabica* during the development of these reproductive structures (SUZUKI and WALLER, 1985).

In these examples from the literature, secondary metabolite biosynthesis has been reported to occur in actively growing and/or expanding organs. This was also observed in this study when hypoxoside biosynthesis and corm growth occurred concurrently. However, the entire growth status of the plant must be considered. The stage of growth at which the rest of the *Camellia sinensis* plants were at, is not discussed but it is to be presumed that flowering would occur once most vegetative growth had ceased for the season.

Because the level of the batatasins (phenolic growth inhibitors) have been reported by IRELAND and PASSAM (1984) to increase rapidly in

Dioscorea alata L. tubers just before the onset of dormancy and decrease during the dormancy period, these researchers conclude that this group of secondary metabolites are playing an inhibitory role. However, they do not correlate batatasin content, which they quantify as a percentage, with tuber growth at this time.

There are accounts that show, that not only the quantity of secondary metabolites vary seasonally, but the ratios of those present are also subject to variation. The tannins in the leaf of *Liquidambar formosana* Hance., a Chinese medicinal plant, vary in composition during the summer months but the maximum concentration occurs in July and August towards the end of summer (HATANO, KIRA, YOSHIZAKI and OKUDA, 1986). There is a qualitative variation in the free flavone aglycones of the aerial parts of *Sideritis lencantha* Cav., a Spanish folk herb, but the greatest yield of flavonoids occurs towards the end of summer (BARBERAN, TOMAS and NUNNEZ, 1985).

In many cases it seems that secondary metabolite production occurs towards the end of the flush of vegetative growth. This situation appears to also exist for hypoxoside production, as the greatest production of the phytochemical occurred after the flush of shoot growth. The observation that biosynthesis and corm growth occurred simultaneously, does not contradict the hypothesis that secondary metabolism and growth are antagonistic, since it was the more mature cells of the corm that were probably biosynthetic, rather than the cells of younger more meristematic parts of the corm. Questions arise as to whether hypoxoside production is the result of "shunt" metabolism and what benefit the plant derives from the extensive production of this phenolic. These questions remain unanswered, requiring that the study of the plant in an ecological context be made.

CHAPTER 4

IN VITRO STUDIES OF HYPOXOSIDE PRODUCTION

4.1 INTRODUCTION

The initial optimism which fuelled the proposal for *in vitro* production of secondary products from plant cell cultures, has been somewhat dampened by the difficulties encountered in attempts to realize this alternative source for important phytochemicals (ELLIS, 1984; STABA, 1985; COLLIN, 1987).

Plants provide the source for many commercially important steroids, alkaloids, flavours, fragrances and terpenes for the pharmaceutical, cosmetic and food industries (STABA, 1985; COLLIN, 1987). Most of the plant species that provide these phytochemicals are situated in tropical climates, which is problematic for the major processors and consumers of the phytochemicals, who tend to be situated in the more temperate regions of the world. The problems encountered include, a reliable and constant source of the phytochemicals, which cannot be guaranteed since this requires cultivation of the plants including breeding of high producing lines, and efficient processing of the plant material upon harvesting. Given that many of the plant species in question are difficult to cultivate, the production of these compounds from cell culture *in vitro*, has been the logical alternative (COLLIN, 1987). Thus far, however, only one successful project for the commercial production of a secondary metabolite, has been reported. This being the production of shikonin, a dye and

bactericide, by the Mitsui Petrochemical Industries (Tokyo) using *Lithospermum* L. cells in 750 liter fermentors (STABA, 1985; COLLIN, 1987).

The initial hindrances to the idea of *in vitro* production of secondary metabolites, has forced researchers to take a new look at the systems they are dealing with. Fresh approaches have taken a more integrated view of the plant cell and are less inclined to impose the bacterial fermentor approach on plant cell cultures (ELLIS, 1984; HEINSTEIN, 1985; STABA, 1985; COLLIN, 1987).

The first set back in attempts to establish cell cultures as a source of secondary products, was the very low production or total absence of these metabolites in the cultured cells. However, by 1982 approximately 30 compounds were known to accumulate in plant culture systems at levels equal to or higher than the parent plant (STABA, 1982). Generally, this low level of production is thought to be inherent in the nature of the secondary metabolic system within plants, in that these processes are developmentally regulated by the plant genotype, with given pathways coming into operation during certain developmental phases, often in specialised tissues (ELLIS, 1984). *In vitro* such developmental phases and specialisation of tissue are not present. Current thought, however, advocates attempts to de-repress those genes that are repressed during the *in vitro* initiation of tissue from high producing explants (HEINSTEIN, 1985). This de-repression can involve use of factors such as, stress and fungal elicitors (ELLIS, 1984). Thus current findings support the following methods for obtaining and/or inducing secondary metabolites *in vitro*:

(i) selection of source plant and explant;

(ii) selection of growth and production media;

(iii) selection of abiotic factors, such as light and temperature;

(iv) identification and regulation of key enzymes; and

(v) isolation, identification and regulation of plant genes (HEINSTEIN, 1985; KURZ and CONSTABEL, 1985; COLLIN, 1987).

Although, the major interest in *in vitro* production of secondary metabolites has been to provide a source of these phytochemicals, these systems also provide a valuable tool for studying the relationship between primary and secondary metabolism (KURZ and CONSTABEL, 1985; COLLIN, 1987). This has been the primary purpose of using an *in vitro* system for investigating hypoxoside production in this study.

As mentioned in earlier chapters, PAGE (1984) and PAGE and VAN STADEN (1987) have reported that hypoxoside was produced at a quantifiable level only in those *in vitro* tissues of *H. hemerocallidea* that displayed root differentiation. They discovered that malformed root-producing callus contained the highest levels of hypoxoside (±0.015 percent) (PAGE and VAN STADEN, 1987) but that after repeated subculturing, this level dropped to approximately 0.005 percent. In the current study the level of hypoxoside detected in malformed tissue, after 10 months, was low (±0.005 percent) while that detected in root-forming callus was approximately 0.135 percent (dry mass) which was higher than that produced (0.004 percent, fresh mass) by the root-producing callus examined by PAGE and VAN STADEN (1987). Thus in the current investigation, root-producing callus was chosen as the *in vitro* system in which to study hypoxoside biosynthesis.

It was decided that the following areas would be investigated for effects on *in vitro* hypoxoside production:

(i) time dependency of production;

(ii) physical factors such as light and temperature;

(iii) nutrient availability;

(iv) supply of plant growth regulators; and

(v) supply of precursors for hypoxoside.

An inverse relationship has commonly been observed between culture growth and metabolite production (KURZ and CONSTABEL, 1985). Batch propagated cultures show a three stage growth cycle comprised of an initial lag phase, which occurs subsequent to inoculation onto new media, followed by a phase of exponential growth, which culminates in a stationary phase, during which cell division has ceased. It has been noted that secondary product formation usually occurs during the late exponential phase and proceeds into the stationary phase. Thus, the concept is held that growth is antagonistic toward secondary product formation (KURZ and CONSTABEL, 1985; COLLIN, 1987). The reasons for the popularity of this hypothesis and possible explanations as to why this relationship exists have been discussed in section 1.8 of Chapter 1.

PAGE (1984) did not examine the relationship between production of hypoxoside and growth for root-producing callus during an 8 week culture period. Thus this was the first area of investigation in the current *in vitro* study.

The effect of light on the production of secondary metabolites in *in vitro* systems, has been extensively studied, but due to the use of

different qualities and quantities of irradiation, interpretation of results has often been difficult (KURZ and CONSTABEL, 1985; COLLIN, 1987).

Generally light tends to stimulate the production of phenolics *in vitro* (HAHLBROCK and WELLMAN, 1970; STICKLAND and SUNDERLAND, 1972a; BUTCHER, 1977). PAGE and VAN STADEN (1987) report that hypoxoside production by malformed root-producing callus, was not favoured in the light; maintaining these cultures in the dark enhanced hypoxoside production. In the current study the effect of various intensities of light on the level of hypoxoside produced by root-producing callus was examined.

The role of temperature in secondary metabolite production *in vitro* has not been well researched. SAHAI and SHULER (1984) reported that while a temperature of 27 ^{o}C favoured production of phenolics in cultured cells of *Nicotiana tabacum* L., lower temperatures were detrimental. IKEDA, MATSUMOTO and NOGUCHI (1976) obtained results for ubiquinone production in *in vitro* cells of *Nicotiana tabacum*. A dramatic decrease in alkaloid production was obtained when cell suspensions of *Catharanthus roseus* (L.) G. Don were cultured below 27 ^{o}C (COURTOIS and GUERN, 1980). COLLIN (1987) has speculated that studies on temperature effects may contribute to a better understanding of the regulatory mechanisms involved in secondary metabolism.

Nutrient availability or media manipulation was one of the first areas in *in vitro* secondary metabolite research to receive attention in the bid to increase *in vitro* production. Many of the media that were developed, initially promoted cell growth *in vitro* but they had a

deleterious effect on secondary product formation. This is an observation that can be linked directly to the antagonistic relationship that often exists between growth and secondary metabolism (KURZ and CONSTABEL, 1985). Most nutrient media for plant tissue cultures are comprised of inorganic nutrients, a carbon source, vitamins, amino acids and growth regulators. Manipulation of these components is usually random and it is difficult to separate the direct effect on secondary metabolite production caused by changing media constituents, from an effect caused by a change in the growth pattern (COLLIN, 1987). Nevertheless, manipulation of media components, does provide valuable information.

The most commonly used carbon source for media is sucrose (KURZ and CONSTABEL, 1985; COLLIN, 1987). Sucrose has been found to be a superior carbon source when compared to glucose and fructose for shikonin biosynthesis in *Lithospermum* cultures (MIZUKAMI, KONOSHIMA and TABATA, 1977), digitoxin production in cell suspensions of *Digitalis purpurea* L. (HAGIMORI, MATSUMOTO and OBI, 1982) and diosgenin production in *Dioscorea deltoidea* Wall cultures (TAL, GRESSEL and GOLDBERG, 1982). Sucrose is generally considered to be the best carbon source for secondary metabolite production (KURZ and CONSTABEL, 1985).

In a number of studies where sucrose has been reduced dramatically, secondary metabolite production has been supressed. *Catharanthus roseus* cell cultures did not produce alkaloids under conditions of sucrose limitation (BALAGUÉ and WILSON, 1982), while under such conditions, anthroquinone production in *Galium mollugo* L. cell cultures decreased (WILSON and BALAGUÉ, 1985). KNOBLOCH and BERLIN (1980) observed that the supressed production of alkaloids in

Catharanthus roseus cell cultures, was alleviated by the addition of 8 percent (weight for volume) sucrose. WILSON and BALAGUÉ (1985) have proposed that during sucrose limitation energy is conserved in cellular maintenance to the detriment of secondary metabolite production.

Increasing the sucrose level of media has, according to some reports, raised secondary metabolite production. An increase in alkaloid production was achieved when the sucrose level of the medium was increased in *Catharanthus roseus* cell cultures during stationary phase (MERILLON, RIDÉAU and CHÉNIEUX, 1984). BHATT, BHATT and SUSSEX (1983) increased the production of the alkaloid, solasodine in *Solanum nigrum* L. cell cultures, by increasing sucrose concentration. High sucrose concentrations have been reported by MATSUMOTO, NISHIDA, NOGUCHI and TAMAKI (1973) to enhance anthocyanin formation in *Populus* L. cell suspension cultures while ZENK, EL-SHAGI and ULBRICH (1977) found that increasing sucrose concentration increased rosmarinic acid production in cell suspension cultures of *Coleus bumei* Benth.. MIZUKAMI, KONOSHIMA and TABATA (1977) obtained enhanced shikonin production in *Lithospermum* callus cultures when sucrose levels were elevated.

PAGE and VAN STADEN (1987) did not obtain a higher production of hypoxoside in malformed root tissue when the sucrose concentration was increased in the medium. The effect of increasing and decreasing the level of sucrose in the medium on hypoxoside production in *H. hemerocallidea* root-producing callus, was examined in this study.

Varying the concentrations of the macronutrients, such as nitrogen (usually supplied as nitrate and ammonium), phosphate and calcium, is widely reported to have an effect on secondary metabolite production *in vitro*.

Nitrogen is usually supplied in a nutrient medium as a nitrate or ammonium salt, although there are reports of other nitrogen sources, such as urea, amino acids and casein hydrolysate, being used. KURZ and CONSTABEL (1985) maintain that the level of nitrogen in a medium has a marked effect on both growth and secondary metabolite production and that the effect of the level of nitrogen on secondary metabolite production has to be correlated with the carbon sources available, since it is the carbon:nitrogen ratio, that is the important factor.

HAGIMORI, MATSUMOTO and OBI (1982) have shown that a reduction by two-thirds of the nitrogen supplied to *Digitalis purpurea* cell suspensions, increased digitoxin production but reduced growth. High and very low levels of nitrogen were observed to decrease both growth and digitoxin production. Thus, it appears that limited reduction in growth favours secondary metabolite formation in this system, while drastic reductions in growth are deleterious to secondary metabolism.

AMORIM, DOUGALL and SHARP (1977) have reported that a decrease in the nitrogen supplied in the medium increased phenolic production in Paul's Scarlet Rose cell cultures. SAHAI and SHULER (1984) have also recorded increased phenolic production brought about by decreasing the nitrogen supply, but in batch cultures of *Nicotiana tabacum*. Rosmarinic acid production was increased in cell suspension cultures of *Anchusa officinalis* L., by decreasing the nitrogen available in the medium (DE-EKNAMKUL and ELLIS, 1985a).

High levels of phosphate are generally thought to inhibit secondary metabolite production (KURZ and CONSTABEL, 1985). COLLIN (1987) draws attention to the fact that the situation is not this simple. KNOBLOCH, BEUTNAGEL and BERLIN (1981) have determined that increased levels of phosphate from zero upwards enhanced growth but repressed putrescine production in *Nicotiana tabacum* cell cultures. A similar trend was noted for alkaloid formation in *Catharanthus roseus* cultures (KNOBLOCH and BERLIN, 1980). HAGIMORI, MATSUMOTO and OBI (1982) observed increased digitoxin formation when the level of phosphate was raised in the medium of *Digitalis purpurea* cultures. A similar response was noted for rosmarinic acid production which was enhanced in *Anchusa officinalis* cultures when the phosphate level was elevated (DE-EKNAMKUL and ELLIS, 1985a).

The effect of phosphate levels is an example of the close balance that is thought to exist between substrate supply to secondary and primary metabolism. It can be argued that when phosphate is available growth is stimulated to the detriment of secondary product formation, but when the level of phosphate is such that substrate availability for growth is more than sufficient, subsequent "shunting" of the excess phosphate into secondary metabolism occurs.

Nitrogen and phosphate are the two macronutrients that are most commonly manipulated, with respect to amount supplied in the media, in *in vitro* secondary metabolite studies. However, DE-EKNAMKUL and ELLIS (1985a) have reported that decreasing the level of calcium available in the medium enhanced production of rosmarinic acid in *Anchusa officinalis* cell cultures.

Of the macronutrients discussed, PAGE and VAN STADEN (1987) investigated only nitrogen. They obtained an increase in hypoxoside production by reducing the level of nitrate and ammonium nitrogen in MURASHIGE and SKOOG'S (1962) medium by half.

In the present study the levels of nitrogen, phosphate and calcium were investigated for their affect on hypoxoside production in root producing callus of *H. hemerocallidea*. The concentration of nitrogen present in MURASHIGE and SKOOG (1962) medium was reduced, while those of phosphate and calcium were both reduced and increased. Each macronutrient mentioned, was examined individually and then in combination with the other two. The effect of sucrose was included in this study.

The plant growth regulators included in incubation media do affect secondary metabolite production. Since they affect growth and cellular differentiation as well, it is not known whether the growth regulators actually trigger secondary metabolism or whether these growth compounds shift cellular metabolism in a direction that favours secondary metabolite production (KURZ and CONSTABEL, 1985; COLLIN, 1987).

It is predominantly growth regulators belonging to the auxins and cytokinins which have been examined for their potential to affect secondary metabolism *in vitro*. This probably results from the fact that it is mainly these two groups of growth regulators that are employed in most *in vitro* systems.

Auxins appear to elicit a greater response in affecting secondary metabolism *in vitro*, than cytokinins. The response to auxin fluctuations is dependent on the system under observation, the type of

auxin applied and the range of concentrations used. In some reports 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA) and naphthaleneacetic acid (NAA) stimulated the production of secondary products. FURUYA, YOSHIKAWA and TAIRA (1984) obtained increased production of saponin in callus cultures of *Panax ginseng* Mey. by increasing 2,4-D, while high concentrations of 2,4-D, IAA and NAA enhanced indole alkaloid production in *Cinchona ledgeriana* Moens tissue *in vitro* (HARKES, KRIJBOLDER, LIBBENGA, WIJNSMA, NSENGIYAREMGE and VERPOORTE, 1985).

However, in *Haplopappus* Cass. corr. Endl. cultures STICKLAND and SUNDERLAND (1972b) and CONSTABEL, SHYLUK and GAMBORG (1971) observed that high concentrations of NAA and 2,4-D reduced anthocyanin production. SAHAI and SHULER (1984) found that low concentrations of 2,4-D promoted total phenol accumulation in cell cultures of *Digitalis purpurea*. An increase in digitoxin levels was obtained by HAGIMORI, MATSUMOTO and OBI (1982) when the levels of 2,4-D were decreased in this *in vitro* tissue.

Generally low concentrations of auxin are considered to favour secondary metabolite production (KURZ and CONSTABEL, 1985; COLLIN, 1987).

The role that the cytokinins play in *in vitro* phytochemical production is not as clear as that of the auxins. Varying the kinetin levels had very little effect on digitoxin formation in *Digitalis purpurea* (HAGIMORI, MATSUMOTO and OBI, 1982). HARKES, KRIJBOLDER, LIBBENGA, WIJNSMA, NSENGIYAREMGE and VERPOORTE (1985) have noted a similar absence of effect with this synthetic cytokinin for anthroquinone production in *Cinchona ledgeriana* cultures. However,

high levels of kinetin were inhibitory for indole alkaloid synthesis in this system (HARKES, KRIJBOLDER, LIBBENGA, WIJNSMA, NSENGIYAREMGE and VERPOORTE, 1985) while high levels of benzyladenine (BA) resulted in an accumulation of alkaloids in *Catharanthus roseus* cultures (ZENK, EL-SHAGI, ARENS, STOCKIGT, WEILER and DEUS, 1977).

PAGE (1984) obtained the optimum growth for root- and shoot-producing callus of *H. hemerocallidea*, by maintaining the calli on the macro- and micro-nutrients of MURASHIGE and SKOOG (1962), supplemented with 0.001 g l^{-1} NAA and 0.001 g l^{-1} BA. In this investigation the effects on hypoxoside production and growth brought about by varying concentrations of the auxins, NAA, IAA and 2,4-D, whilst the cytokinin compliment of the medium was kept constant at 0.001 g l^{-1} BA, in root-producing callus of *H. hemerocallidea*, were examined. The effects of the various cytokinins, BA, zeatin and kinetin at a range of concentrations in combination with 0.001 g l^{-1} NAA, were also examined.

If the accumulation of a secondary metabolite *in vitro* is dependent on precursor availability, a factor that is apparently governed by the demand for the precursor by primary metabolism, then the exogenous addition of the precursor to the *in vitro* system should promote the biosynthesis of the metabolite in question. An aspect of such a study that should be considered, is the possible toxicity of the applied precursor when fed in levels exceeding the endogenously regulated norm of the tissue.

In fact precursor addition has been shown to enhance secondary metabolite production in a number of *in vitro* systems. MIZUKAMI, KONOSHIMA and TABATA (1977) observed that addition of L-phenylalanine,

a precursor for shikonin, markedly stimulated shikonin derivative formation in *Lithospermum erythrorhizon* Sieb. & Zucc. cultures, while the acids, *t*-cinnamic acid and *p*-coumaric, acid were toxic to cell growth and inhibited shikonin derivative formation at levels above 10^{-5}M.

An increase of 90-fold for the yield of alkaloids from cell suspension cultures of *Cinchona pubescens* Lamb, was obtained by KOBLITZ, KOBLITZ, SCHMAUDER and GRÖGER (1983) when the precursor L-tryptophan was supplied in the medium. These researchers also noted that addition of the precursor at high levels had an adverse effect on growth. HAY, ANDERSON, ROBERTS and PHILLIPSON (1986) determined that addition of L-tryptophan to root organ suspension cultures of *Cinchona ledgeriana* resulted in a 5-fold increase in quinine and quinidine production but had a deleterious effect on growth.

The effect of precursor feeding on hypoxoside production *in vitro* was examined by supplying *H. hemerocallidea* root-producing callus with various concentrations of L-phenylalanine, *t*-cinnamic acid, *p*-coumaric acid and caffeic acid.

4.2 MATERIALS AND METHODS

4.2.1 Material

Root-producing callus was initiated from corm explants according to the method of PAGE (1984) described in section 2.2.1.1.

The root-producing callus was maintained on 50 ml basal medium in 100 ml conical flasks with non-absorbent cotton wool stoppers. The basal medium (BM), on which the callus was maintained, consisted of MURASHIGE and SKOOG (1962) micro- and macro-nutrients (Table 2.2.1) supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol, 0.001 g l^{-1} naphthaleneacetic acid (NAA) and 0.001 g l^{-1} benzyladenine (BA). The BM was solidified with one percent agar at pH 5.8. Media were autoclaved at 121 ^{o}C and one bar-pressure for 20 minutes. All subsequent manipulations were carried out aseptically on a laminar flow bench. The callus was incubated at 23 ± 2 ^{o}C at a light intensity of 0.4 $\mu E\ m^{-2}\ s^{-1}$. The callus was subcultured onto fresh BM at 8 weekly intervals.

