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THE BIOCHEMICAL AND CYTOKININ CHANGES
IN THE DEVELOPING AND GERMINATING SEEDS
OF *PODOCARPUS HENKELII* STAFF. /

By
A
Malcolm Caulton Dodd _{or}—

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PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg 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.

M. C. Dodd

Malcolm Caulton Dodd

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Finally, to my wife Lynette, my grateful thanks for her steadfast support, encouragement and patience throughout the period of study.

for Lynette

ABSTRACT

A review of the literature revealed that there is a lack of depth in our knowledge of gymnospermous seeds with regard to the development and germination processes. The phytohormones, particularly the cytokinins have been implicated in these processes. The seeds of *Podocarpus henkelii* were thus selected as experimental material for studying the biochemical and cytokinin-like changes associated with development and germination.

The development of these seeds was also followed at the ultrastructural level. These studies revealed that cellular detail within the female gametophyte only began to form in December (early summer), approximately six weeks after fertilization had taken place. At this time some reserve protein was evident and the embryo sporophyte consisted of only a few pro-suspensor and pro-embryo cells. Concurrently, the cytokinin levels were fairly high in the female gametophyte but low in the epimatium. In both seed components two cytokinin-like compounds predominated which co-chromatographed with the free base cytokinin zeatin and its ribonucleoside.

The second sample was taken in late January (mid-summer) by which time the embryo sporophyte had developed rapidly into a readily distinguishable seed component. The cellular detail indicated that much cell division had recently taken place and that the cells were currently increasing in size and accumulating starch and lipid. In the female gametophyte the soluble sugars were at the maximum level recorded during these

experiments and the level of starch was increasing. The extractable cytokinin content of the seed was high at this time, particularly in the embryo sporophyte. In all three seed components cytokinin-like compounds which co-chromatographed with zeatin and ribosylzeatin were present. These high levels of cytokinin coincided with the rapid increase in both fresh and dry mass of the embryo sporophyte and female gametophyte.

Ultrastructural studies of the third sample collected in mid-March (early autumn) showed that cellular changes were associated mainly with increases in cell size and the accumulation of food reserves, particularly starch. The cytokinin levels had decreased in all three seed components at this time. There was an increase in the cytokinin which co-chromatographed with glucosylzeatin in the female gametophyte. The seeds matured in late April (autumn) and had the unusual features of not drying out during maturation. Fresh seeds collected from the ground had a moisture content of ca. 62 per cent. The main food reserve was starch with relatively small amounts of protein and lipid also present.

The seeds of *Podocarpus henkeli* germinated readily after scarification in the absence of water provided that their moisture content remained ca. 60 per cent. Seeds in which the moisture content had fallen below ca. 54 per cent required additional water for germination. The moisture content of the seeds fell rapidly under natural conditions and viability was lost below a moisture content of ca. 34 per cent. Unscarified seeds of 52 per cent moisture content

placed under moist conditions at a constant 25°C took 23 weeks to achieve 68 per cent germination. These experiments showed that although the epimatium limited water uptake by the seeds it did not prevent moisture loss to the atmosphere. This appears to be the main factor contributing to the seed's inefficiency as a propagule. A small degree of after-ripening was recorded with the embryo sporophyte increasing in size with storage. This appeared to contribute to the increased rate of germination of the scarified seeds. An interesting feature of the seeds of *Podocarpus henkelii* is that they have the ability to fix atmospheric carbon, which is subsequently translocated from the epimatium to the female gametophyte and embryo sporophyte. The mature seeds were stored at 4°C for six weeks during which time little change had occurred at the ultrastructural level. Protein vacuoles in the embryo sporophyte had disappeared and in all three seed components cytokinin levels were low. Three days after scarification and the start of incubation, little change in cellular detail was apparent as limited rehydration was necessary due to the high moisture content. The cytokinin levels in the embryo sporophyte and epimatium had increased, whilst the levels in the female gametophyte had decreased at this time. In the embryo sporophyte lipid mobilization had commenced with these reserves apparently being metabolized within vacuoles. The rate of respiration measured in terms of increases in CO₂ evolution, increased 60 hours after the start of the incubation period, just 12 hours before ten per cent germination was recorded.

Germination was accompanied by a large increase in the levels of cytokinins in the female gametophyte and embryo sporophyte. The cytokinins detected co-chromatographed with the free base cytokinin zeatin and its riboside, ribosyl-zeatin. Concurrently, marked ultrastructural changes were recorded with increases in the amounts of dictyosomes, endoplasmic reticulum and the formation of polyribosomes, all of which are indicative of increased metabolic activity. Similar increases in the female gametophyte were of a lower order and occurred only after nine days of incubation. By this time the levels of cytokinins had decreased considerably.