4.2.2 Experimental Design

In all experiments described, the root-producing callus was innoculated onto the BM described in 4.2.1, except where experimental changes are mentioned. For each treatment three replicates were used and ±0.75g (fresh mass) root-producing callus was inoculated onto 30 ml BM in 50 ml conical flasks stoppered with non-absorbent cotton wool bungs. The root-producing callus was maintained under the incubation conditions outlined in 4.2.1, except where experimental design differs. After 8 weeks the callus from each treatment was

harvested by flash freezing with liquid nitrogen and freeze drying for 24 h. Dried callus was milled, weighed and stored at -20 ^{o}C until analysis.

From each replicate, in each treatment, for each experiment, 0.05 g of dried and milled material was extracted and assayed for hypoxoside as described in 2.2.1.1. The mean percentage hypoxoside and callus dry mass were calculated in each case and least significant differences were determined at $p < 0.05$ percent except in the case of the time experiment where standard errors were determined at a 95 percent confidence level.

Time study

Root-producing callus was subcultured and maintained on the normal BM under the described conditions. The callus was harvested at the following time intervals: 0, 2, 4, 5, 6, 7, and 8 weeks, respectively and assayed for hypoxoside.

Light study

Root-producing callus was subcultured and maintained on the normal BM under the described conditions, except for a change in light regime. The callus was incubated at the following light intensities, 0, 0.4 and 35 $\mu E\ m^{-2}\ s^{-1}$ for 8 weeks. The photoperiod in the case of the latter two intensities was 16 hours. The callus was harvested after 8 weeks and prepared for analysis.

Temperature study

Root-producing callus was subcultured onto fresh BM and incubated at a light intensity of 0.4 $\mu E\ m^{-2}\ s^{-1}$ at the following temperatures for 8

weeks: 10, 20 and 30 ^{o}C. Upon termination of the experiment the callus was harvested and prepared for analysis.

Nutrient study

Root-producing callus was subcultured onto fresh BM, the ratio of components of which had been altered in a number of ways. The callus was incubated for 8 weeks prior to harvesting. The alterations of the BM included the following treatments:

(i) **Sucrose** was adjusted to either 20 or 40 g l^{-1} with the inclusion of a 30 g l^{-1} control.

(ii) **Nitrogen** was adjusted to a half and a quarter of the normal concentration present in the MURASHIGE and SKOOG (1962) medium by adding half and quarter amounts of the ammonium nitrate and potassium nitrate (Table 2.2.1) that is usually included. A control with the normal nitrogen levels was included.

(iii) **Phosphate** concentrations were adjusted to one and a half, a half and a quarter times the normal level by altering the potassium ortho-phosphate component of the MURASHIGE and SKOOG (1962) medium. A control containing the usual level of phosphate was included.

(iv) **Calcium** concentrations were altered to one and a half, a half and a quarter times the normal value by altering the calcium chloride component of the MURASHIGE and SKOOG (1962) medium accordingly. A control was included.

Combined nutrient study

Root-producing callus was subcultured onto BM in which the concentrations of sucrose, ammonium nitrate and potassium nitrate, potassium orthophosphate and calcium chloride had been altered in

Table 4.2.1: Alterations made to the composition of the BM for the study of the effect of combined nutrients on hypoxoside production by root-producing callus of *H. hemerocallidea*.

TREATMENT	BM ALTERATIONS			
	Sucrose $20g\ l^{-1}$	Nitrogen $\frac{1}{4}$ strength	Phosphate $\frac{1}{2}$ strength	Calcium $\frac{1}{2}$ strength
A	-	-	-	-
B	+	-	-	-
C	-	+	-	-
D	-	-	+	-
E	-	-	-	+
F	+	+	-	-
G	+	-	+	-
H	+	-	-	+
I	-	+	+	-
J	-	+	-	+
K	-	-	+	+
L	+	+	+	-
M	+	+	-	+
N	-	+	+	+
O	+	-	+	+
P	+	+	+	+

various permutations as outlined in Table 4.2.1. After 8 weeks incubation, the callus in each treatment (A to P) was harvested and analysed.

Hormonal study

Since PAGE (1984) obtained the best growth of root-producing callus on BM supplemented with 0.001 g l^{-1} BA and 0.001 g l^{-1} NAA it was decided to examine the effect of this concentration of BA and NAA with different concentrations of other auxins and cytokinins, respectively.

BA and auxins: The concentration of cytokinin in the form of BA was maintained at 0.001 g l^{-1} in the BM. The auxins, IAA, NAA and 2,4-D were individually combined into the BM. Each of these auxins were tested at the following concentrations: 0, 0.5, 2.0 and 5.0 mg l^{-1}. IAA was added aseptically to the BM in a filter sterilised form after autoclaving of the medium but before solidification of the agar occurred. Callus incubated on these various treatment media was harvested after 8 weeks and analysed.

NAA and cytokinins: The concentration of NAA was maintained at 0.001 g l^{-1} throughout. The cytokinins, kinetin, BA and zeatin, were individually included into the BM. Each of these cytokinins were added to the BM in the following amounts: 0, 0.5, 2.0 and 5.0 mg l^{-1}. The callus innoculated onto these various treatments was harvested after 8 weeks incubation and analysed.

Precursor study

Root-producing callus was innoculated onto fresh BM to which one of the following potential precursors had been added: L-phenylalanine, *t*-cinnamic acid, *p*-coumaric acid and caffeic acid. Each of these

compounds were added in the following concentration range: 0, 10^{-5}, 10^{-4} and 10^{-3} Molar. The potential precursors were all added aseptically as filter sterilised aqueous solutions after autoclaving of the BM but prior to solidification of the medium. Callus incubated on these various treatment media were harvested after 8 weeks and analysed.

4.3 RESULTS

Time study

The growth of the root-producing callus, during the period of the 8 week subculture (Figure 4.3.1), showed an initial two week lag phase after inoculation, followed by a phase of exponential growth which continued up until the seventh week, after which the growth began to slow down.

The percentage of hypoxoside present in this *in vitro* tissue (Figure 4.3.1) increased during the first two weeks before growth occurred. However, between the end of the second and fourth week as the callus entered an exponential growth phase, the percentage of hypoxoside dropped significantly, remaining at a low level until the end of the seventh week. As growth slowed down in the eighth week the percentage hypoxoside increased, reaching a level comparable with the level the root-producing callus contained upon inoculation.

Light study

Incubation in complete darkness reduced the growth of root-producing callus (Figure 4.3.2) in comparison to callus grown in the light. However, the reduction in growth was not significant and increasing the light intensity did not cause any further increase in growth.

The percentage hypoxoside recovered from callus grown in the dark (Figure 4.3.2) was higher than that recovered in light grown callus. This difference was only significant between callus grown in total darkness and callus grown at a light intensity of 35 $\mu E\ m^{-2}\ s^{-1}$. Increasing light intensity appeared to be detrimental for the accumulation of hypoxoside, but this effect was not significant.

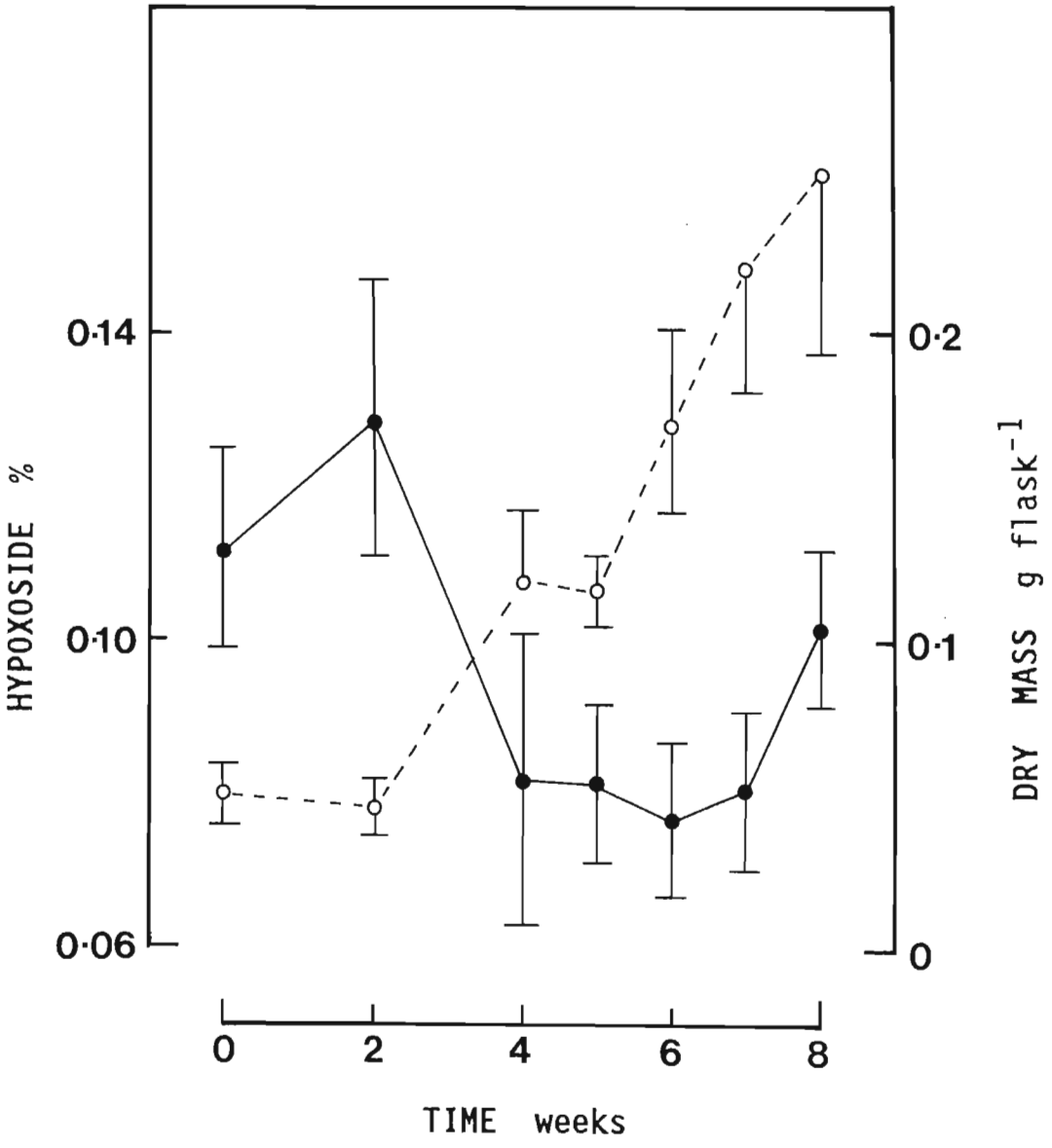


Figure 4.3.1: The percentage hypoxoside (dry mass)(●—●) detected in, and the dry mass (O--O) of, root-producing callus of *H. hemerocallidea* over an 8 week period of culture. The bars indicate the confidence limit of each mean calculated at $p < 0.05$

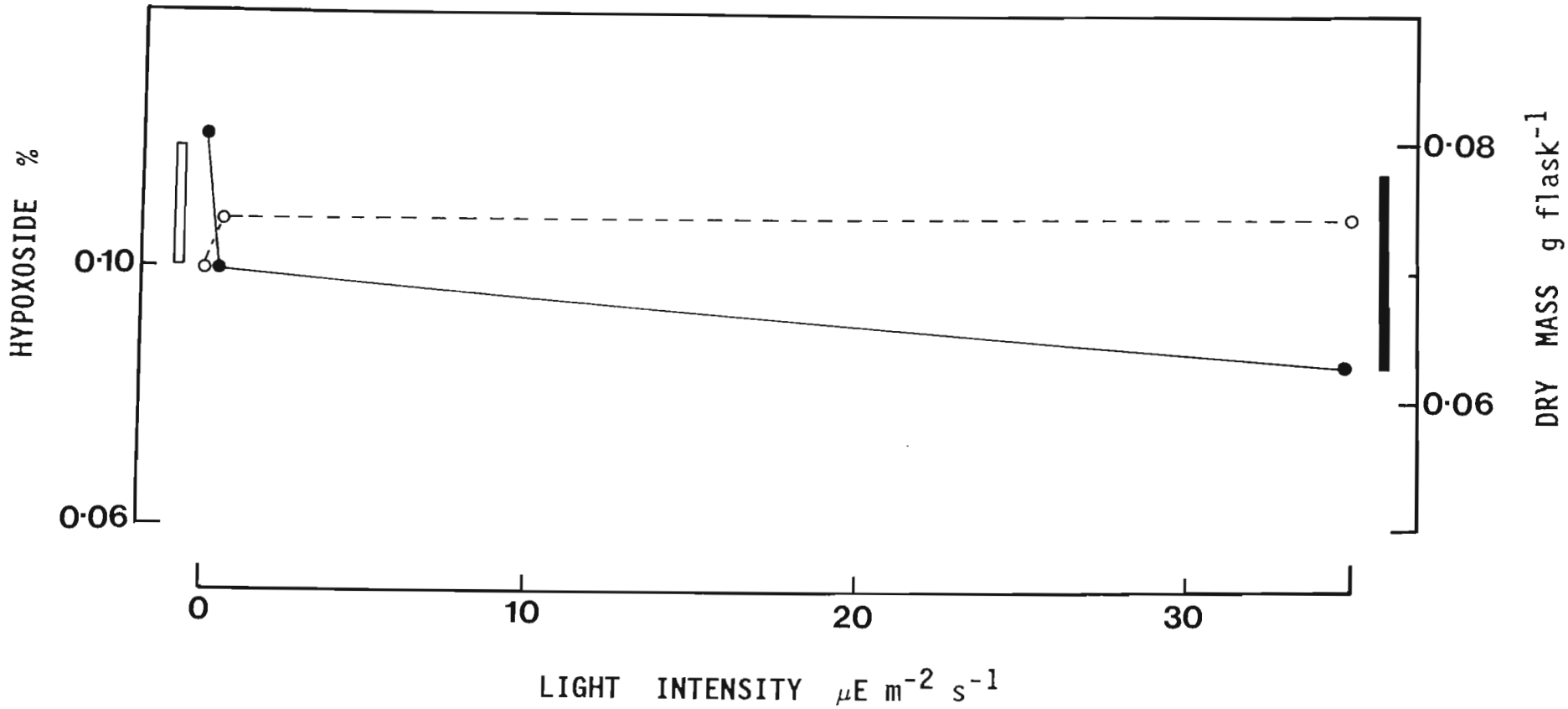


Figure 4.3.2: The percentage hypoxoside (dry mass)(●—●) detected in, and the dry mass (O--O) of, root-producing callus of *H. hemerocallidea* grown under different light intensities and harvested after an 8 week culture period. Solid bars indicate the least significant difference calculated for the percentage hypoxoside ▌ and the dry mass of the callus ▯ at $p < 0.05$.

Temperature study

Low temperatures inhibited growth of the root-producing callus (Figure 4.3.3) while increasing the temperature to 30 ^{o}C did not affect growth. Decreasing the temperature did not reduce the percentage hypoxoside present in the culture tissue (Figure 4.3.3). While raising the incubation temperature to 30 ^{o}C slightly enhanced the percentage of hypoxoside recovered from the tissue, the effect was not significant.

Nutrient study

Sucrose: Altering the percentage of sucrose in the BM did not affect growth of the callus compared to the control (Figure 4.3.4). Reducing the sucrose to 2 percent did enhance the percentage hypoxoside but not significantly so (Figure 4.3.4). Increasing the sucrose to 4 percent had no effect on the hypoxoside percentage in the culture tissue.

Nitrogen: Reducing the nitrogen component of the BM by a half and a quarter, significantly increased growth, particularly at the former level (Figure 4.3.4). The percentage hypoxoside recovered from callus cultured on these media did not differ significantly from the control, however (Figure 4.3.4).

Phosphate: Reducing the phosphate component of the BM to one half and one quarter, significantly reduced growth of the callus, but in the case of the reduction of phosphate by half the enhancement of the percentage of hypoxoside recovered from the callus, was significant (Figure 4.3.4). The reduction by a quarter did not have such a large effect on the percentage of this phytochemical recovered from the callus. Increasing the phosphate concentration by half increased

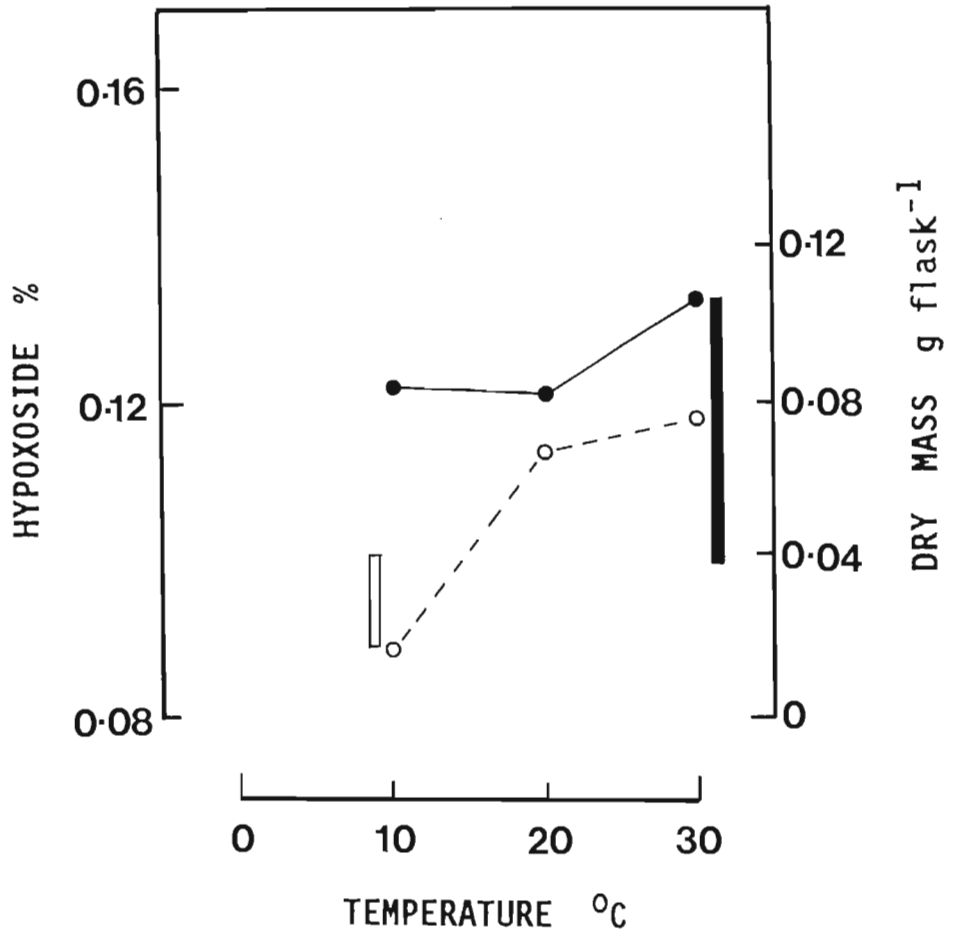
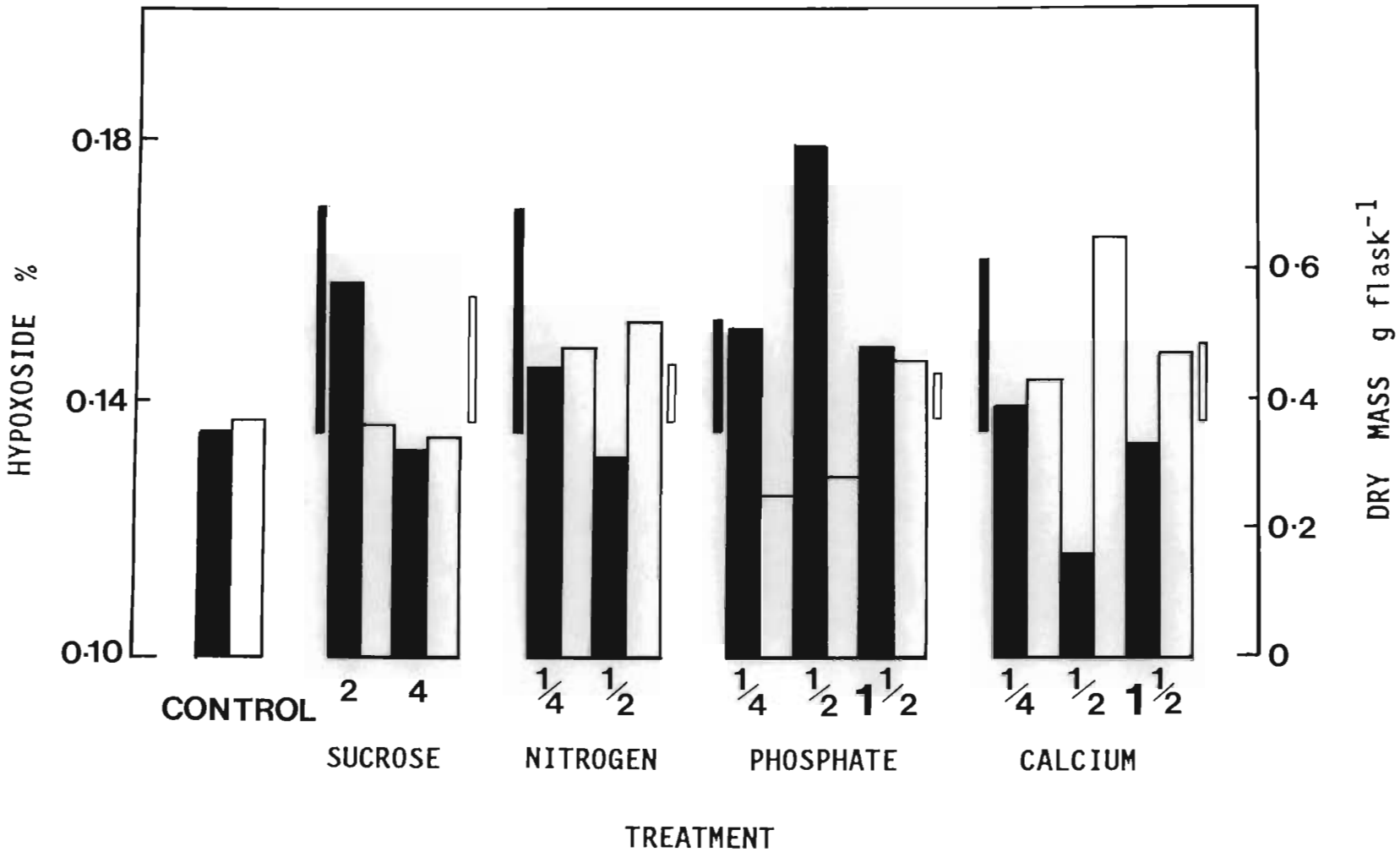


Figure 4.3.3: The percentage hypoxoside (dry mass)(●—●) detected in, and the dry mass (O--O) of root-producing callus of *H. hemerocallidea* incubated at different temperatures over an 8 week culture period. Solid bars indicate the least significant difference calculated for the percentage hypoxoside ▌ and the dry mass of the callus ▯ at $p < 0.05$.

Figure 4.3.4: The percentage hypoxoside (dry mass) ■ detected in, and the dry mass □ of, root-producing callus of *H. hemerocallidea* incubated for 8 weeks on BM in which the concentrations of sucrose, nitrogen, phosphate and calcium had been altered from the normal levels present in the MURASHIGE and SKOOG (1962) medium. Solid bars indicate the least significant difference calculated for the percentage hypoxoside ▌ and the dry mass of the callus ▯ at $p < 0.05$.


HYPOXOSIDE %
0·18
0·14
0·10
DRY MASS g flask⁻¹
0·6
0·4
0·2
0
CONTROL
2
4
¼
½
¼
½
1½
¼
½
1½
SUCROSE
NITROGEN
PHOSPHATE
CALCIUM
TREATMENT

growth significantly and increased the percentage of hypoxoside, but this increase was not significant.

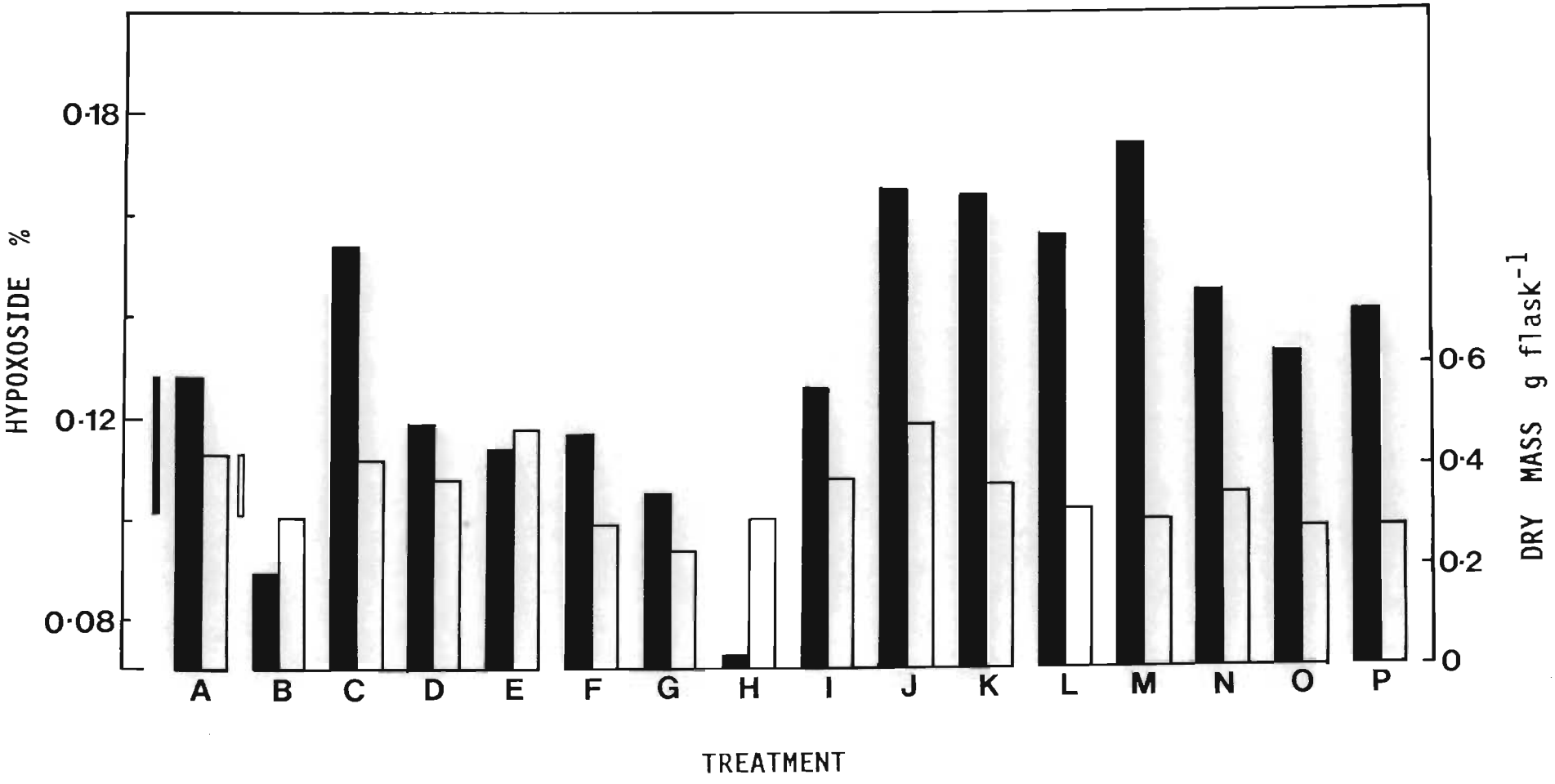
Calcium: Reduction of the calcium concentration by half, significantly increased growth, but reduced the percentage of hypoxoside recovered from the callus (Figure 4.3.4). This decrease, while large was not significantly different from the control. Decreasing the calcium concentration by a quarter did not have any effect on growth or the percentage of hypoxoside recovered from callus incubated on such a medium. Increasing the calcium concentration in the BM by half, slightly increased growth and slightly reduced the percentage hypoxoside but in each case this difference was not significant.

Combined nutrients

The root-producing callus was subcultured onto various combinations of sucrose, nitrogen, phosphate and calcium, using the concentration for each nutrient which gave the greatest percentage hypoxoside in the previous experiment. In the cases where just one of these nutrient levels were altered, the results obtained differed from the previous experiment (Figure 4.3.5). Reduction of sucrose to 2 percent significantly reduced both the growth and percentage hypoxoside of the callus. Reducing the nitrogen level to one quarter did not alter the growth of the callus but resulted in a large increase in the percent hypoxoside recovered. This increase was not, however, significantly different from the control. Decreasing the concentration of phosphate and calcium to one half that usually present in the basal medium, in both cases, had no effect on either the growth or the level of hypoxoside of the root producing callus.

Figure 4.3.5: The percentage hypoxoside (dry mass) ■ detected in, and the dry mass □ of, root-producing callus of *H. hemerocallidea* incubated for 8 weeks on BM in which the concentrations of sucrose, nitrogen, phosphate and calcium which had been altered from the normal MURASHIGE and SKOOG (1962) levels and were combined in a number of permutations. Solid bars indicate the least significant difference calculated for the percentage hypoxoside ▌ and the dry mass of the callus ▯ at $p < 0.05$.

A= control
B= 20 g l^{-1} sucrose
C= ¼ nitrogen
D= ½ phosphate
E= ½ calcium
F= 20 g l^{-1} sucrose, ¼ nitrogen
G= 20 g l^{-1} sucrose, ½ phosphate
H= 20 g l^{-1} sucrose, ½ calcium
I= ¼ nitrogen, ½ phosphate
J= ¼ nitrogen, ½ calcium
K= ½ phosphate, ½ calcium
L= 20 g l^{-1} sucrose, ¼ nitrogen, ½ phosphate
M= 20 g l^{-1} sucrose, ¼ nitrogen, ½ calcium
N= ¼ nitrogen, ½ phosphate, ½ calcium
O= 20 g l^{-1} sucrose, ½ phosphate, ½ calcium
P= 20 g l^{-1} sucrose, ¼ nitrogen, ½ phosphate, ½ calcium


HYPOXOSIDE %
0·18
0·12
0·08
DRY MASS g flask⁻¹
0·6
0·4
0·2
0
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
TREATMENT

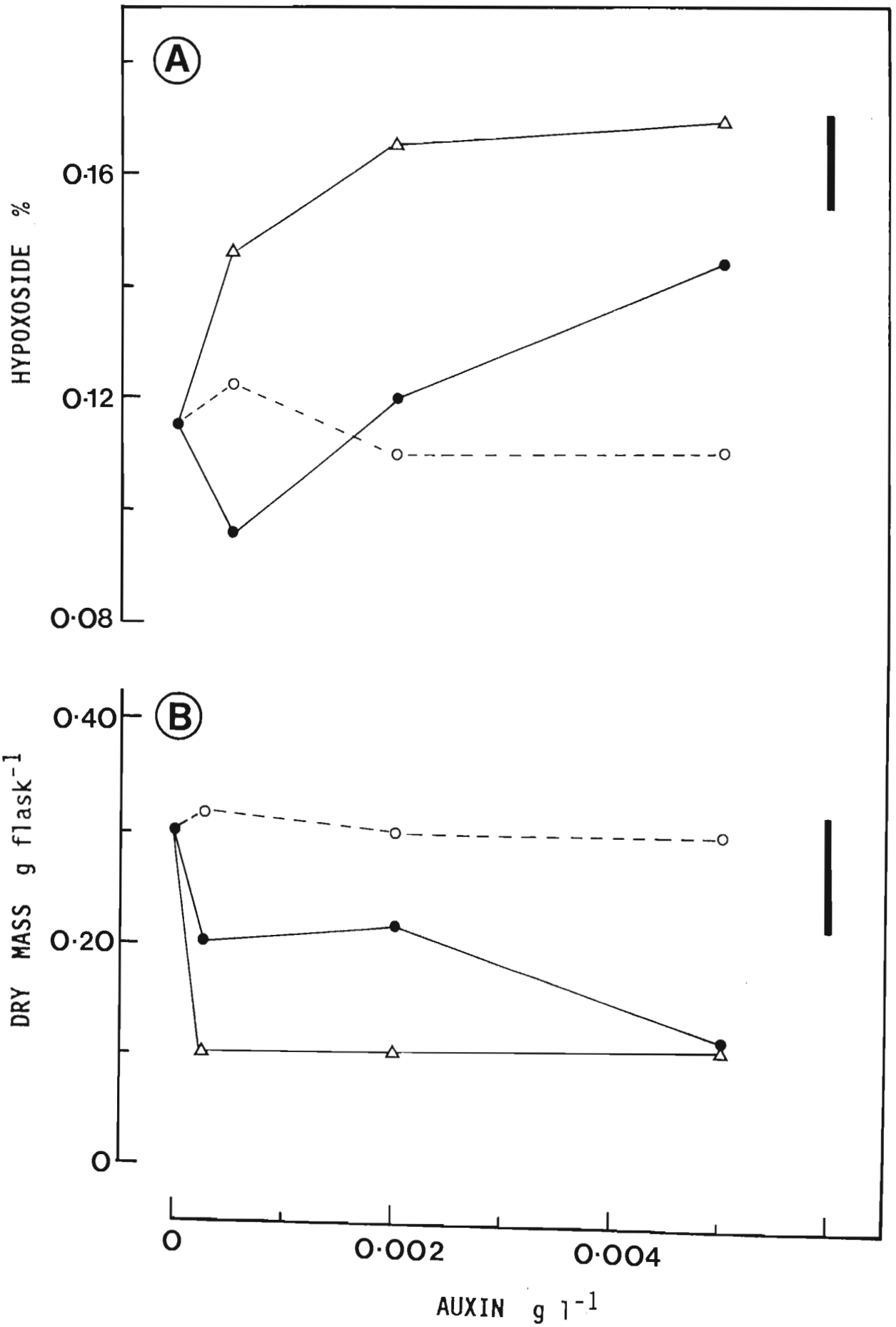
The reduction of two nutrients at a time (Figure 4.3.5), in the case of sucrose and nitrogen, and of sucrose and phosphate, reduced the production of hypoxoside but not significantly so, while in both cases growth was significantly inhibited. Reduction of both sucrose and calcium had a very deleterious effect on both hypoxoside content and growth of the callus. Reducing nitrogen and phosphate simultaneously had no effect on either the growth or percentage hypoxoside present. Reducing nitrogen and calcium, and phosphate and calcium, had no effect on growth of the callus, but significantly enhanced the hypoxoside production by this callus.

The reduction of three nutrients simultaneously (Figure 4.3.5), inhibited growth of the callus in all cases, but only significantly in the treatments where sucrose, nitrogen and phosphate, and sucrose, phosphate and calcium, were decreased. In all the treatments, the production of hypoxoside by the callus, was enhanced, but only significantly so in those treatments where sucrose, nitrogen and phosphate and sucrose, nitrogen and calcium were reduced. Reduction of all four nutrients at once inhibited growth significantly and enhanced hypoxoside production. Enhancement of the latter, however, was not statistically significant.

Hormonal study

BA and auxins: Addition of NAA and 2,4-D to the basal medium inhibited growth of the callus (Figure 4.3.6 B). In the case of NAA this inhibition was not significant at 0.0005 g l^{-1} or 0.002 g l^{-1} but was significant at 0.005 g l^{-1}. Inhibition by 2,4-D was significant at all concentrations. IAA neither inhibited nor promoted growth of the callus. Thus, NAA and 2,4-D retarded callus growth whilst IAA did not. The effect of 2,4-D on the percentage hypoxoside recovered from

Figure 4.3.6: The percentage hypoxoside (dry mass)(**A**) detected in, and the dry mass (**B**) of, root-producing callus of *H. hemerocallidea* incubated for 8 weeks on BM supplemented with 0.001 mg l^{-1} BA and various concentrations of the auxins IAA (O--O), NAA (●—●) and 2,4-D (△--△). The solid bars in each case indicate the least significant difference calculated for each parameter at $p < 0.05$.


A
HYPOXOSIDE %
0·16
0·12
0·08
B
DRY MASS g flask^{-1}
0·40
0·20
0
0
0·002
0·004
AUXIN g l^{-1}

the root producing callus was a promotive one, with the level of hypoxoside being significantly enhanced at all concentrations (Figure 4.3.6 A). NAA retarded the production of this phytochemical at 0.0005 g l^{-1} but was promotive at 0.005 g l^{-1}. IAA had no effect on the level of hypoxoside present in the callus.

Therefore NAA and 2,4-D enhanced hypoxoside production, the latter at lower concentrations than the former, but both retarded growth. The effect of IAA on these two parameters was minimal.

NAA and cytokinins: BA and zeatin both enhanced callus growth, significantly, at all concentrations (Figure 4.3.7 B). This effect being greatest in the case of BA. The effect of kinetin on callus growth was promotive but not significantly so.

The effect of the cytokinins on the production of hypoxoside by the root-producing callus was ambiguous (Figure 4.3.7 A). BA apparently promoted the level of hypoxoside significantly, at 0.0005 g l^{-1} and 0.005 g l^{-1} whilst the effect of zeatin and kinetin, which gave enhanced production at 0.0005 g l^{-1} and 0.002 g l^{-1} respectively, was not significant.

Therefore the cytokinins, particularly BA, promote callus growth whilst their effect on the level of hypoxoside produced by the callus, varies but not to any significant extent.

Precursor studies

Inclusion of L-phenylalanine, at different concentrations, into the BM produced various responses in the amount of callus growth (Figure 4.3.8 A). At 10^{-5} M it was inhibitory but at 10^{-4} M, stimulatory.

Figure 4.3.7: The percentage hypoxoside (dry mass) (**A**) detected in, and the dry mass (**B**) of, root-producing callus of *H. hemerocallidea* incubated for 8 weeks on BM supplemented with 0.001 mg l^{-1} NAA and various concentrations of the cytokinins BA (●—●), kinetin (O--O) and zeatin (Δ-Δ). The solid bars in each case indicate the least significant difference calculated for each parameter at $p < 0.05$.

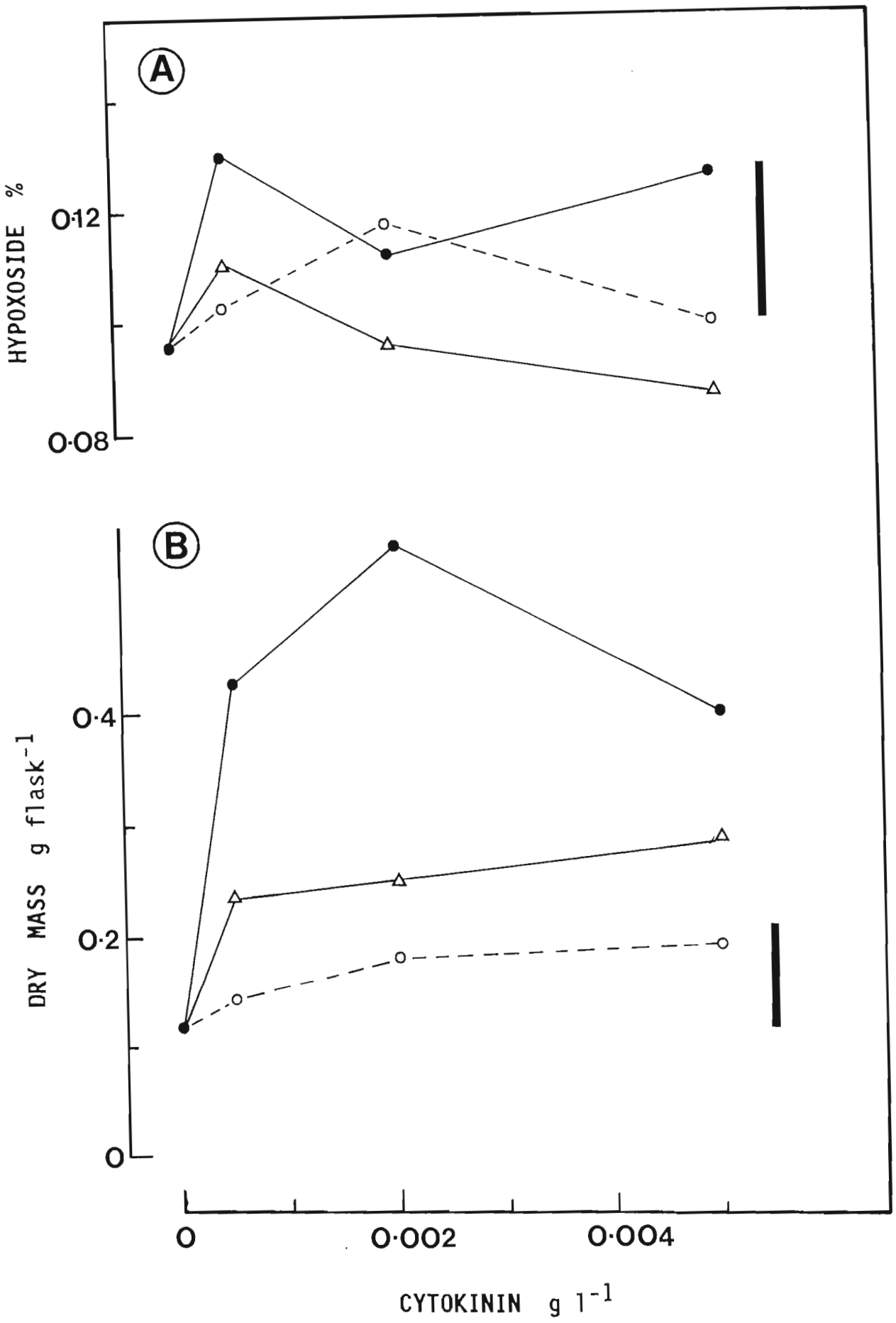
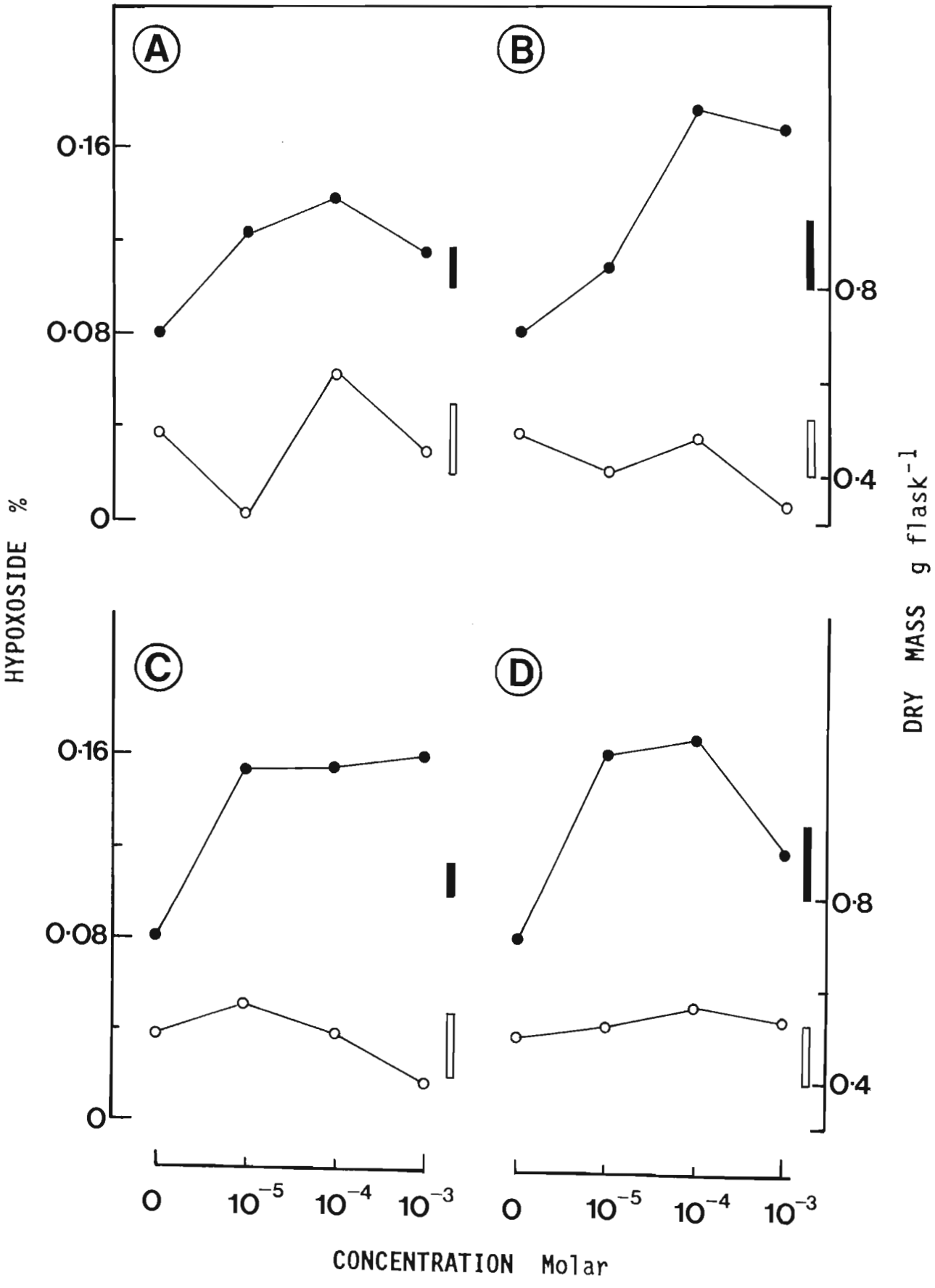