After 12 days of incubation 65 per cent of the seeds had germinated. As much of the food reserves in the female gametophyte and embryo sporophyte remained, it is suggested that these reserves are utilized for subsequent seedling establishment rather than for germination. The actual role that cytokinins play in the development and germination of these seeds is not clear. High levels of this phytohormone coincide with periods of food deposition and mobilization suggesting that they play an important part in these processes. The results of the biochemical, cytokinin and ultrastructural studies are discussed in relation to the developmental and germination processes and are compared to the data of other seeds.

CONTENTS

	Page
PREFACE	i
ACKNOWLEDGEMENTS	ii
ABSTRACT	iv
LIST OF TABLES	x
LIST OF FIGURES	xii
LIST OF PLATES	xv
INTRODUCTION	1
MATERIALS AND METHODS	36
CHAPTER ONE THE ULTRASTRUCTURE AND BIOCHEMISTRY OF DEVELOPING <i>PODOCARPUS HENKELII</i> SEEDS	60
CHAPTER TWO CYTOKININS IN DEVELOPING <i>PODOCARPUS</i> <i>HENKELII</i> SEEDS	107
CHAPTER THREE GERMINATION AND VIABILITY STUDIES IN <i>PODOCARPUS HENKELII</i> SEEDS	135
CHAPTER FOUR THE ULTRASTRUCTURE AND BIOCHEMISTRY OF GERMINATING <i>PODOCARPUS HENKELII</i> SEEDS	157
CHAPTER FIVE CYTOKININS IN THE GERMINATING SEEDS OF <i>PODOCARPUS HENKELII</i> AND METABOLISM OF APPLIED {8- ¹⁴ C} ZEATIN	197
CONCLUSION	240
REFERENCES	246

LIST OF TABLES

	Page
TABLE 1. Basal medium for the soybean callus bioassay	51
TABLE 1.1. Measurements of the size and mass of developing <i>Podocarpus henkelii</i> seeds	62
TABLE 1.2. Percentage contribution of each seed component on a fresh mass basis	63
TABLE 1.3. Percentage contribution of each seed component on a dry mass basis	63
TABLE 1.4. Differences between the fatty acids detected in the female gametophyte and embryo sporophyte	77
TABLE 1.5. Changes in the fatty acids of the embryo sporophyte and female gametophyte during development	87
TABLE 1.6. The soluble sugars of the seed components of developing <i>Podocarpus henkelii</i> seeds	92
TABLE 1.7. The free amino acids detected in developing <i>Podocarpus henkelii</i> seed components	97
TABLE 2.1. Cytokinin activity detected in the seed components of developing <i>Podocarpus henkelii</i> seeds	109
TABLE 2.2. Cytokinin activity detected in the seed components of developing <i>Podocarpus henkelii</i> seeds expressed as zeatin equivalents per dry seed component	109
TABLE 3.1. Changes in moisture content of <i>Podocarpus henkelii</i> seeds under different storage conditions	141
TABLE 3.2. The effect of desiccation on the moisture content of <i>Podocarpus henkelii</i> seeds	145
TABLE 3.3. Absorption peaks of pigments extracted from the epimatium of <i>Podocarpus henkelii</i> seeds	145
TABLE 3.4. Radioactivity detected in seeds of <i>Podocarpus henkelii</i> incubated in $^{14}\text{CO}_2$	149
TABLE 3.5. Radioactivity associated with sugars extracted from <i>Podocarpus henkelii</i> seeds after incubation in $^{14}\text{CO}_2$	149

	Page
TABLE 3.6. Changes in moisture content of <i>Podocarpus henkelii</i> seeds during incubation	152
TABLE 4.1. Size and mass measurements of germinating <i>Podocarpus henkelii</i> seeds	160
TABLE 4.2. Changes in the fatty acids of the embryo sporophyte and female gametophyte during germination	177
TABLE 4.3. Soluble sugars of the seed components of germinating <i>Podocarpus henkelii</i> seeds	182
TABLE 4.4. The free amino acids detected in germinating <i>Podocarpus henkelii</i> seed components	184
TABLE 4.5. $^{14}\text{CO}_2$ evolved from <i>Podocarpus henkelii</i> seeds treated with ^{14}C sucrose	190
TABLE 4.6. Radioactivity detected in seeds of <i>Podocarpus henkelii</i> after treatment with ^{14}C sucrose	192
TABLE 4.7. Radioactivity detected in scarified and incubated seeds of <i>Podocarpus henkelii</i> after treatment with ^{14}C sucrose	193
TABLE 4.8. Radioactivity detected in seeds of <i>Podocarpus henkelii</i> after treatment with ^{14}C linoleic acid	194
TABLE 5.1. Cytokinin activity detected in the seed components of germinating <i>Podocarpus henkelii</i> seeds	200
TABLE 5.2. Cytokinin activity detected in the seed components of germinating <i>Podocarpus henkelii</i> seeds expressed as zeatin equivalents per dry seed component	200
TABLE 5.3. Radioactivity detected in seed components of scarified <i>Podocarpus henkelii</i> seeds treated with {8- ^{14}C } zeatin applied to the epimatium	226
TABLE 5.4. Radioactivity detected in seed components of scarified <i>Podocarpus henkelii</i> seeds treated with {8- ^{14}C } zeatin applied to the female gametophyte	227