A
HYPOXOSIDE %
0·12
0·08
B
DRY MASS g flask^{-1}
0·4
0·2
0
0
0·002
0·004
CYTOKININ g l^{-1}

Figure 4.3.8: The percentage hypoxoside (dry mass) (●—●) detected in, and the dry mass (O--O) of root-producing callus of *H. hemerocallidea* which was incubated for an 8 week culture period on BM supplemented with various concentrations of L-phenylalanine (**A**), *t*-cinnamic acid (**B**), *p*-coumaric acid (**C**) and caffeic acid (**D**). The solid bars in each case indicate the least significant difference calculated for each parameter at $p < 0.05$.


A
B
C
D
0·16
0·08
0
0·8
0·4
HYPOXOSIDE %
DRY MASS g flask^{-1}
0
10^{-5}
10^{-4}
10^{-3}
CONCENTRATION Molar

These differences were not significant. The effect of increasing the concentration of *t*-cinnamic acid (Figure 4.3.8 B), included in the BM, was generally an inhibitory one, but this was not significant. The inclusion of *p*-coumaric acid in the medium was initially slightly stimulatory (Figure 4.3.8 C) for growth but became inhibitory at high concentrations. These effects were not significant. Caffeic acid had no effect on growth of the callus (Figure 4.3.8 D).

The addition of all of the potential precursors to the BM was promotive for hypoxoside production at all concentrations (Figure 4.3.8). The effect of L-phenylalanine was greatest at 10^{-4} M, as was the effect of *t*-cinnamic acid. *p*-Coumaric acid was equally effective at all of the concentrations tested, while the greatest response for hypoxoside production caused by caffeic acid occurred at 10^{-5} M and 10^{-4} M.

Therefore, while inclusion of these potential precursors into the BM did not substantially affect growth of the callus, the effect on hypoxoside production was one of a significant enhancement in all cases.

4.4 DISCUSSION

The growth of the root-producing callus of *H. hemerocallidea*, during an eight week subculture period, followed the pattern observed for most plant tissue culture systems, be they tissue or cell suspension cultures. This pattern included a lag phase, followed by an exponential growth phase and terminated with a stationary phase. As has been recorded for the production of secondary metabolites in many other systems, there exists an inverse relationship between growth and hypoxoside production. This situation has been found to exist for the *in vitro* production of other phenolic compounds, although conflicting reports have been made in some cases.

FORREST (1969) reported an inverse correlation between polyphenol (anthocyanin and flavonols) synthesis and the growth rate of cultures of *Camellia sinensis* L. Tannin production by *Acer pseudoplatanus* L. cell suspensions was reported to follow a similar pattern and the observation was made that tannin accumulation occurred when net protein synthesis was complete and nitrogen had been depleted in the medium (WESTCOTT and HENSHAW, 1976). AMORIM, DOUGALL and SHARP (1977) detected phenols in cell cultures of Paul's Scarlet Rose throughout the culture period. However, the greatest production of total phenolics occurred during the stationary phase.

Formation of rosmarinic acid, a phenolic compound, was initiated at the end of the exponential phase of growth in cell cultures of *Anchusa officinalis* (DE-EKNAMKUL and ELLIS, 1984).

Reports on the production of the phenolic pigments, betacyanins, by cell suspension cultures are contradictory. BERLIN, SIEG, STRACK,

BOKERN and HARMS (1986) obtained maximum betacyanin production from cells of *Chenopodium rubrum* L. in the late exponential phase of growth, after initiation of synthesis had occurred during mid-exponential growth. However, a peak of betacyanin accumulation occurred in mid logarithmic phase in cell cultures of *Phytolacca americana* L. (SAKUTA, TAKAGI and KOMAMINE, 1986).

Conflicting reports for the production of anthroquinones by cell cultures of *Cinchona ledgeriana* have also been made. ROBINS, PAYNE and RHODES (1986) claimed that these phenolic derivatives are produced late in the growth cycle of the cell cultures, while WIJNSMA, VERPOORTE, HARKES, VAN VLIET, TEN HOOPEN and SVENDSEN (1986) reported an increase of the anthroquinones during the growth phase.

While the production of hypoxoside follows the trends observed for most secondary metabolite production, in that production occurred in late exponential phase, the production of hypoxoside in the lag phase was an unusual observation. TABATA (1977) classifies the growth and production of secondary metabolites into three main groups.

(i) Production occurring concurrently with cell growth.

(ii) Production delayed until cell division decreases or stops.

(iii) Production showing a biphasic curve with a lag behind the growth curve.

MIYASAKA, NASU, YAMAMOTO and YONEDA (1985) have described a fourth type of production for the production of ferruginol by cell cultures of *Salvia miltiorrhiza* Bunge.. These researchers reported two phases of production during the subculture period, one in the lag phase and

another in the stationary phase. They concluded that ferruginol production was inversely related to active cell division.

In the current study a similar situation was observed for hypoxoside production, with production occurring in both the lag and stationary phases but not during the actively growing stages.

While maintaining *H. hemerocallidea* root-producing callus in the light was slightly more beneficial for growth, the effect was not significant. Although, many reports exist where light has been reported to enhance the production of phenolic compounds, particularly anthocyanins (STICKLAND and SUNDERLAND, 1972a; SCHMIDT and MOHR, 1981), cinnamic acids and flavonoids (HAHLBROCK and WELLMAN, 1973; GRISEBACH and HAHLBROCK, 1974), the current data, with regard to hypoxoside production, supports the findings of PAGE and VAN STADEN (1987), in that this process is promoted in the dark. The accumulation of the phenolic betacyanin is stimulated by the dark in callus cultures of *Portulaca grandiflora* Hook., according to ENDRESS, JÄGER and KREIS (1984). The results of BERLIN, SIEG, STRACK, BOKERN and HARMS (1986) differ, in that betacyanin synthesis in cell suspension of *Chenopodium rubrum* declined in the dark, but was restimulated when returned to the light. These two examples illustrate that the biosynthesis of a phenolic compound in two different species can be under different controlling factors which are obviously dependent on the plant and its environment.

The production of ferruginol, by cell suspension cultures of *Salvia miltiorrhiza*, was inhibited by light and MIYASAKA, NASU, YAMAMOTO and YONEDA (1985) have drawn attention to the fact that this compound was found only in the roots of the intact plant. A similar situation has

been found in *H. hemerocallidea* where hypoxoside is confined to the subterranean portions of the plant. Although *in vitro* there was a concommitant decrease in growth associated with dark grown callus, it was not substantial and while increasing light intensity did not enhance growth, it did inhibit hypoxoside production slightly.

The inhibition of growth of the root-producing callus at 10 ^{o}C was not surprising, but at this low temperature hypoxoside production continued, with the tissue maintained at this temperature, having a similar capacity for production compared to that maintained at 20 ^{o}C. Raising the temperature to 30 ^{o}C enhanced production of hypoxoside only slightly. SAHAI and SHULER (1984) obtained temperature enhanced production of total phenolics in *Nicotiana tabacum* cell suspensions. A similar effect was observed for ubiquinone synthesis by tobacco cells (IKEDA, MATSUMOTO and NOGUCHI, 1977). SAHAI and SHULER (1984) associated the increased phenolic production with the increased metabolic activity at higher temperatures.

The sucrose concentrations tested did not apparently affect hypoxoside production. The first nutrient experiment indicated that increasing sucrose concentration had no effect while, decreasing it enhanced production of the phenolic slightly. However, in a subsequent experiment, reduction of sucrose led to a substantial decrease in hypoxoside production. In the former experiment the changes in sucrose concentration did not affect growth, whilst in the latter growth was inhibited. This inhibition of growth indicates, that in this experiment, the sucrose level was limiting for growth, whilst in the previous experiment it did not have the same effect. Thus, in the later experiment, the fact that the sucrose level was limiting could have been the result of available energy and carbohydrates being

channelled into growth, leaving an insufficient supply of carbohydrates for hypoxoside formation.

The addition of sucrose has, in many *in vitro* systems, been reported to enhance the production of secondary metabolites, presumably because excess carbohydrate becomes available as a source for precursors. Increasing the sucrose concentration from 2 percent to 4 percent increased phenolic accumulation by three times in cell suspensions of *Acer pseudoplatanus* (WESTCOTT and HENSHAW, 1976). Sucrose limitation in chemostat cultures of *Galium mollugo*, supressed anthroquinone production (WILSON and BALAGUÉ, 1985). Increasing sucrose to 8 percent in *Cinchona ledgeriana* cell suspensions, increased anthroquinone yields in one report (WIJNSMA, VERPOORTE, HARKES, VAN VLIET, TEN HOOPEN and SVENDSEN, 1986) and produced no effect in another (ROBINS, PAYNE and RHODES, 1986). Other reports indicate that there is an optimum sucrose concentration, above or below which, secondary product formation is inhibited. HAGIMORI, MATSUMOTO and OBI (1982) observed this to be the case for digitoxin formation by shoot forming callus of *Digitalis purpurea*. Here, the optimum sucrose concentration occurred in the range of 3 to 5 percent. The production of rosmarinic acid by cell suspension of *Anchusa officinalis*, was also sucrose sensitive with the optimum production of the phenolic occurring at 3 percent sucrose (DE-EKNAMKUL and ELLIS, 1985a).

The effect of increasing sucrose levels from 2 to 10 percent in *Chenopodium rubrum* suspension cultures, was deleterious for betacyanin production, reducing the levels of these pigments ten-fold (BERLIN, SIEG, STRACK, BOKERN and HARMS, 1986).

Thus, the effect of sucrose on secondary metabolite production, varies considerably from system to system and as was evident in the current

study may vary considerably within the same system, at different times. The effect elicited by sucrose on secondary product formation, however, appears to be closely linked to growth. Available carbohydrates have to be partitioned between primary and secondary metabolism. They provide carbon skeletons for metabolites and the energy required for the construction of these molecules.

The effect of reducing the nitrogen concentration in the BM was not large. Growth was not affected and hypoxoside production was slightly enhanced with a reduction to one quarter nitrogen in both experiments. The effect of nitrate on secondary metabolite production has been widely studied and in most cases appears to be linked to the carbohydrate status of the medium. Nevertheless, there are varied reports on its action *in vitro*.

AMORIM, DOUGALL and SHARP (1977) observed that the phenolic production, by cell suspensions of Paul's Scarlet Rose, remained unaffected by increasing the nitrate concentration at low glucose concentrations. At higher glucose concentrations, the level of phenolics produced was higher but the effect of increasing nitrate was detrimental.

This antagonism between high nitrogen levels and polyphenol production has been observed in other systems. WESTCOTT and HENSHAW (1976) observed a supression of phenolic production by increasing the médium nitrogen in suspension cultures of *Acer pseudoplatanus*. SAHAI and SHULER (1984) noted a similar effect in cell suspension cultures of *Nicotiana tabacum*. Work with rosmarinic acid production by *Anchusa officinalis* cell suspensions, also showed that increasing the nitrate levels decreased phenolic production, whilst decreases in the

nitrogen, up to a point, were favourable, after which the decreases became very inhibitory (DE-EKNAMKUL and ELLIS, 1985a). The production of the non-phenolic cardiac glycoside, digitoxin, by shoot forming cultures of *Digitalis purpurea* was effected similarly by nitrogen concentration (HAGIMORI, MATSUMOTO and OBI, 1982).

In the case of betacyanin formation in cell suspensions of *Chenopodium rubrum*, altering the nitrate in the medium had no effect on phenolic production, while WIJNSMA, VERPOORTE, HARKES, VAN VLIET, TEN HOOPEN and SVENDSEN (1986) reported that anthroquinone production was enhanced by increasing the nitrate levels in cultures of *Cinchona ledgeriana*.

The slight enhancement of hypoxoside production, produced *in vitro* in *H. hemerocallidea* tissue observed in this study and by PAGE and VAN STADEN (1987), which was brought about by reducing the nitrogen level, can be explained by the restriction in nitrogen availability for protein synthesis. This may have caused a reduction in protein synthesis which allowed a channelling of phenylalanine and energy into secondary metabolism. However, consideration of the growth of the root callus in both experiments conducted in this study, showed that a reduction in nitrogen either enhanced or did not affect growth. If a reduction in medium nitrogen was resulting in a greater shunting of carbohydrates into secondary metabolism then, reducing the sucrose and nitrogen simultaneously, should have reduced both growth and hypoxoside production, which it did; significantly in the case of hypoxoside production. However, the effect of nitrogen concentration did not appear to be a strong one.

The effect of increasing phosphate in the medium in the first experiments was promotive for the growth of *H. hemerocallidea* callus and inhibitory for growth when reduced. Reduction of phosphate in these studies enhanced hypoxoside production. In the subsequent study reducing the phosphate level had no significant effect on growth or hypoxoside production. These contradictory results once again point to the variability of nutrient studies *in vitro*. In other systems phosphate has been found to be an important nutrient as far as secondary metabolism is concerned.

DE-EKNAMKUL and ELLIS (1985a) reported that high phosphate levels favoured the formation of rosmarinic acid by *Anchusa officinalis* cell cultures, while WILSON and BALAGUÉ (1985) found that phosphate limitation in *Gallium mollugo* cell cultures hindered anthroquinone formation. Digitoxin formation, by *Digitalis purpurea* cultures, was favoured at higher concentrations of phosphate, when compared to lower concentrations. In other systems, increased phosphate has been reported to supress secondary metabolism, for example, alkaloid formation by *Peganum harmala* L. callus and cell cultures (NETTLESHIP and SLAYTUR, 1974; SASSE, HECKENBERG and BERLIN, 1982). However, anthroquinone formation by *Cinchona ledgeriana* cell suspensions (ROBINS, PAYNE and RHODES, 1986) and betacyanin production by *Chenopodium rubrum* suspension cultures (BERLIN, SIEG, STRACK, BOKERN and HARMS, 1986), have remained unchanged by alterations of phosphate concentration in the respective media.

The role that phosphate plays in hypoxoside biosynthesis in *H. hemerocallidea* root callus is not clear.

The effect of calcium concentration on hypoxoside production *in vitro* in *H. hemerocallidea* callus was inhibitory at half the level normally present in the BM but growth at this concentration was substantially enhanced. At lower and higher concentrations neither growth nor hypoxoside production were affected. The reduction in hypoxoside synthesis at the half dilution appears, therefore to be a result of increased growth and a partitioning of energy and nutrients into primary metabolism at the expense of secondary product formation.

In other systems the absence of calcium has been found to be inhibitory for product formation. Examples of these include rosmarinic acid production by *Anchusa officinalis* cell cultures (DE-EKNAMKUL and ELLIS, 1985a) and the production of the harman alkaloids by *Peganum harmala* cell cultures (SASSE, HECKENBERG and BERLIN, 1982).

The results obtained for the combined reduction of nutrients, indicated that a reduction of sucrose coupled with either nitrogen, phosphate or calcium was deleterious for both growth and hypoxoside production. This implies that under the conditions of reduced carbohydrate supply, sufficient energy and precursors were available only for the operation of primary metabolism. The lack of effect of reducing nitrogen and phosphate simultaneously, was unexpected as it was though that the reduction of these nutrients, in the presence of sufficient carbohydrate, would cause a shift from primary to secondary metabolism. Decreasing phosphate and calcium and nitrogen and calcium definitely enhanced secondary metabolism but using growth as an indication appeared not to affect primary metabolism. Surprisingly reduction of sucrose and two other factors such as nitrogen and phosphate, nitrogen and calcium, or phosphate and calcium enhanced secondary metabolism as well as reducing growth. This suggests that

the effect of sucrose combined with other nutrients is not simply a case of precursors and energy being shunted either into primary or secondary metabolism.

The effect of the plant growth regulators, particularly the auxins, in this particular study, contradict much of the literature, with regard to both growth of the root-producing callus and production of the secondary metabolite, hypoxoside, by it.

Generally, only low concentrations of auxin are considered to be stimulatory for secondary metabolite production *in vitro* (KURZ and CONSTABEL, 1985; COLLIN, 1987), yet the findings of this investigation indicate that the converse is true for the synthetic auxins, NAA and 2,4-D, whilst the effect of IAA on the production of hypoxoside was minimal. It is important to note that, where an auxin promoted hypoxoside production, growth of the root-producing callus was inhibited. This implies that the classic antagonistic relationship between growth and secondary metabolism was operating in the root-producing callus, with regard to hypoxoside production. Only the inclusion of IAA, which had no effect on hypoxoside production, did not inhibit growth. IAA is known, however, to be unstable both without and within tissue when applied exogenously.

While, 2,4-D and NAA were the most beneficial as far as the accumulation of hypoxoside was concerned, their effect on growth was deleterious. In most reports on the production of phenolic compounds, 2,4-D and NAA are cited as favourable growth regulators for growth but inhibitory for secondary product accumulation.

The accumulation of phenolics by suspension cultures of *Acer pseudoplatanus* has been shown to be inhibited by increasing concentrations of 2,4-D. Reduction of the concentration of 2,4-D allowed an increase in phenolic production without reducing the growth in the cultures (WESTCOTT and HENSHAW, 1976). Furthermore, decreasing the concentration of 2,4-D also resulted in an increase in phenylalanine ammonia lyase activity which was concommitant with increased production of the phenolics. IBRAHIM and EDGAR (1976) reported that high levels of NAA were inhibitory both for phenolic accumulation and phenylalanine ammonia lyase activity in cell suspensions of *Perilla ocymoides* L. The accumulation of phenolics in cell suspension cultures of *Acer pseudoplatanus* was totally supressed by the addition of 2,4-D at as low a level as 0.4 mg l^{-1} (PHILLIPS and HENSHAW, 1977). In this study the addition of 2,4-D stimulated the incorporation of phenylalanine into protein in preference to the phenolics. This finding, according to PHILLIPS and HENSHAW (1977), supports the hypothesis that precursor competition is thought to exist between primary and secondary metabolism.

The phenolic compound 3,4-dihydroxyphenylalanine (DOPA), produced by callus cultures of *Stizolobium hassjoo* Piper & Tracy, did not accumulate favourably at the levels of 2,4-D (0.25 mg l^{-1}) which produced optimum growth (OBATA-SASAMOTO and KOMAMINE, 1983). Decreasing the level of 2,4-D to below 0.025 mg l^{-1} improved the production of the phenolic compound. These authors also reported that the lower levels of 2,4-D supressed protein synthesis, while higher concentrations of the auxin promoted protein synthesis.

The effect of 2,4-D upon anthocyanin production in carrot cell suspensions, was one of inhibition combined with a concurrent decrease

in the activity of phenylalanine ammonia lyase (OZEKI and KOMAMINE, 1985, 1986). When this auxin was excluded from the medium the phenylalanine ammonia lyase activity increased as did anthocyanin production, while cell division was supressed. Upon addition of 2,4-D, however, the activity of phenylalanine ammonia lyase was immediately reduced and all division resumed. However, the supression of anthocyanin production by gibberellic acid (GA_3), which had no effect on cell division, suggests that anthocyanin production may have occurred independently of cell division (OZEKI and KOMAMINE, 1986).

DE-EKNAMKUL and ELLIS (1985b) reported that the production of the phenolic, rosmarinic acid, by cell suspensions of *Anchusa officinalis*, was increased by the addition of low concentrations of 2,4-D and NAA; the latter having a more marked effect. At higher concentrations this effect was lost. Addition of these two auxins also proved to be beneficial for growth of the cultures. The effect of IAA addition, however, was an inhibitory one for rosmarinic acid production, whilst growth of the culture remained unaffected. It must be mentioned that the production of rosmarinic acid in this system was restricted to the linear phase of growth (DE-EKNAMKUL and ELLIS, 1984), thus the typical antagonistic relationship observed between growth and secondary metabolism was not present.

The production of anthroquinones by cell suspensions of *Cinchona ledgeriana* was promoted by low levels of 2,4-D, but was inhibited at concentrations greater than 0.5 mg l^{-1}, while at these higher concentrations, growth was stimulated (ROBINS, PAYNE and RHODES, 1986). Substitution of 2,4-D by NAA or IAA proved to be more beneficial for anthroquinone production, with NAA giving the greatest increase in accumulation of these phenolics.

Thus, in most of the examples cited, the auxins, particularly 2,4-D followed by NAA and IAA to a much less extent, favoured production of phenolic compounds *in vitro* only at low concentrations. Higher concentrations resulted in increased growth and reduced phenolic accumulation. The converse situation was observed in *H. hemerocallidea* root-producing callus. It is possible that the nature of the callus can provide an explanation for this. In most cases cited the culture systems employed were cell suspensions. In those where such a system was not used the *in vitro* tissue was of shoot origin, for example DOPA production by *Stizolobium hassjoo*. The system employed in this study, utilised a callus culture that displayed considerable organogenesis, in that roots were present.

It has been extensively reported that root tissue is more sensitive to auxin concentration than shoot tissue (GOODWIN, 1978) and thus, the response obtained may be one where growth of the cultures was inhibited through their sensitivity to auxin concentrations resulting in the excess energy and precursors being channelled into secondary metabolism.

The fact that decreasing or increasing the auxin concentrations, in some *in vitro* systems, resulted in a concommitant increase (WESTCOTT and HENSHAW, 1976) or decrease (OBATA-SASAMOTO and KOMAMINE, 1983) in phenylalanine ammonia lyase activity, respectively, indicates that in these systems, the effect of auxin on secondary metabolism, may not only have be through the stimulation of primary metabolism, brought about by the enhancement of cell division.

How the auxin effect is mediated in root-producing callus of *H. hemerocallidea* is not known. The enhancement of secondary metabolism may be the result of inhibited cell division, and therefore an enhancement of the availability of precursors for secondary product formation, and/or a more direct effect on the enzymes involved in these secondary processes, either via their effect on activation or synthesis of these enzymes.

Although, the general effect of increasing concentrations of cytokinins, was to increase growth of the root-producing callus, these growth regulators, apparently had no distinct effect on hypoxoside production. The postulation that growth and secondary metabolism are antagonistic clearly does hold in all cases for hypoxoside production by root-producing callus of *H. hemerocallidea*.

This is not the only example where the effect of cytokinins on growth and phenolic production *in vitro* does not uphold the hypothesis that growth and secondary metabolism are antagonistic. The accumulation of caffeic acid, both in free and ester forms, was promoted by high kinetin concentrations in cell suspension cultures of *Perilla ocymoides* and a concommitant increase in growth was reported (IBRAHIM and EDGAR, 1976). Increasing concentrations of kinetin, promoted both growth and DOPA accumulation in callus cultures of *Stizolobium hassjoo* (OBATA-SASAMOTO and KOMAMINE, 1983).

The cytokinins, BA, isopentenyladenosine and zeatin, promoted anthocyanin synthesis in cell suspensions of *Daucus carota* L., but only in the absence of 2,4-D, which induced cell division (OZEKI and KOMAMINE, 1986). However, further experiments where the effects of gibberellic acid and the use of inhibitors of anthocyanin synthesis

were examined, led these authors to conclude that anthocyanin synthesis and cell division probably occur independently of one another.

Varying the concentration of BA in cell suspension cultures of *Cinchona ledgeriana*, had little effect on the accumulation of anthroquinones, while zeatin riboside had a slightly promotive effect (ROBINS, PAYNE and RHODES, 1986). BA had little effect on growth of the cell suspensions, although an absence of it from the medium was slightly inhibitory, in this system. In the case of rosmarinic acid synthesis by cell suspension cultures of *Anchusa officinalis* the cytokinins, BA, zeatin and kinetin were similar in their effect, in that, increasing their concentration led to a decrease in both rosmarinic acid accumulation and growth of the cultures (DE-EKNAMKUL and ELLIS, 1985b).

Thus, the antagonistic relationship that was observed between hypoxoside production and growth of the root producing callus for the auxins, was not evident in the treatments where cytokinins were examined. It therefore appears that hypoxoside production may not be controlled simply by precursor availability, which is dependent on the demands of primary metabolism, and the rate of cell division and growth.

The growth of the root-producing callus in the presence of phenylalanine, *t*-cinnamic acid, *p*-coumaric acid and caffeic acid was not greatly affected by the presence of these phytochemicals. However, at concentrations higher than 10^{-3} M, it appeared that the addition of higher concentrations of these precursors could become inhibitory for callus growth.

In most cases where the shikimate derivatives have been supplied to *in vitro* systems as precursors to boost secondary metabolite production, their effect on growth has been deleterious. SAHAI and SHULER (1984) reported a substantial decrease in growth by the addition of phenylalanine at 0.01 percent ($\pm 6 \times 10^{3}$ M) to low, and high inoculum density suspension cultures of *Nicotiana tabacum*. However, a concommitant increase in the percentage total phenolics produced in this system, was observed by these authors.

The addition of the suspected precursors, *p*-coumaric acid and tryamine to suspension cultures of *Colchicum autumnale* L., was reported to enhance the levels of the alkaloid, colchicine, without any substantial effect on growth. The addition of 0.1 percent phenylalanine or tyrosine, produced increased alkaloid formation in cultured tissue of *Datura tatula* L., but a substantial reduction in growth was observed (SAIRAM and KHANNA, 1971). This effect was also observed by KOBLITZ, KOBLITZ, SCHMAUDER and GRÖGER (1983) and HAY, ANDERSON, ROBERTS AND PHILLIPSON (1986) for growth of cultured tissue of various species of *Cinchona* L. when the quinoline alkaloid precursor L-tryptophan, was included in the medium. While alkaloid production was boosted dramatically, growth was inhibited. KOBLITZ, KOBLITZ, SCHMAUDER and GRÖGER (1983) concluded that it is not clear whether the effect of boosted alkaloid production, caused by the addition of L-tryptophan, is just one of precursor availability. The conclusion drawn by HAY, ANDERSON, ROBERTS and PHILLIPSON (1986) is that this increase is, in part, the result of improved availability of precursor.

In most of the accounts cited, addition of precursor has stimulated secondary metabolite production, but inhibited growth. This fits the

hypothesis that secondary metabolite production and growth are antagonistic. When precursors are exogenously supplied it is possible that the increased concentration of these primary intermediates triggers secondary metabolism at the expense of primary metabolism hence the reduction in growth of cultures. This implies that the added compounds do not only act as precursors, but affect general metabolism in some other way so that addition may be both an indirect and direct effect.

In the root-producing callus of *H. hemerocallidea*, the addition of the suspected precursors did not inhibit growth, suggesting that this addition did not result in a clear switch from primary to secondary metabolism. The addition of these shikimate derivatives enhanced hypoxoside biosynthesis, but whether this was just due to greater precursor availability or not, is debatable in the light of the findings that both *p*-coumaric and caffeic acid are poor precursors for hypoxoside. Since the addition in all cases produced a similar response in hypoxoside production, one would assume that their effect in enhancing this process is similar.

However, until more is known about the biosynthetic processes of hypoxoside formation, it is difficult to predict how and at what level the effect of manipulating nutrients, growth regulators and probable precursors is elicited.

The *in vitro* studies on the production of hypoxoside production indicate that in many ways this process is one typical of secondary metabolism, as is shown by the growth pattern and hypoxoside production. However, in many instances the hypothesis that hypoxoside production is the result of shunt metabolism or is antagonistic

towards growth, is not upheld. It is clear that hypoxoside production in root-producing callus of *H. hemerocallidea* is influenced by many factors both physical, nutritional and hormonal. The level at which these factors, which affected hypoxoside production, operated in the metabolism of the plant tissue, is unknown.

CHAPTER 5

CONCLUSION

The shikimate pathway contributes at least one of the two aromatic moieties that contribute to the hypoxoside molecule in *H. hemerocallidea* tissue, *in vivo* and *in vitro*. Phenylalanine and *t*-cinnamic acid are the two shikimate derivatives, which under the systems tested, proved to be the most efficient precursors for hypoxoside both *in vivo* and *in vitro*. A possible role for the acetate pathway in hypoxoside biosynthesis could not be found. The data thus, supports the postulation that the hypoxoside molecule is formed by the joining of two cinnamate units with the loss of a carbon atom (MARINI-BETTOLO, PATAMIA, NICOLETTI, GALEFFI and MESSANA, 1982) rather than the hypothesis that the molecule is derived from the head-to-tail condensation of acetate units onto a propenylic moiety to give the second aromatic ring. How the joining of the two cinnamate units with a resulting loss of a carbon atom occurs is open to speculation.

Despite the findings that, endogenously, both *in vivo* and *in vitro*, caffeic and *p*-coumaric acids, were in most cases, the predominant hydroxycinnamic acids and/or were the acids in which radioactivity accumulated when either ^{14}C-phenylalanine, ^{14}C-*t*-cinnamic acid or ^{14}C-carbon dioxide were applied, these acids proved to be poor precursors for hypoxoside in the systems tested. The poor participation of these acids in this process could be due to a number of factors including poor transport to the site of synthesis, channelling off into primary metabolism for the purposes of cell wall formation (primary wall, lignification and suberisation), difficulty of conversion to a

required active form or lack of involvement in hypoxoside biosynthesis directly, with joining of the aromatic moieties occurring prior to hydroxylation.

Of the organs the corm accumulates hypoxoside to the greatest extent and it is also the major site for biosynthesis of the molecule. The ability of the roots to produce this phytochemical is not disregarded, but the biosynthetic potential of the leaves is.

In agreement with the report of PAGE (1984), the upper region of the corm has a greater concentration of hypoxoside than the lower. This region of the corm was also found to be the most active biosynthetically.

Electron microscopic studies revealed that osmiophilic bodies of a densely granular nature were aggregated in the vacuoles of cells rich in amyloplasts. Light microscopic investigations showed that these cells contributed to most of the ground tissue of the corm. The presence of rough and tubular endoplasmic reticulum and vesicles in association with osmiophilic material within the cytoplasm of these cells, suggests high synthetic and/or transport activity. Since plasmodesmata were not frequently observed it is most probable that these cells were synthetically active. It is envisaged that these cells are the site for the accumulation and biosynthesis of hypoxoside and that since the greatest number of this type of cell was found in the upper region of the corm, an explanation is provided for the observation that this was the most biosynthetically active region of the corm.

It seems that the availability of precursors for hypoxoside biosynthesis is primarily dependent on the shikimate pathway which is predominant in the leaves. The application of ^{14}C-carbon dioxide revealed that cinnamic acid and the hydroxycinnamic acids became radioactively labelled in the leaves initially, followed by labelling in the roots and then the corm. The radioactivity accumulated, initially, in *p*-coumaric acid in all of the organs. It is not likely that the radioactivity was transported in this form, as *p*-coumaric acid was poorly translocated in comparison to cinnamic acid and the remaining hydroxycinnamic acids. Since it is the first hydroxycinnamic acid to become labelled it is postulated that the transport form of the shikimate derivatives from the leaves to the roots and corm is phenylalanine and/or cinnamic acid which are rapidly converted to the more metabolically stable *p*-coumaric and caffeic acids for sequestering. Whether these latter two acids are at all involved naturally in the process of hypoxoside synthesis in the corm tissue, is not known. Therefore whether phenylalanine and/or cinnamic acid are serving directly as precursors for hypoxoside, is unclear.

The seasonal fluctuation in the hypoxoside synthesis, indicates that the biosynthesis of this phytochemical follows the pattern typical for secondary metabolites. Most of the synthesis and accumulation occurred after the corms had broken winter dormancy and produced a flush of leaves. Only once the growth of the shoots slowed did hypoxoside biosynthesis occur to any substantial degree. Thus once the growth requirements of the leaves had been satisfied, the shikimate derivatives became available for hypoxoside biosynthesis. Once the leaves had fully senesced this process stopped while the plant was dormant.

Therefore, the most satisfactory time at which plants could be harvested for their corms, would be when the leaves are dying back at the onset of dormancy, as this is when the levels of hypoxoside are the highest.

The *in vitro* studies further substantiate the observation that hypoxoside biosynthesis occurs only once growth begins to slow down. This would appear to indicate that hypoxoside is a shunt metabolite, formed when excess primary intermediates, caused by slowing of growth, are shunted into secondary metabolism. This is a simplistic interpretation as further *in vitro* studies, including carbohydrate and mineral availability, hormonal effects and potential precursor availability, showed that the triggering and control of hypoxoside biosynthesis is dependent on factors other than just an accumulation of the primary intermediates of primary metabolism which serve as precursors for the shikimate pathway. While the availability of sucrose, nitrogen, phosphate and calcium did influence hypoxoside production, there was not always a concommitant decrease in growth with increased hypoxoside production when these nutrients were reduced. Manipulating the nutrients in combinations further illustrated that the view that restriction of a nutrient will cause a shunt into secondary metabolism does not hold in every case.

The *in vitro* studies, with regard to light and temperature, agreed with the site of synthesis and seasonal investigations. Since hypoxoside is apparently synthesised only in the subterranean portions of the plant, it is not surprising to find that this process occurs more efficiently in the dark. It was also not surprising to find that

hypoxoside biosynthesis, a process which occurred in mid- to late summer in intact plants, was favoured by a temperature of 30 ^{o}C over that of 20 ^{o}C.

It was beyond the scope of this study to elucidate the role of hypoxoside in plants of *H. hemerocallidea*. Such an investigation would involve studying the species in an ecological context. Suffice to say that hypoxoside is a typical secondary metabolite in many respects, in that it accumulates in the vacuoles of the corm cells towards the end of growth of the shoot and is not apparently utilised by the plant. However, many aspects of this study require further attention, particularly the biosynthetic derivation of the phytochemical.

REFERENCES

AKAHORI, A. YASUDA, F. OKUNO, I. TOGAMI, M. OKANISHI, T. and IWAO, T. 1969. Studies on the steroidal components of domestic plants - LVI - Changes in the sapogenin composition of *Dioscorea tokoro* in its first season's growth from seed. *Phytochemistry* 8:45-50.

AMANN, M., WANNER, G. and ZENK, M.H. 1986. Intracellular compartmentation of two enzymes of berberine biosynthesis in plant cell cultures. *Planta* 167:310-320.

AMORIM, H.V., DOUGALL, D.K. and SHARP, W.R. 1977. The effect of carbohydrate and nitrogen concentration on phenol synthesis in Paul's Scarlet Rose cells grown in tissue culture. *Physiologia Plantarum* 39:91-95.

ARAGAO CRAVEIRO, A., DA COSTA PRADO, A., GOTTLIEB, O.R. and WELERSON DE ALBUQUERQUE, P.C. 1970. Diarylheptanoids of *Centrolobium* species. *Phytochemistry* 9:1869-1875.

ARMHEIN, N. and ZENK, M.H. 1971. Untersuchungen zur Rolle der Phenylalanin-ammonium-lyase (PAL) bei der Regulation der Flavonoidsynthese im Buchweizen (*Fagopyrum esculentum* Moench.) *Zeitschrift für Pflanzenphysiologie* 64:145-168.

ASAKAWA, Y. 1970. Chemical constituents of *Alnus firma* (Betulaceae). I. Phenyl propane derivatives isolated from *Alnus firma*. *Nihon Kagaku-Kai (Chemical Society of Japan Bulletin)* 43:2223-2229

ASHIHARA, H. and KUBOTA, H. 1986. Patterns of adenine metabolism and caffeine biosynthesis in different parts of tea seedlings. *Physiologia Plantarum* 68:275-281.

BAGGE, P. and LARSSON, C. 1986. Biosynthesis of aromatic amino acids by highly purified spinach chloroplasts - Compartmentation and regulation of the reactions *Physiologia Plantarum* 68:641-647.

BAKER, J.G. 1878. A synopsis of the Hypoxidaceae. *Journal of the Linnean Society* 17:93-126.

BAKER, J.G. 1896. Amaryllidaceae. *Flora Capensis* 6:171-246.

BALAGUÉ, C. and WILSON, G. 1982. Growth and alkaloid biosynthesis by cell suspensions of *Catharanthus roseus* in a chemostat under sucrose and phosphate limiting conditions. *Physiologie Végétale* 20:515-522.

BANERJI, A. and CHINTALWAR, G.J. 1984. Biosynthesis of bakuchiol from cinnamic and *p*-coumaric acids. *Phytochemistry* 23:1605-1606.

BARBERAN, F.A.T., TOMAS, F. and NUNEZ, J.M. 1985. Seasonal variation of free flavone aglycones from *Sideritis leucantha* (Laminaceae) *Zeitschrift für Naturforschung* 40c:914-916.

BARCLAY, A.S. and PERDUE, R.E. 1976. Distribution of anticancer activity in higher plants. *Cancer Treatment Reports* 60:181-1113.

BARIK, B.R., KUNDU, A.B. and DEY, A.K. 1987. Two phenolic constituents from *Alpinia galanga* rhizome. *Phytochemistry* 26:2126-2127.

BATE-SMITH, E.C. 1956. Chromatography and systematic distribution of ellagic acid. *Chemistry and Industry BIF Review* :32-33.

BEECHER, C.W.W., SARG, T.M. and EDWARDS, J.M. 1983. Occurrence and biosynthesis of 9-phenylphenalenones in callus tissue of *Lachnanthes tinctoria*. *Journal of Natural Products* 46:932-933.

BEGLEY, M.J., CAMPBELL, R.V.M., CROMBIE, L., TUCK, B. and WHITING D.A. 1971. Constitution and absolute configuration of *meta, meta*-bridged, strained biphenyls from *Myrica nagi*; X-ray analysis of 16-bromomyricanol. *Journal of the Chemistry Society C*:3634-3637.

BEGLEY, M.J. and WHITING, D.A. 1970. X-ray study of 16-bromomyricanol; the structure of myricanol, a natural *m,m*-bridged bent biphenyl. *Chemical Communications* :1207-1208.

BENTHAM, G. and HOOKER, J.D. 1883. *Genera Plantarum*. Vol. 3. Reeve. London.

BERACIERTA, A.P. and WHITING, D.A. 1976. Synthesis of the (±)-dimethylethers of agatharesinol, sequirin-A and hinokiresinol, related norlignins of Coniferae. *Tetrahedron Letters* 27:2367-2370.

BERACIERTA, A.P. and WHITING, D.A. 1978. Stereoselective total synthesis of the (±)-di-o-methyl ethers of agatharesinol, sequirin-A and hinokiresinol, and of (±)-tri-o-methyl-sequirin-E, characteristic norlignins of Coniferae. *Journal of the Chemical Society Perkin Transactions I* 3:1257-1263.

BERLIN, J. 1988. Formation of secondary metabolites in cultured plant cells and its impact on pharmacy. In: *Biotechnology in Agriculture and Forestry. 4. Medicinal and Aromatic Plants I.* Bajaj, Y.P.S. (ed). Springer-Verlag, Berlin, Heidelberg, New York.

BERLIN, J., SIEG, S., STRACK, D., BOKERN, M. and HARMS, H. 1986. Production of betalins by suspension cultures of *Chenopodium rubrum* L. *Plant Cell Tissue and Organ Culture* 5:163-174.

BEWS, J.W. and VANDERPLANK, J.E. 1930. Storage and other carbohydrates in a Natal succulent and a Natal geophyte and their behaviour before, during and after the winter resting season. *Annals of Botany* 44:689-719.

BHATT, P.N., BHATT, D.P. and SUSSEX, I. 1983. Studies on some factors affecting solasodine contents in tissue cultures of *Solanum nigrum*. *Physiologia Plantarum* 57:159-162.

BICKEL, H., PALME, L. and SCHULTZ, G. 1978. Incorporation of shikimate and other precursors into aromatic amino acids and prenylquinones of isolated spinach chloroplasts. *Phytochemistry* 17:119-124.

BICKEL, H. and SCHULTZ, G. 1979. Shikimate pathway regulation in suspensions of intact spinach chloroplasts. *Phytochemistry* 18:498-499.

BINDROO, B.B., BHAT, B.K. and KACHROO, P. 1984. Seasonal periodicity of diosgenin production in *Dioscorea deltoidea* Wall. *Beiträge zur Biologie der Pflanzen* 60:333-339.

BIRCH, A.J. and DONOVAN, F.W. 1953. Studies in relation to biosynthesis. I. Some possible routes of derivatives of orcinol and phloroglucinol. *Australian Journal of Chemistry* 6:360-368.

BITSCH, A., TRIHBES, R. and SCHULTZ, G. 1984. Compartmentation of phenylacetic and cinnamic acid synthesis in spinach. *Physiologia Plantarum* 61:617-621.

BLUM, U. and DALTON, B.R. 1985. Effects of ferulic acid, an allelopathic compound, on leaf expansion of cucumber seedlings grown in nutrients culture. *Journal of Chemical Ecology* 11:279-301.

BLUM, U., DALTON, B.R. and RAWLINGS, J.O. 1984. Effects of ferulic acid and some of its microbial metabolic products on radicle growth of cucumber. *Journal of Chemical Ecology* 10:1169-1191.

BLUM, U., DALTON, B.R. and SHANN, J.R. 1985a. Effects of various mixtures of ferulic acid and some of its microbial metabolic products on cucumber leaf expansion and dry matter in nutrient culture. *Journal of Chemical Ecology* 11:619-641.

BLUM, U., DALTON, B.R. and SHANN, J.R. 1985b. Effects of ferulic and *p*-coumaric acids in nutrient culture of cucumber leaf expansion as influenced by pH. *Journal of Chemical Ecology* 11:1567-1582.

BLUNDEN, G., HARDMAN, R. and HIND, F.J. 1970. Seasonal variation in sapogenin yields from *Dioscorea sylvatica* and *D. hondurensis*. *Planta Medica* 19:19-21.

BONNER, J. and GALSTON, W.A. 1952. *Principles of Plant Physiology*. Freeman-Cooper. San Francisco.

BRISSON, J.D., ROBB, J. and PETERSON, R.L. 1976. Phenolic localisation by ferric chloride and other compounds. *Microscopical Society of Canada* 3:174-175.

BROWN, S.A. 1981. Coumarins. In: *The Biochemistry of Plants: Secondary Plant Products*. Vol.7. Conn, E.E. (ed.). Academic Press. London. pp.269-300.

BROWN, S.A. 1985. Biosynthesis of 6,7-dihydroxycoumarin in *Cichorium intybus*. *Canadian Journal of Biochemistry and Cell Biology* 63:292-295.

BROWN, S.A. and NEISH, A.C. 1955. Studies of lignin biosynthesis using isotopic carbon. IV. Formation from some aromatic monomers. *Canadian Journal of Biochemistry* 33:948-962.

BROWN, S.A., RIVETT, D.E.A. and THOMPSON, H.J. 1984. Elaboration of the 6,7,8 oxygenation pattern of simple coumarins: Biosynthesis of puberulin in *Agathosoma puberula* Fourc. *Zeitschrift für Naturforschung* 39c:31-37.

BRYANT, A.T. 1966. *Zulu Medicine and Medicine Men*. C. Struik. Cape Town.

BUCHHOLZ, B., REUPKE, B., BICKEL, H. and SCHULTZ, G. 1979. Reconstruction of amino acid synthesis by combining spinach chloroplasts with other leaf organelles. *Phytochemistry* 18:1109-1111.

BU'LOCK, J.D. 1961 Intermediary metabolites and antibiotic synthesis. *Advances in Applied Microbiology* 3:293-342.

BU'LOCK, J.D. 1965. *Biosynthesis of Natural Products: An Introduction to Secondary Metabolism.* McGraw-Hill. Maidenhead.

BU'LOCK, J.D. 1980 Mycotoxins as secondary metabolites. In: *The Biosynthesis of Mycotoxins.* Steyn, P.S. (ed.). Academic Press. London. pp.1-16.

BU'LOCK, J.D. and POWELL, H.M. 1964. Secondary metabolism: an explanation in terms of induced enzyme synthesis. *Experientia* 21:55-56.

BUTCHER, D.N. 1977 Secondary products in tissue culture. In: *Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture.* Reinert, J. and Bajaj, Y.P.S. (eds.). Springer-Verlag. Berlin. pp.668-693.

BYNG, G.S., KANE, J.F. and JENSEN, R.A. 1982. Diversity in the routing and regulation of complex biochemical pathways as indicators of microbial relatedness. *Critical Reviews in Microbiology* 9:227-252.

CAMM, E.L. and TOWERS, G.H.N. 1973. Phenylalanine ammonia lyase. *Phytochemistry* 12:961-973.

CAMPBELL, R.V.M., CROMBIE, L., TUCK, B. and WHITING, D.A. 1970. Isolation and structure of new *meta*-bridged biphenyls from *Myrica nagi*. *Chemical Communications* :1206-1207.

CARLSON, J.E. and WIDHOLM, J.M. 1978. Separation of two forms of anthranilate synthetase from 5-methyl-tryptophan-susceptible and -resistant cultured *Solanum tuberosum* cells. *Physiologia Plantarum* 44:251-255.

CHAPMAN, D.J. and RAGAN, M.A. 1980. Evolution of biochemical pathways: Evidence from comparative biochemistry. *Annual Review of Plant Physiology* 31:639-678.

CHEADLE, V.I. 1937. Secondary growth by means of a thickening ring in certain monocotyledons. *Botanical Gazette* 98:535-555.

CO, H. and MARKAKIS, P. 1966. Precursors in the biosynthesis of pelargonidin 3-glucoside. *Phytochemistry* 5:755-759.

COLLIN, H.A. 1987. Determinants of yield of secondary products in plant tissue cultures. *Advances in Botanical Research* 13:145-187.

CONNELLY, J.A. and CONN, E.E. 1986. Tyrosine biosynthesis in *Sorghum bicolor*: Isolation and regulatory properties of arogenate dehydrogenase. *Zeitschrift für Naturforschung* 41c:69-78.

CONSTABEL, F. 1968. Gerbstoffproduktion der Calluskulturen von *Juniperis communis* L. *Planta* 79:58-64.

CONSTABEL, F., SHYLUK, J.P. and GAMBORG, D.L. 1971. The effect of hormones on anthocyanin accumulation in cell cultures of *Haplopappus gracilis*. *Planta* 96:306-316.

CORNFORTH, J.W. 1973. The logic of working with enzymes. *Chemistry Society Reviews* 2:1-20.

COTTLE, W. and KOLATTUKUDY, P.E. 1982. Biosynthesis, deposition and partial characterisation of potato suberin phenolics. *Plant Physiology* 69:393-399.

COURTOIS, D. and GUERN, J. 1980. Temperature response of *Catharanthus roseus* cells cultivated in liquid medium. *Plant Science* 17:473-482.

CRUZADO, H.J., DELPIN, H. and ROARK, B.A. 1965. Sapogenin production in relation to age of tuber in two *Dioscorea* sp. *Turrialba* 15:25-28.

CZINCHI, U. and KINDL, H. 1975. Formation of *p*-coumaric and o-coumaric acid from L-phenylalanine by microsomal membrane-bound enzyme complexes. *Planta* 125:115-125.

CZINCHI, U. and KINDL, H. 1977. Phenylalanine ammonia lyase and cinnamic acid hydroxylases as assembled consecutive enzymes on microsomal membranes of cucumber cotyledons: Cooperation and subcellular distribution. *Planta* 134:133-143.

DAHLGREN, R.M.T. and CLIFFORD, H.T. 1982. *The Monocotyledons : A Comprehensive Study*. Academic Press. London.

DAHLGREN, R.M.T., CLIFFORD, H.T. and YEO, P.F. 1985. *The Families of the Monocotyledons: structure, evolution and taxonomy*. Springer. Berlin.

DAVIS, B.D. 1955. Intermediates in amino acid biosynthesis. *Advances in Enzymology* 16:247-312.

DE-EKNAMKUL, W. and ELLIS, B.E. 1984. Rosmarinic acid production and growth characteristics of *Anchusa officinalis* cell suspension cultures. *Planta Medica* 4:346-350.

DE-EKNAMKUL, W. and ELLIS, B.E. 1985a. Effects of macronutrients on growth and rosmarinic acid formation in cell suspension cultures of *Anchusa officinalis*. *Plant Cell Reports* 4:46-9.

DE EKNAMKUL, W. and ELLIS B.E. 1985b. Effects of auxins and cytokinins on growth and rosmarinic acid formation. *Plant Cell Reports* 4:50-53.

DEL MORAL, R. and MULLER C.H. 1970. The allelopathic effects of *Eucalyptus camaldulensis*. *American Midland Naturalist* 83:254-282.

DENNIFF, P., MACLEOD, I. and WHITING, D.A. 1980. Studies in the biosynthesis of [6]-gingerol, pungent principle of ginger (*Zingiber officinale*). *Journal of the Chemistry Society Perkin Transactions I* 12:2637-2644.

DEUS, B. and ZENK, M.H. 1982. Exploitation of plant cells for the production of natural compounds. *Biotechnology and Bioengineering* 24:1965-1974.

DEUS-NEUMANN, B. and ZENK, M.H. 1984. A highly selective alkaloid uptake system in the vacuoles of higher plants. *Planta* 162:250-260.

DHAR, M.L., DHAR, M.M., DHAWAN, B.N., MEHROTRA, B.N. and RAY, C. 1968. Screening of Indian plants for biological activity: Part 1. *Indian Journal of Experimental Biology* 6:232-247.

DOUGALL, D.K. 1979. Factors affecting the yields of secondary products in plant tissue cultures. In: *Plant Cell and Tissue Culture*. Sharp, W.R., Larsen, P.O., Paddock, E.F. and Raghavan, V. (eds.). Ohio State University Press. Columbus. pp.727-743.

DREWES, S.E., HALL, A.J., LEARMONTH, R.A. and UPFOLD, U. 1984. Isolation of hypoxoside from *Hypoxis rooperi* and the synthesis of (E)-1,5-*bis*(3',4'-dimethoxyphenyl)pent-4-en-1-yne. *Phytochemistry* 23:1313-1316.

DUDLEY, M.W., DUEBER, M.T. and WEST, C.A. 1986. Biosynthesis of the macrocyclic diterpene casbene in castor bean (*Ricinus communis* L.) seedlings. *Plant Physiology* 81:335-342.

DYER, R.A. 1976. *The Genera of Southern African Flowering Plants. Vol.2. Gymnosperms and Monocotyledons.* Department of Agriculture and Technical Services. Pretoria.

EDWARDS, J.M., SCHMITT, R.C. and WEISS, U. 1972. Biosynthesis of a 9-phenylperinaphthanone by *Lachnanthes tinctoria*. *Phytochemistry* 11:1717-1720.

ELLIS, B.E. 1984. Probing secondary metabolism in plant cell cultures. *Canadian Journal of Botany* 62:2912-2917.

ENDRESS, R., JÄGER, A. and KREIS, W. 1984. Catecholamine biosynthesis dependent on the dark in betacyanin-forming *Portulaca* callus. *Journal of Plant Physiology* 115:291-295.

ENGELSMA, G. 1968. Photoinduction of phenylalanine deaminase in gerkin seedlings. III. Effects of excision and irradiation on enzyme development in hypocotyl segments. *Planta* 82:355-268.

ENZELL, C.R., HIROSE, Y. and THOMAS, B.R. 1967. The chemistry of the order Araucariales. 6. Absolute configurations of agatharesinol, hinokiresinol and sugiresinol. *Tetrahedron Letters* 9:793-798.

ENZELL, C.R. and THOMAS, B.R. 1965. The chemistry of the order Araucariales. 2. The wood resin of *Agathis australis*. *Acta Chemica Scandinavica* 19:913-919.

ENZELL, C.R. and THOMAS, B.R. 1966. The chemistry of the order Araucariales. 5. Agatharesinol. *Tetrahedron Letters* 22:2395-2402.

ENZELL, C.R., THOMAS, B.R. and WAHLBERG, I. 1967. The chemistry of the order Araucariales. 7. Mass spectrometric fragmentation of diphenylpentanneresinols. *Tetrahedron Letters* 23:2211-2217.

ESAU, K. 1977. *Anatomy of Seed Plants.* John Wilely and Sons. New York. London.

FERNANDES, A. and NEVES, J.B. 1961. Sur la caryologie de quelques monocotylédones africaines. *Rendus IV^e Reunion AETFAT*:439-464.

FIEDLER, E. and SCHULTZ, G. 1985. Localisation, purification and characterization of shikimate oxidoreductase-dehydroquinate hydrolyase from the stroma of spinach chloroplasts. *Plant Physiology* 79:212-218.

FLOSS, H.G. 1979. The shikimate pathway. In: *Biochemistry of Plant Phenolics. Recent Advances in Phytochemistry.* Vol. 12. Swain, T., Harborne, J.B. and Van Sumere, C.F. (eds.). Plenum Press, New York, London. pp.59-90.

FORKMANN, G., HELLER, W. and GRISEBACH, H. 1980. Anthocyanin biosynthesis in flowers of *Mathiola incana*, flavanone 3'- and flavonoid 3'-hydroxylases. *Zeitschrift fur Naturforschung* 35c:691-695.

FORKMANN, G. and STOTZ, G. 1981. Genetic control of flavonone 3-hydroxylase activity and flavonoid 3'-hydroxylase activity in *Antirrhinum majus* (Snapdragon). *Zeitschrift fur Naturforschung* 36c:411-416.

FORREST, G.I. 1969. Studies on the polyphenol metabolism of tissue cultures derived from the tea plant (*Camellia sinensis* L.) *Biochemistry Journal* 113:765-772.

FOSTER, J.W. 1947. Some introspections on mold metabolism. *Bacteriological Reviews* 11:167-188.

FISCHER and MEYER. 1845. Cited in: HILLIARD, O.M. and BURTT, B.L. 1986. Notes on some plants of southern Africa chiefly from Natal. XII. *Notes Royal Botanic Garden Edinburgh* 43:189-228.

FRAENKEL, G.S. 1959. The raison d'etre of secondary plant substances. *Science* 129:1466-1470.

FRISCHKNECHT, P.M., ELLER, B.M. and BAUMANN, T.W. 1982. Purine alkaloid formation and carbon dioxide gas change in dependence of development and of environmental factors in leaves of *Coffea arabica* L. *Planta* 156:296-301.

FRY, S.C. 1982. Phenolic components of primary cell wall: Feruloylated disaccharides of D-galactose and L-arabinones from spinach polysaccharide. *Biochemical Journal* 203:493-504.

FURUYA, T., YOSHIKAWA, T. and TAIRA, M. 1984. Biotransformation of codeinone to codeine by immobilized cells of *Papaver somnifera*. *Phytochemistry* 23:999-1001.

GEERINCK, D. 1968. Considerations taxonomiques au suget des Haemodoraceae et des Hypoxidaceae (Monocotyledones). *Bulletin of the Royal Botanical Society of Belgium* 39:47-82.

GEISSMAN, T.A. and CROUT, D.H.G. 1969. *Organic Chemistry of Secondary Plant Products.* Freeman-Cooper. San Francisco.

GOERS, S.K. and JENSEN, R.A. 1984. The differential allosteric regulation of two chorismate mutase isoenzymes of *Nicotiana silvestris*. *Planta* 12:117-124.

GOODWIN, P.B. 1978. Phytohormones and growth and development of organs of the vegetative plant. In: *Phytohormones and Related Compounds - A Comprehensive Treatise*. Vol. 2. Letham, D.S., Goodwin, P.B. and Higgins, T.J.V. (eds.). Elsevier. Amsterdam. pp.31-173.

GREGOR, H.D. 1976. Studies on phenylalanine ammonia-lyase from caster bean endosperm. 1. Subcellular localisation and induction of the enzyme. *Zeitschrift fur Pflanzenphysiologie* 77:454-463.

GRISEBACH, H. 1957. Zur Biogenese des Cyanidins. *Zeitschrift für Naturforschung* 12b:227-231.

GRISEBACH, H. 1981. Lignins. In: *The Biochemistry of Plants. Secondary Plant Products*. Vol.7. Conn, E.E. (ed.). Academic Press. London. pp.457-476.

GRISEBACH, H. and HAHLBROCK, K. 1974. Enzymology and regulation of flavonoid and lignin biosynthesis in plants and plant cell suspension cultures. In: *Metabolism and Regulation of Secondary Plant Products - Recent Advances in Phytochemistry*. Vol.8. Runeckles, V.C. and Conn, E.E. (eds.). Academic Press. New York. pp.21-52.

GROB, K. and MATILE, P. 1979. Vacuolar localisation of glucosinolates in horse-radish root cells. *Plant Science Letters* 14:327-335.

GROSS, G.G. 1979. Recent advances in the chemistry and biochemistry of lignin. In: *Biochemistry of Plant Phenolics. Recent Advances in Phytochemistry*. Vol.12. Swain, T., Harborne, J.B. and Van Sumere, C.F. (eds.). Plenum Press. London. pp.177-220.

GROSS, G.G. 1981. Phenolic acids. In: *The Biochemistry of Plants: Secondary Plant Products*. Vol.7. Conn, E.E. (ed.). Academic Press. London. pp.301-316.

GUNNING, B.E.S. and STEER, M.W. 1975. *Ultrastructure and Biology of Plant Cells*. Edward Arnold. London.

HAGIMORI, M., MATSUMOTO, T. and OBI, Y. 1982. Studies on the production of *Digitalis* cardenolide by plant tissue culture. III. Effects of nutrients on digitoxin formation by shoot-forming cultures of *Digitalis purpurea* L. grown in liquid media. *Plant and Cell Physiology* 23:1205-1211.

HAGMANN, M.L., HELLER, W. and GRISEBACH, H. 1983. Induction and characterisation of a microsomal flavonoid 3'-hydroxylase from parsley cell cultures. *European Journal of Biochemistry* 134:547-554.

HAHLBROCK, K. 1972. Isolation of apigenin from illuminated cell suspension cultures of soybean, *Glycine max*. *Phytochemistry* 11:165-167.

HAHLBROCK, K. 1981. Flavonoids. In: *The Biochemistry of Plants. Secondary Plant Products.* Vol.7. Conn, E.E. (ed.). Academic Press, London and New York. pp.425-456.

HAHLBROCK, K. and WELLMAN, E. 1970. Light induced flavone biosynthesis and activity of phenylalanine-ammonia-lyase and UDP-apiose synthetase in cell suspension cultures of *Petroselinum hortense*. *Planta* 94:236-239.

HAHLBROCK, K. and WELLMAN, E. 1973. Light-independent induction of enzymes related to phenylpropanoid metabolism in cell suspension cultures from parsley. *Biochimica et Biophysica Acta* 304:702-706.

HAMBURGER, M. and HOSTETTMANN, K. 1985. Hydroxycinnamic acid esters from *Polygala chamaebuxus*. *Phytochemistry* 24:1793-1797.

HAMMERTON, R.D., SMITH, M.T. and VAN STADEN, J. 1989. Factors influencing seed variability and germination in *Hypoxis hemerocallidea*. *Seed Science and Technology*. In press.

HAMMERTON, R.D. and VAN STADEN, J. 1988. Seed germination in *Hypoxis hemerocallidea*. *South African Journal of Botany*. In press.

HANSON, K.R. and HAVIR, E.A. 1979. An introduction to the enzymology of phenylpropanoid biosynthesis. In: *Biochemistry of Plant Phenolics. Recent Advances in Phytochemistry*. Vol.12. Plenum Press. New York. pp.91-138.

HARBORNE, J.B. 1977. *Introduction to Ecological Biochemistry*. Academic Press. London.

HARBORNE, J.B. 1980. Plant phenolics. In: *Secondary Plant Products. Encyclopedia of Plant Physiology*. Vol.8. Bell, E.A. and Charlwood, B.V. (eds.). Springer-Verlag. Berlin. pp.329-402.

HARBORNE, J.B. and CORNER, J.J. 1961. Plant polyphenols. 4. Hydroxycinnamic acid-sugar derivatives. *Biochemistry Journal* 81:242-250.

HARBORNE, J.B. and SIMMONDS, N.W. 1964. The natural distribution of phenolic aglycones. In: *Biochemistry of Phenolic Compounds*. Harborne, J.B. (ed.) Academic Press. New York. pp.77-128.

HARKES, P.A.A., KRIJBOLDER, L., LIBBENGA, K.R., WIJNSMA, R. NSENGIYAREMGE, T. and VERPOORTE, R. 1985. Influence of various media constituents on the growth of *Cinchona ledgeriana* tissue cultures and the production of alkaloids and anthroquinones therein. *Plant Cell Tissue and Organ Culture* 4:199-214.

HARMON, A.D., EDWARDS, J.M. and HIGHET, R.J. 1977. The biosynthesis of 2,5,6-trihydroxy-9-phenylphenalenone by *Lachnanthes tinctoria*. Incorporation of 1-13C-phenylalanine. *Tetrahedron Letters* 51:4471-4474.

HARTLEY, R.D., JONES, E.C. and WOOD, T.M. 1976. Carbohydrates and carbohydrate esters of ferulic acid released from cell walls of *Lolium multiflorum* by treatment with cellulolytic enzymes. *Phytochemistry* 15:305-307.

HARTWELL, J.L. 1967. Plants used against cancer. A survey. *Journal of Natural Products (Lloydia)* 30:379-439.

HASHIMOTO, T., TORI, M. and ASAKAWA, Y. 1986. Five new diarylheptanoids from the male flowers of *Alnus sieboldiana*. *Chemical and Pharmaceutical Bulletins* 34:1846-1849.

HASLAM, E. 1974. *The Shikimate Pathway*. Butterworths. London.

HASLAM, E. 1981. Vegetable tannins. In: *The Biochemistry of Plants. Secondary Plant Products*. Vol.7. Conn, E.E. (ed.). Academic Press. London. pp.527-556.

HASLAM, E. 1985. *Metabolism and Metabolites*. Clarendon Press. Oxford.

HASLAM, E. 1986. Secondary metabolism - fact and fiction. *Natural Product Reports* 3:217-249.

HATANO, T., KIRA, R., YOSHIZAKI, M. and OKUDA, T. 1986. Seasonal changes in the tannins of *Liquidambar formosana* reflecting their biogenesis. *Phytochemistry* 25:2787-2789.

HAUPT, A.W. 1930. A gelatin fixative for paraffin sections. *Stain Technology* 5:97-98.

HAY, C.A., ANDERSON, L.A., ROBERTS, M.F. and PHILLIPSON, J.D. 1986. *In vitro* cultures of *Cinchona* species. Precursor feeding of *C. ledgeriana* root organ suspension cultures with L-tryptophan. *Plant Cell Reports* 5:1-4.

HAYAT, M.A. 1981. *Fixation for Electron Microscopy*. Academic Press. New York.

HEGENAUER, R. 1963. *Chemotaxonomic der Pflanzen*. Band 2. Birkhauser-Verlag. Basel. pp.234-236.

HEIDE, L. and LEISTNER, E. 1983. Enzyme activities in extracts of anthroquinone-containing cells of *Gallium mollugo*. *Phytochemistry* 22:659-662.

HEIDEMAN, M.E. 1979. *Taxonomic Studies in the Genus Hypoxis L. (Hypoxidaceae) on the Witwatersrand*. M.Sc. Thesis. University of the Witwatersrand. Johannesburg.

HEINSTEIN, P.F. 1985. Future approaches to the formation of secondary natural products in plant suspension cultures. *Journal of Natural Products (Lloydia)* 48:1-9.

HERBERT, R.B. 1981. *The Biosynthesis of Secondary Metabolism*. Chapman and Hall. London.

HERRMANN, K. 1976. Flavonols and flavones in food plants: a review. *Journal of Food Technology* 11:433-448.

HILLIARD, O.M. and BURTT, B.L. 1986. Notes on some plants of southern Africa chiefly from Natal. XII. *Notes Royal Botanic Garden Edinburgh* 43:189-228.

HIROSE, Y., OISHI, N., NAGAKI, H. and NAKATSUKA, T. 1965. The structure of hinokiresinol. *Tetrahedron Letters* 41:3665-3668.

HOMEYER, B.C. and ROBERTS, M.F. 1984. Alkaloid sequestration by *Papaver somniferum* latex. *Zeitschrift für Naturforschung* 39c:876-881.

HOPP, W. and SEITZ, H.U. 1987. The uptake of acylated anthocyanin into isolated vacuoles from a cell suspension culture of *Daucus carota*. *Planta*. 170:74-85.

HOROWITZ, N.H. 1945. On the evolution of biochemical syntheses. *Proceedings of the National Academy of Science USA* 31:153-157.

HUTCHINSON, A., TAPER, C.D. and TOWERS, G.H.N. 1959. Studies of phloridzin in *Malus*. *Canadian Journal of Biochemistry and Physiology* 37:901-910.

HUTCHINSON, C.R. 1986. Biological methods for studying the biosynthesis of natural products. *Natural Product Reports* 3:133-151.

HUTCHINSON, J. 1959. *The Families of Flowering Plants*. Clarendon. Oxford.

HYODO, H., KURODA, H. and YANG, S.F. 1978 Induction of phenylalanine ammonia-lyase and increase in phenolics in lettuce leaves in relation to the development of russet spotting caused by ethylene. *Plant Physiology* 62:31-35.

HYODO, H. and YANG, S.F. 1971. Ethylene enhanced synthesis of phenylalanine ammonia-lyase in pea seedlings. *Plant Physiology* 47:765-770.

IBRAHIM, R.K. and EDGAR, D. 1976. Phenolic synthesis in *Perilla* cell suspension cultures. *Phytochemistry* 15:129-131.

IKEDA, T., MATSUMOTO, T. and NOGUCHI, M. 1976. Effects of nutritional factors on the formation of ubiquinone by tobacco plant cells in suspension culture. *Agricultural and Biological Chemistry* 40:1765-1770.

IKEDA, T., MATSUMOTO, T. and NOGUCHI, M. 1977. Effects of inorganic nitrogen sources and physical factors on the formation of ubiquinone by tobacco plant cells in suspension culture. *Agricultural and Biological Chemistry* 41:1197-1201.

ILLI, V., MARTIN, E.H. and PEGEL, K.H. 1976. Constituents of the monocotyledon *Hypoxis rooperi*. Family Hypoxidaceae. (An investigation of *H. rooperi*, part II). Unpublished.

INOUE, T., KENMOCHI, N., FURUKAWA, N., and FUJITA, M. 1987. Biosynthesis of acerogenin A, a diarylheptanoid from *Acer nikoense*. *Phytochemistry* 26:1409-1411.

IRELAND, C.R. and PASSAM, H.C. 1984. The level and distribution of phenolic plant growth inhibitors in yam tubers during dormancy. *New Phytology* 97:233-242.

ITOKAWA, H., AIYAMA, R., and IKUTA, A. 1981. A pungent diarylheptanoid from *Alpinia oxyphylla*. *Phytochemistry* 20:769-771.

JANAKI, N. and BOSE, J.L. 1967. An improved method for the isolation of curcumin from turmeric (*Curcuma longa* L.) *Journal of the Indian Chemical Society* 44:985-986.

JANZEN, D.H. 1969. Coevolution. *Science* 165:415.

JANISZOWSKA, W. 1987. Intracellular localization of tocopherol biosynthesis in *Calendula officinalis*. *Phytochemistry* 26:1403-1407.

JENSEN, R.A. 1986. The shikimate/arogenate pathway: Link between carbohydrate metabolism and secondary metabolism. *Physiologia Plantarum* 66:164-168.

JENSEN, R.A. and PIERSON, D.L. 1975. Evolutionary implications of different types of microbial enzymology for L-tyrosine biosynthesis. *Nature* 254:667-671.

JENSEN, W.A. 1962. *Botanical Histochemistry*. Freeman. San Francisco.

JONSSON, L.M.V., DONKER-KOOPMAN, W.E. and SCHRAM, A.W. 1984. Turnover of anthocyanins and tissue compartmentation of anthocyanin biosynthesis in flowers of *Petunia hybrida*. *Journal of Plant Physiology* 115:29-37.

JURD, L. and GEISSMAN, T.A. 1956. Absorption spectra of metal complexes of flavonoid compounds. *Journal of Organic Chemistry* 21:1395-1401.

KAMSTEEG, J., VAN BREDERODE, J. and VAN NIGTEVECHT, G. 1980. Genetic and biochemical evidence that the hydroxylation pattern of the anthocyanin B-ring in *Silene dioica* is determined at the *p*-coumaroyl-coenzyme A stage. *Phytochemistry* 19:1459-1462.

KAPOOR, L.D., KAPOOR, S.L., SRIVASTAVA, S.N., SINGH, A. and SHARMA, P.C. 1971. Survey of Indian plants for saponins, alkaloids and flavonoids. II. *Journal of Natural Products (Lloydia)* 34:94-102.

KARNICK, C.R. 1968. Seasonal periodicity of sapogenin production in *Dioscorea deltoidea* Wall and *Dioscorea prazeri* Prain & Burkill. *Planta Medica* 16:269-272.

KLISCHIES, M. and ZENK, M.H. 1978. Stereochemistry of C-methylation in the biosynthesis of rhododendrin in *Alnus* and *Betula*. *Phytochemistry* 17:1281-1284.

KNOBLOCH, K.H. and BERLIN, J. 1980. Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus* (L.) G. Don. *Zeitschrift für Naturforschung* 35c:551-556.

KNOBLOCH, K.H., BEUTNAGEL, G. and BERLIN, J. 1981. Influence of accumulated phosphate on culture growth and formation of cinnamoyl putrescines in medium-induced cell suspension cultures of *Nicotiana tabacum*. *Planta* 153:582-585.

KNOGGE, W. and WEISSENBÖCK, G. 1986. Tissue-distribution of secondary phenolic biosynthesis in developing primary leaves of *Avena sativa* L. *Planta* 167:196-205.

KOBLITZ, H., KOBLITZ, D. SCHMAUDER, H-P. and GRÖGER, D. 1983 Studies on tissue cultures of the genus *Cinchona* L. Alkaloid production in cell suspension cultures. *Plant Cell Reports* 2:122-125.

KOJIMA, M. and URITANI, I. 1972. Elucidation of the structure of a possible intermediate in chlorogenic acid biosynthesis in sweet potato root tissue. *Plant Cell Physiology* 13:1075-1084.

KOJIMA, M. and URITANI, I. 1973. Studies on chlorogenic acid biosynthesis in sweet potato root tissue in special reference to the isolation of a chlorogenic acid intermediate. *Plant Physiology* 51:768-771.

KOUKOL, J. and CONN, E.E. 1961. Metabolism of aromatic compounds in higher plants. 4. Purification and properties of phenylalanine deaminase of *Hordeum vulgare*. *Journal of Biological Chemistry* 236:2692-2698.

KREUZALER, F. and HAHLBROCK, K. 1973. Flavonoid glycosides from illuminated cell suspension cultures of *Petroselinum hortense*. *Phytochemistry* 12:1149-1153.

KRISHNA RAO, R.V., ALI, N., and REDDY, M.N. 1978. Occurrence of both sapogenin and alkaloid lycorine in *Curculigo orchiodes*. *Indian Journal of Pharmaceutical Sciences* 40:104-105.

KUBO, M., NAGAI, M. and INOUE, T. 1983. Studies on the constituents of Aceraceae plants. IV. Carbon-13 nuclear magnetic resonance spectra of acerogenin A, rhododendrol and related compounds, and structure of aceroside IV from *Acer nikoense*. *Chemical and Pharmaceutical Bulletins* 31:1917-1922.

KURZ, W.G.W. and CONSTABEL, F. 1985. Aspects affecting biosynthesis and biotransformation of secondary metabolites in plant cell cultures. *CRC Critical Reviews in Biotechnology* 2:105-118.

LEETE, E. 1983. Failure of 3-hydroxy-3-phenylpropanoic acid and cinnamic acid to serve as precursors of tropic acid in *Datura inoxia*. *Phytochemistry* 22:933-935.

LEIGH, R.A., AP REES, T., FULLER, W.A. and BANFIELD, J. 1979. The location of acid invertase activity and sucrose in the vacuoles of storage roots of beetroot (*Beta vulgaris*). *Biochemical Journal* 178:539-547.

LEISTNER, E. 1981. Biosynthesis of plant quinones. In: *The Biochemistry of Plants. Secondary Plant Products*. Vol.7. Conn, E.E. (ed.). Academic Press, London. pp.403-424.

LEWIS, W.H. and ELVIN-LEWIS, P.F. 1977. *Medical Botany. Plants Affecting Man's Health*. John Wiley. New York. pp.330.

LIAAEN-JENSEN, S. 1979. Carotenoids- A chemosystematic approach. *Pure and Applied Chemistry* 51:661-675.

LOOMIS, W.D. and BATTAILE, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5:423-438.

LUCKNER, M. 1980. Expression and control of secondary metabolism. In: *Secondary Plant Products*. Bell, E.A. and Charlwood, B.V. (eds.). Springer-Verlag. Heidelberg. pp.23-63.

LUH, B.S. and PHITHAKPOL, B. 1972. Characteristics of polyphenoloxidase related to browning in cling peaches. *Journal of Food Science* 37:264-268.

LÜTKE-BRINKHAUS, F., LIEDVOGEL, B. and KLEINIG, H. 1984. On the biosynthesis of ubiquinones in plant mitochondria. *European Journal of Biochemistry* 141:537-541.

MALTERUD, K.E. and ANTHONSEN, T. 1980. 13-oxomyricanol, a new [7.0]-metacyclophane from *Myrica nagi*. *Phytochemistry* 19:705-707.

MANITTO, P. 1981. *Biosynthesis of Natural Products*. Ellis Harwood. Chichester. pp.1.

MANN, J. 1987. *Secondary Metabolism*. Clarendon Press. Oxford.

MARINI-BETTOLO, G.B., NICOLETTI, M., MESSANA, I., GALEFFI, C., MSONTHI, J.D. and CHAPYA, W.A. 1985. Research on African medicinal plants. X. Glucosides of *Hypoxis nyasica* Bak. The structure of nyasoside, a new glucoside biologically related to hypoxoside. *Tetrahedron Letters* 41:665-670.

MARINI-BETTOLO, G.B., PATAMIA, M., NICOLETTI, M., GALEFFI, C. and MESSANA, I. 1982. Research on African medicinal plants. II. Hypoxoside, a new glycoside of uncommon structure from *Hypoxis obtusa* Buch. *Tetrahedron Letters* 11:1683-1687.

MARKOTTER, E.I. 1936. Die lewensgeskiedenis van sekere geslagte van die Amaryllidaceae. *Annals of the University of Stellenbosch*. A2.14.

MATHEW, A.G. and PARPIA, H.A.B. 1971. Food browning as a polyphenol reaction. *Advances in Food Research* 19:75-145.

MATILE, P. 1974. Das toxische Kompartiment der Pflanzenzelle. *Naturwissenschaften* 71:18-24.

MATILE, P. 1978. Biochemistry and function of vacuoles. *Annual Review of Plant Physiology* 29:193-213.

MATSUMOTO, T., NISHIDA, K., NOGUCHI, M. and TAMAKI, E. 1973. Some factors affecting the anthocyanin formation by *Populus* cells in suspension culture. *Agricultural and Biological Chemistry* 37:561-567.

MAYER, A.M. and HAREL, E. 1979. Review: Polyphenol oxidase in plants. *Phytochemistry* 18:193-215.

McCLURE, J.W. 1979. The physiology of phenolic compounds in plants. In: *Biochemistry of Plant Phenolics. Recent Advances in Phytochemistry*. Vol. 12. Swain, T., Harborne, J.B. and Van Sumere, C.F. (eds.(Plenum Press. New York. pp.525-556.

McPHERSON, J.K. 1971. Allelopathic constituents of chaparral shrub *Adenostoma fasciculatum*. *Phytochemistry* 10:2925-2933.

MEINS, F. 1983. Heritable variation in plant cell culture. *Annual Review of Plant Physiology* 34:327-346.

MERILLON, J.M., RIDÉAU, M. and CHÉNIEUX, J.C. 1984. Influence of sucrose levels on ajmalicine, serpentine and tryptamine in *Catharanthus roseus* cells *in vitro*. *Planta Medica* 50:497-501.

METTAL, U., BOLAND, W., BEYER, P. and KLEINIG, H. 1988. Biosynthesis of monoterpene hydrocarbons by isolated chromoplasts from daffodil flowers. *European Journal of Biochemistry* 170:613-616.

MINAMIKAWA, T. and URITANI, I. 1965a. Phenylalanine ammonia-lyase in sliced sweet potato roots. *Journal of Biochemistry* 57:678-688.

MINAMIKAWA, T. and URITANI, I. 1965b. Phenylalanine ammonia-lyase in sweet potato roots : inhibition by phenylpropanoids. *Journal of Biochemistry* 58:53-59.

MISHRA, A.K., HARIBAL, M.M. and SABATA, B.K. 1985. Garuganin III, a macrocyclic biphenyl ether from *Garuga pinnata*. *Phytochemistry* 24:2463-2465.

MIYASAKA, H., NASU, M., YAMAMOTO, T. and YONEDA, K. 1985. Production of ferruginol by cell suspension cultures of *Salvia miltiorrhiza*. *Phytochemistry* 24:1931-1933.

MIZUKAMI, H., KONOSHIMA, M. and TABATA, M. 1977. Effect of nutritional factors on shikonin derivative formation in *Lithospermum* callus cultures. *Phytochemistry* 16:1183-1987.

MOLDEREZ, M., NAGELS, L. and PARMENTIER, F. 1978. Time-course tracer studies on the metabolism of cinnamic acid in *Cestrum poeppigii*. *Phytochemistry* 17:1747-1750.

MÖLLER, B. and HERMANN, K. 1983. Quinic acid esters of hydroxycinnamic acids in stone and pome fruit. *Phytochemistry* 22:477-481.

MOORE, T. 1852. Cited in: HILLIARD, O.M. and BURTT, B.L. 1986. Notes on some plants of southern Africa chiefly from Natal. XII. *Notes Royal Botanic Garden Edinburgh* 43:184-228.

MOSKOWITZ, A.H. and HRAZDINA, G. 1981. Vacuolar contents of fruit subepidermal cells from *Vitis* species. *Plant Physiology* 68:686-692.

MOUSDALE, D.M. and COGGINS, J.R. 1985. Subcellular localisation of the common shikimate-pathway enzymes in *Pisum sativum*. *Planta* 163:241-249.

MUELLER, W.C. and BECKMAN, C.H. 1974. Ultrastructure of the phenol-storing cells in the roots of banana. *Physiological Plant Pathology* 4:187-190.

MUELLER, W.C. and GREENWOOD, A.D. 1978. The ultrastructure of phenolic storing cells fixed with caffeine. *Journal of Experimental Botany* 29:757-764.

MUHITCH, M.J. and FLETCHER, J.S. 1985. Influence of culture age and spermidine treatment on the accumulation of phenolic compounds in suspension cultures. *Plant Physiology* 78:25-28.

MULLER, C.H. 1969. The "co" in co-evolution. *Science* 164:197-198.

MURASHIGE, T. and SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* 15:473-497.

NASH, D.T. and DAVIES, M.E. 1972. Some aspects of growth and metabolism of Paul's Scarlet Rose cell suspensions. *Journal of Experimental Botany* 23:75-91.

NAGAI, M., KUBO, M. FUJITA, M., INOUE T. and MATSUO, M. 1978 Studies on the constituents of Aceraceae plants II. Structure of aceroside, a glucoside of a novel cyclic diarylheptanoid from *Acer nikoense* Maxim. *Chemical and Pharmaceutical Bulletins* 9:2805-2810.

NEISH, A.C. 1961. Formation of *m*- and *p*-coumaric acids by enzymatic deamination of the corresponding isomers of tyrosine. *Phytochemistry* 1:1-24.

NEISH, A.C. 1964. Major pathways of biosynthesis of phenols. In: *Biochemistry of Phenolic Compounds*. Harborne, J.B. (ed.). Academic Press. New York. pp.295-360.

NEL, 1914. Cited in: HILLIARD, O.M. and BURTT, B.L. 1986. Notes on some plants of southern Africa chiefly from Natal. XII. *Notes Royal Botanic Garden Edinburgh* 43:189-228.

NETTLESHIP, L. and SLAYTOR, M. 1974. Adaptation of *Peganum harmala* callus to alkaloid production. *Journal of Experimental Botany* 25:1114-1123.

NEUMANN, D., KRAUSS, G., HIEKE, M. and GRÖGER, D. 1983. Indole alkaloid formation and storage in cell suspension cultures of *Catharanthus roseus*. *Planta Medica* 48:20-23.

NOMURA, M., TOKOROYAMA, T. and KUBOTA, T. 1981. Biarylheptanoids and other constituents from wood of *Alnus japonica*. *Phytochemistry* 20:1097-1104.

OBATA-SASAMOTO, H. and KOMAMINE, A. 1983. Effect of callus conditions on DOPA accumulation in a callus culture of *Stizolobium hassjoo*. *Journal of Medicinal Plant Research* 49:120-123.

OZEKI, Y. and KOMAMINE, A. 1985. Changes in activities of enzymes involved in general phenylpropanoid metabolism during the induction and reduction of anthocyanin synthesis in carrot suspension culture as regulated by 2,4-D. *Plant and Cell Physiology* 26:903-911.

OZEKI, Y. and KOMAMINE, A. 1986. Effects of growth regulators on the induction of anthocyanin synthesis in carrot suspension cultures. *Plant and Cell Physiology* 27:1361-1368.

PAGE, Y.M. 1984. *The Regeneration of* Hypoxis rooperi *S. Moore and Production of Hypoxoside* In Vitro. Ph.D. Thesis. University of Natal. Pietermaritzburg.

PAGE, Y.M. and VAN STADEN, J. 1984. *In vitro* propagation of *Hypoxis rooperi*. *Plant Cell, Tissue and Organ Culture* 3:359-362.

PAGE, Y.M. and VAN STADEN, J. 1986. *In vitro* propagation of *Hypoxis rooperi* from flower buds. *South African Journal of Botany* 52:261-264.

PAGE, Y.M. and VAN STADEN, J. 1987. *In vitro* production of hypoxoside. *Plant Cell, Tissue and Organ Culture* 9:131-136.

PEGEL, K.H. 1976a. An investigation of *Hypoxis rooperi* (Family Hypoxidaceae). Part I. Unpublished.

PEGEL, K.H. 1976b. A note on the non-toxic nature of the Hypoxidaceae family and especially the *Hypoxis* genus. Part III. Unpublished.

PEGEL, K.H. 1979. Active plant extracts of Hypoxidaceae and their use. *United States Patent*:4160860.

PHILLIPS, R. and HENSHAW, G.G. 1977. The regulation of phenolics in stationary phase cell cultures of *Acer pseudoplatanus* L. *Journal of Experimental Botany* 28:785-794.

PLIENINGER, H., SCHWARZ, B., JAGGY, H., HUBER-PATZ, U., RODEWALD, H., IRNGARTINGER, H. and WEINGES, K. 1986. Isolierung Strukturaufklärung und Synthese von (Z,Z-4,4'-1,4-pentadien-1,5-diyl)diphenol einem ungewöhnlichen Naturstoff aus den Böttern des Ginkgo-Baumes (*Ginkgo biloba* L.) *Liebigs Annalen der Chemie* 1986:1772-1778.

POTGIETER, M. WENFELER, G.L. and DREWES, S.E. 1987. Synthesis of rooperol [1,5-bis(3',4'-dihydroxyphenyl)pent-1-en-4-yne]. *Phytochemistry* 27:1101-1104.

PRIDHAM, J.B. 1963. *Enzyme Chemistry of Phenolic Compounds*. Pergamon Press. Oxford.

RAFFAUF, R.F. 1970. *A Handbook of Alkaloids and Alkaloid-containing Plants*. Wiley-Interscience. New York.

RAMIREZ-MARTINEZ, J.R. and LUH, B.S. 1973. Phenolic compounds in frozen avocados. *Journal of Science of Food and Agriculture*. 24:219-225.

RAMSTAD, E. 1953. Über das Vorkommen und die Verbreitung von Chelidonsaure in einigen Pflanzenfamilien. *Pharmaceutica Acta Helvetiae* 28:45-57.

RAO, P.S. and BERI, R.M. 1950. Studies on plant mucilages III. Mucilage from the tubers of *Curculigo orchiodes*. *Proceedings of the Indian Academy of Science*. 34a:27-31.

RASMUSSEN, J.A. and EINHELLIG, F.A. 1977. Synergistic inhibitory effects of *p*-coumaric and ferulic acids on germination and growth of grain sorghum. *Journal of Chemical Ecology* 3:197-205.

RAVINDRANATH, V. and SATYANARAYANA, M.N. 1980. An asymmetrical diarylheptanoid from *Curcuma longa*. *Phytochemistry* 19:2031-2032.

REINERT, J., CLAUSS, H. and VON ARDENNE, R. 1964. Anthocyanbildung in Gewebekulturen von *Haplopappus gracilis* in licht verschiedener Qualität. *Naturwissenschaften* 51:87.

REISCH, J. and MÖLLMANN, H. 1974. Wirkstoffextrakt aus *Hypoxis* species und verfahren zu seiner Gewinnung. *German Offenlegungsschrift*: 2251695.

REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* 17:208-212.

RHODES, M.J.C. and WOOLTORTON, L.S.C. 1971. The effect of ethylene on respiration and on the activity of phenylalanine ammonia lyase in swede and parsnip root tissue. *Phytochemistry* 10:1989.

RHODES, J.M. and WOOLTORTON, L.S.C. 1978. The biosynthesis of phenolic compounds in wounded plant storage tissue. In: *Biochemistry of Wounded Plant Tissue*. Kahl, G. (ed.). Gruyter. Berlin. pp.243-286.

RHODES, M.J.C., WOOLTORTON, L.S.C. and HILL, A.C. 1981. Changes in phenolic metabolism in fruit and vegetable tissues under stress. In: *Recent Advances in the Biochemistry of Fruits and Vegetables*. Friend, J. and Rhodes, M.J.C. (eds.). Academic Press. London. pp.193-220.

RIBÉREAU-GAYON, P. 1972. *Plant Phenolics*. Oliver and Boyd. Edinburgh.

RILEY, H.P. 1963. *Families of Flowering Plants of Southern Africa*. University of Kentucky Press. pp.227.

RIOV, J., MONSELISE, S.P. and KAHAN, R.S. 1968. Effect of gamma radiation on phenylalanine ammonia lyase activity and accumulation of phenolics in citrus fruit peel. *Radiation Botany* 8:463-466.

ROBARDS, A.W. 1970. *Electron Microscopy and Plant Ultrastructure*. McGraw-Hill. London.

ROBINS, R.J., PAYNE, J. and RHODES, M.J.C. 1986. The production of anthroquinones by cell suspension cultures of *Cinchona ledgeriana*. *Phytochemistry* 25:2327-2334.

ROBINSON, T. 1980. *The Organic Constituents of Higher Plants*. Cardus Press. North Amherst.

ROUGHLEY, P.J. and WHITING, D.A. 1971. Diarylheptanoids: The problems of biosynthesis. *Tetrahedron Letters* 40:3741-3746.

ROUGHLEY, P.J. and WHITING, D.A. 1973. Experiments in the biosynthesis of curcumin. *Journal of the Chemistry Society Perkins Transactions I* :2379-2388.

RUBIN, J.L. and JENSEN, R.A. 1985. Differentially regulated isozymes of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase from seedlings of *Vigna radiata* (L.) Wilczek. *Plant Physiology* 79:711-718.

RUIS, H. and KINDL, H. 1971. Formation of α,β-unsaturated carboxylic acids from amino acids in plant peroxisomes. *Phytochemistry* 10:2627-2631.

SAHAI, O.P. and SHULER, M.L. 1984. Environmental parameters influencing phenolic production by batch cultures of *Nicotiana tabacum*. *Biotechnology and Bioengineering* 26:111-120.

SAIRAM, T.V. and KHANNA, P. 1971. Effect of tyrosine and phenylalanine on growth and production of alkaloids in *Datura tatula*. *Journal of Natural Products (Lloydia)* 34:170-171.

SAKUTA, M., TAKAGI, T. and KOMAMINE, A. 1986. Growth related accumulation of betacyanin in suspension cultures of *Phytolacca americana* L. *Journal of Plant Physiology* 125:337-344.

SASSE, F., HECKENBERG, U. and BERLIN, J. 1982. Accumulation of β-carboline alkaloids and serotonin by cell cultures of *Peganum harmala* L. *Plant Physiology* 69:400-404.

SATO, M. 1967. Metabolism of phenolic substances by the chloroplasts III. Phenotase as an enzyme concerning the formation of esculetin. *Phytochemistry* 6:1363-1373.

SATO, M. and HASEGAWA, M. 1972. Biosynthesis of dihyroxycoumarins in *Daphne odora* and *Cichorium intybus*. *Phytochemistry* 11:657-662.

SATO, T. KIUCHI, F. and SANKAWA, U. 1982. Inhibition of phenylalanine ammonia-lyase by cinnamic acid derivatives and related compounds. *Phytochemistry* 21:845-850.

SAUNDERS, J.A. 1979. Investigations of vacuoles isolated from tobacco. I. Quantitation of nicotine. *Plant Physiology* 64:74-78.

SCHLEPPHORST, R. and BARZ, W. 1979. Metabolism of aromatic acids in plant cell suspension cultures. *Planta Medica* 36:333-342.

SCHMAUDER, H.P. and GRÖGER, D. 1986. Biosynthesis of phenylalanine and tyrosine in *Claviceps*. *Planta Medica* 5:395-397.

SCHMIDT, R. and MOHR, H. 1981. Lag-phase and rate of synthesis in light-mediated anthocyanin synthesis. *Planta* 151:541-543.

SCHULZE-SIEBERT, D., HEINEKE, D., SCHARF, H. and SCHULTZ, G. 1984. Pyruvate-derived amino acids in spinach chloroplasts: Synthesis and regulation during photosynthetic carbon metabolism. *Plant Physiology* 76:465-471.

SCHUSTER, B. and HERMANN, K. 1985. Hydroxybenzoic and hydroxycinnamic acid derivatives in soft fruits. *Phytochemistry* 24:2761-2764.

SHANN, J.R. and BLUM, U. 1987. The utilisation of exogenously supplied ferulic acid in lignan biosynthesis. *Phytochemistry* 26:2977-2982.

SHOYAMA, Y., MATSUMOTO, M. and NISHIOKA, I. 1987. Phenolic glycosides from diseased roots of *Rehmania glutinosa* var. *purpurea*. *Phytochemistry* 26:983-986.

SKRZYPCZAKOWA, L. 1970. Flawonoidy I biosyntetyczme pokrewne zwiazki jako czynniki taksonomiczne klasy jednolisciennych. *Wiadomosci Botaniczne* 14:55-63.

SMITH, C.A. 1966. *Common Names of South African Plants. Department of Agricultural Technical Services. Botanical Survey Memoir No.35.* Government Printer. Pretoria. pp.544.

SPRIBILLE, R. and FORKMANN, G. 1982. Chalcone synthesis and hydroxylation of flavonoids in 3'-position with enzyme preparations from flowers of *Dianthus caryophyllus* L. (carnation). *Planta* 155:176-182.

STABA, E.J. 1982. Production of useful compounds from plant tissue cultures. In: *Plant Tissue Culture.* Fujiwara, A. (ed.). Japanese Association for Plant Tissue Culture. Tokyo.

STABA, E.J. 1985. Milestones in plant tissue culture systems for the production of secondary products. *Journal of Natural Products (Lloydia)* 48:203-209.

STAFFORD, H.A. 1974. The metabolism of aromatic compounds. *Annual Review of Plant Physiology* 25:459-486.

STAFFORD, H.A. and LEWIS, L.L. 1977 Interference by a phenylacetate pathway in isotopic assays for phenylalanine ammonia lyase in leaf extracts. *Plant Physiology* 60:830-834.

STAFLEU, F.A. 1972. *International Code of Botanical Nomenclature.* Uitgeversmaatschappij. Utrecht.

STAFLEU, F.A. and COWAN, R.S. 1976. *Taxonomic Literature.* Vol.1. Bohn, Scheltema and Holkema. Utrecht. pp.364.

STAHL, E. 1888. Cited in: FRAENKEL, G.S. 1959. The raison d'etre of secondary plant substances. *Science* 129:1466-1470.

STICKLAND, R.G. and SUNDERLAND, N. 1972a. Photocontrol of growth, and of anthocyanin and chlorogenic acid production in cultured callus tissue of *Haploppus gracilis*. *Annals of Botany* 36:671-685.

STICKLAND, R.G. and SUNDERLAND, N. 1972b. Production of anthocyanins flavonols and chlorogenic acid by cultured callus tissues of *Haplopappus gracilis*. *Annals of Botany* 36:443-457.

STOTZ, G., SPRIBILLE, R. and FORKMANN, G. 1984. Flavonoid biosynthesis in flowers of *Verbena hybrida*. *Journal of Plant Physiology* 116:173-183.

STRACK, D. 1977. Sinapic acid ester fluctuations in cotyledons of *Rhaphanus sativus*. *Zeitschrift für Pflanzenphysiologie* 84:139-145.

STRACK, D. and SHARMA, V. 1985. Vacuolar localisation of the enzymatic synthesis of hydroxycinnamic acid esters of malic acid protoplasts from *Raphanus sativus* leaves. *Physiologia Plantarum* 65:45-50.

STUART, G.A. 1977. *Chinese Materia Medica. Vegetable Kingdom.* Gordon Press. New York. pp.212.

SUGA, T., ASAKAWA, Y. and IWATA, N. 1971. 1,7-Diphenyl-1,3-heptadien-5-one : a new ketone from *Alnus pendula* (Betulaceae). *Chemistry and Industry* :766.

SUGIMOTO, Y., SUGIMURA, Y. and YAMADA, Y. 1988. Production of bisbenzylisoquinoline alkaloids in cultured roots of *Stephania cepharantha*. *Phytochemistry* 27:1379-1381.

SÜTFELD, R., KEHREL, B. and WIERMANN, R. 1978. Characterisation, development and localisation of "flavanone synthase" in tulip anthers. *Zeitschrift für Naturforschung* 33c:841-846.

SÜTFLED, R. and WIERMANN, R. 1981. Purification of chalcone synthase from tulip anthers and comparison with the synthase from *Cosmos* petals. *Zeitschrift für Naturforschung* 36c:30-34.

SUZUKI, T. 1985. Purine alkaloids in *Camellia sinensis* flowers. *Agricultural and Biological Chemistry* 49:2803-2805.

SUZUKI, T. and WALLER, G.R. 1984. Biosynthesis and biodegration of caffeine, theobromine and theophylline in *Coffea arabica* L. fruits. *Journal of the Science of Food and Agriculture* 35:66-70.

SUZUKI, T. and WALLER, G.R. 1985. Purine alkaloids of the fruits of *Camellia sinensis* L. and *Coffea arabica* L. during fruit development. *Annals of Botany* 56:537-542.

TABATA, M. 1977. Recent advances in the production of medicinal substances by plant cell cultures. In: *Plant Tissue Culture and Its Biotechnological Application.* Barz, W., Reinhard, E. and Zenk, M.H. (eds.). Springer. Berlin. pp.3-16.

TAL, B., GRESSEL, J. and GOLDBERG, I. 1982. The effect of media constituents on growth and diosgenin production by *Dioscorea deltoidea* cells grown in batch cultures. *Planta Medica* 44:111-115.

THOMAS, R. 1961. Studies on the biosynthesis of fungal metabolites. 3. The biosynthesis of fungal perinaphthenones. *Biochemical Journal* 78:807-813.

THOMAS, R. 1971. The biosynthesis of the plant phenalenone haemocorin. *Chemical Communications* :739-740.

THOMPSON, M.F. 1972. *Anatomical and Cytological Studies in the Hypoxidaceae.* M.Sc. Thesis. University of Stellenbosch. Stellenbosch.

THOMPSON, R.H. 1964. Structure and reactivity of phenolic compounds. In: *Biochemistry of Phenolic Compounds.* Harborne, J.B. (ed.). Academic Press. London. pp.1-32.

TSUSHIDA, T. and TAKEO, T. 1979. Changes of caffeine content in tea seedlings during germination and development. *Chagyo-Gijutsu Kenkyu* 57:38-42.

TUNTIWACHWUTTIKUL, P., PANCHAROEN, O., KANJANAPOTHI, D., PANTHONG, A., TAYLOR, W.C. and REUTRAKUL, V. 1986. Zingiberaceous plants. In: *Folk Medicine : The Art and the Science.* Steiner, R.P. (ed.). American Chemical Society. Washington D.C. pp.191-204.

UEHARA, S-I., YASUDA, I., AKIYAMA, K., MORITA, H., TAKEYA, K. and ITOKAWA, H. 1987. Diarylheptanoids from the rhizomes of *Curcuma xanthorrhiza* and *Alpinia officinarum. Chemical and Pharmaceutical Bulletins.* 35:3298-3304.

UNDERHILL, E.W., WATKIN, J.E. and NEISH, A.C. 1957. Biosynthesis of quercetin in buckwheat. Part 1. *Canadian Journal of Biochemistry and Physiology* 35:219-228.

VAN STADEN, J. 1981. Inhaltsstoffe der *Hypoxis rooperi* einer wertvollen Arzneipflanze des südlichen Afrikas. Sitosterine und Zytokinins im cormus. *Der Deutsche Apotheker* 33:460-466.

VAN STADEN, J., PAGE, Y.M. and FORSYTH, C. 1986. Antagonistic effect of hypoxoside on kinetin-induced growth of soybean callus. *South African Journal of Botany* 52:408-412.

VICKERY, M.L. and VICKERY, B. 1981. *Secondary Plant Metabolism.* Macmillan Press. London.

WAGNER, G.J. 1979. Content and vacuole/extravacuole distribution of neutral sugars, free amino acids and anthocyanins in protoplasts. *Plant Physiology* 64:88-93.

WALFORD, S.N. 1979. *Hypoxis rooperi* (Hypoxidaceae), eine bisher in Europa wenig bekannte Heilpflanze Afrikas. *Der Deutsche Apotheker*, 31:642-648.

WALL, M.E., KRIDER, M.M., KREWSON, C.F., EDDY, C.R., WILLAMAN, J.J., CORRELL, D.S. and GENTRY, G.S. 1954. *Steroidal sapogenins. XIII. Supplementary table of data for steroidal sapogenins.* United States Department of Agriculture Research Services, Circular AIC-363.

WALLER, G.R. and DERMER, O.C. 1981. Enzymology of alkaloid metabolism in plants and microorganisms. In: *The Biochemistry of Plants. Secondary Plant Products.* Vol. 7. Conn, E.E. (ed.). Academic Press. London. pp.317-402.

WALTON, D.C. and SONDHEIMER, E. 1968. Effects of abscisin II on phenylalanine ammonia-lyase activity in excised bean axes. *Plant Physiology* 43:467-469.

WANG, T.S.C., YANG, T. and CHUANG, T. 1967. Soil phenolic acids as plant growth inhibitors. *Soil Science* 103:239-246.

WARREN, K.S. 1972. Medicinal substances. *UK Patent*:1259503.

WATT, J.M. and BREYER-BRANDWIJK, M.G. 1962. *The Medicinal and Poisonous Plants of Southern and Eastern Africa.* Livingstone. Edinburgh. pp.39-41.

WEISS, U. and EDWARDS, J.M. 1969. Pigments of *Lachnanthes tinctoria* Ell. (Haemodoraceae). I. Isolation and photolysis of some 9-phenylperinaphthenones. *Tetrahedron Letters* 49:4325-4328.

WEISSENBÖCK, G. 1975. Aktivitätsverlauf der Phenylalanin-, Tyrosin-ammonium-lyase (PAL, TAL) und Chalkon-flavanon-isomerase in Vergleich zur C-glycosylfavon-Akkumulation im wachsenden Hafersprofs (*Avena sativa* L.) bei Belichtung und Dunkelheit. *Zeitschrift für Pflanzenphysiologie* 74:226-254.

WESTCOTT, R.J. and HENSHAW, G.G. 1976. Phenolic synthesis and phenylalanine ammonia-lyase activity in suspension cultures of *Acer pseudoplatanus* L. *Planta* 131:67-73.

WIJNSMA, R., VERPOORTE, R., HARKES, P.A.A., VAN VLIET, T.B., TEN HOOPEN, H.J.G. and SVENDSEN, A.B. 1986. The influence of initial sucrose and nitrate concentrations on the growth of *Cinchona ledgeriana* cell suspension cultures and the production of alkaloids and anthraquinones. *Plant Cell, Tissue and Organ Culture* 7:21-30.

WILLAMAN, J.J. and HUI-LIN, L. 1970. Alkaloid bearing plants and their contained alkaloids 1957-1968. *Journal of Natural Products Supplement* 33:93-94.

WILSENACH, R. 1967. Cytological observations on *Hypoxis*: I. Somatic chromosomes and meiosis in some *Hypoxis* species. *South African Journal of Botany* 33:75-84.

WILSENACH, R. and PAPENFUS, J.N. 1967. Cytological observations of *Hypoxis* II. Pollen germination, pollen tube growth and haploid chromosome numbers in some *Hypoxis* species. *South African Journal of Botany* 133:111-116.

WILSENACH, R. and WARREN, J.L. 1967. Cytological observations of *Hypoxis* III. Embryo sac development in *Hypoxis rooperi* and *H. filiformis*. *South African Journal of Botany* 33:133-140.

WILSON, G. and BALAGUÉ, C. 1985. Biosynthesis of anthroquinone by cells of *Galium mollugo* L. grown in a chemostat with limiting sucrose or phosphate. *Journal of Experimental Botany* 36:485-493.

WINK, M. and HARTMANN, T. 1981. Sites of enzymatic synthesis of quinolizidine alkaloids and their accumulation in *Lupinus polyphyllus*. *Zeitschrift für Pflanzenphysiologie* 102:337-344.

WINK, M. and HARTMANN, T. 1982. Diurnal fluctuations of quinolizidine alkaloid accumulation in legume plants and photomixotropic cell suspension cultures. *Zeitschrift für Naturforschung* 37:369-375.

WOOD, S.E. 1976. *A contribution to the knowledge of the genus* Hypoxis *L. (Hypoxidaceae) in Natal, South Africa.* M.Sc. Thesis. University of Natal, Pietermaritzburg.

WOODRUFF, H.B. 1966. The physiology of antibiotic production: the role of the producing organism. *Symposium of the Society of Genetics and Microbiology* 16:22-46.

WRIGHT, W.G. 1963. *The Wild Flowers of Southern Africa.* Nelson. Johannesburg.

YAMAKI, S. 1984. Isolation of vacuoles from immature apple fruit flesh and compartmentation of sugars, organic acids, phenolic compounds and amino acids. *Plant and Cell Physiology* 25:151-166.

YEOMAN, M.M., MIEDZYBRODZKA, M.B., LINDSEY, K. and McLAUCHLAN, W.R. 1980. The synthetic potential of cultured cells. In: *Plant Cell Cultures: Results and Perspectives*. Sala, F., Parisi, B., Cella, R. and Ciferri, O. (eds.). Elsevier. North Holland. pp.327-344.

ZENK, M.H. 1979. Recent work on cinnamoyl CoA derivatives. In: *Biochemistry of Plant Phenolics. Recent Advances in Phytochemistry*. Vol 12. Swain, T., Harborne, J.B., and Van Sumere, C.F. (eds.). Plenum Press. New York. pp.139-176.

ZENK, M.H. 1985. Enzymology of benzylisoqinone alkaloid formation. In: *The Chemistry and Biology of Isoquinoline Alkaloids* Phillipson, J.D., Roberts, M.F. and Zenk, M.H. (eds.). Springer, Berlin, Heidelberg, New York, Tokyo.

ZENK, M.H., EL-SHAGI, H., ARENS, H., STOCKIGT, J., WEILER, E.W. and DEUS, B. 1977. Formation of the indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus*. In: *Plant Tissue Culture and its Biotechnological Application*. Barz, W., Reinhard, E. and Zenk, M.H. (eds.). Springer-Verlag, New York.

ZENK, M.H., EL-SHAGI, H. and ULBRICH, B. 1977. Production of rosmarinic acid by cell suspension cultures of *Coleus bumei*. *Naturwissenschaften* 64:585-586.

ZIMMERMAN, M.H. and TOMLINSON, P.B. 1970. The vascular system in the axis of *Dracaena fragrans* (Agavaceae). 2. Distribution and development of secondary vascular tissue. *Journal of the Arnold Arboretum* 51:478-491.