LIST OF FIGURES

	Page
FIGURE 1. The seed structure of <i>Podocarpus henkelii</i>	5
FIGURE 2. The scarified region of <i>Podocarpus henkelii</i> seeds	38
FIGURE 3. The areas of seed sampled for electron microscopy	40
FIGURE 4. Flow diagram of biochemical extraction procedures	41
FIGURE 5. The areas of seed to which ^{14}C labelled compounds were applied	56
FIGURE 1.1. Change in fresh mass of developing <i>Podocarpus henkelii</i> seeds.	71
FIGURE 1.2. Change in dry mass of developing <i>Podocarpus henkelii</i> seeds	71
FIGURE 1.3. The lipid content of developing <i>Podocarpus henkelii</i> seeds	86
FIGURE 1.4. The percentage ethanol soluble fraction of developing <i>Podocarpus henkelii</i> seeds	86
FIGURE 1.5. The soluble sugar content of developing <i>Podocarpus henkelii</i> seeds	94
FIGURE 1.6. The free amino acid content of developing <i>Podocarpus henkelii</i> seeds	94
FIGURE 1.7. The protein content of developing <i>Podocarpus henkelii</i> seeds	103
FIGURE 1.8. The starch content of developing <i>Podocarpus henkelii</i> seeds	103
FIGURE 2.1. The cytokinin content of developing <i>Podocarpus henkelii</i> seeds	112
FIGURE 2.2. Bioassay results of the epimatium during development	114
FIGURE 2.3. Bioassay results of the female gametophyte during development	116
FIGURE 2.4. Bioassay results of the embryo sporophyte during development	118

	Page
FIGURE 2.5. Bioassay results from January extracts of the female gametophyte separated by column chromatography	121
FIGURE 2.6. Bioassay results from January extracts of the embryo sporophyte separated by column chromatography	125
FIGURE 2.7. Bioassay results from April extracts of the embryo sporophyte separated by column chromatography	131
FIGURE 2.8. Bioassay results from April extracts of the female gametophyte separated by column chromatography	133
FIGURE 3.1. Germination of <i>Podocarpus henkelii</i> seeds	140
FIGURE 3.2. Water uptake by seeds of <i>Podocarpus henkelii</i> during germination	140
FIGURE 3.3. Increase in fresh mass of <i>Podocarpus henkelii</i> seeds during incubation	144
FIGURE 3.4. The increase in CO ₂ evolution and water uptake of germinating <i>Podocarpus henkelii</i> seeds	144
FIGURE 3.5. Absorption spectra of pigments from the leaves and epimatium of <i>Podocarpus henkelii</i>	147
FIGURE 4.1. Germination of <i>Podocarpus henkelii</i> seeds used for biochemical analyses	159
FIGURE 4.2. Lipid content of the germinating <i>Podocarpus henkelii</i> seeds	176
FIGURE 4.3. The percentage ethanol soluble fraction of germinating <i>Podocarpus henkelii</i> seeds	180
FIGURE 4.4. The soluble free sugar content of germinating <i>Podocarpus henkelii</i> seeds	180
FIGURE 4.5. The protein content of germinating <i>Podocarpus henkelii</i> seeds	187
FIGURE 4.6. The starch content of germinating <i>Podocarpus henkelii</i> seeds	187
FIGURE 5.1. The cytokinin content of germinating <i>Podocarpus henkelii</i> seeds	202

	Page
FIGURE 5.2. Bioassay results of the embryo sporophyte during germination	205
FIGURE 5.3. Bioassay results of the female gametophyte during germination	207
FIGURE 5.4. Bioassay results of the epimatium during germination	209
FIGURE 5.5. Bioassay results from extracts of the embryo sporophyte on day three of incubation, separated by column chromatography	212
FIGURE 5.6. Bioassay results from extracts of the female gametophyte on day three of incubation, separated by column chromatography	214
FIGURE 5.7. Bioassay results from extracts of the embryo sporophyte on day six of incubation, separated by column chromatography	217
FIGURE 5.8. Bioassay results from extracts of the female gametophyte on day six of incubation, separated by column chromatography	221
FIGURE 5.9. The three fractions of the paper chromatograms of ^{14}C zeatin treated seeds	224
FIGURE 5.10. Radioactivity and bioassay results from fraction A of the female gametophyte treated with $\{8\text{-}^{14}\text{C}\}$ zeatin	232
FIGURE 5.11. Radioactivity and bioassay results from fraction B of the female gametophyte treated with $\{8\text{-}^{14}\text{C}\}$ zeatin	234
FIGURE 5.12. Radioactivity and bioassay results from fraction C of the female gametophyte treated with $\{8\text{-}^{14}\text{C}\}$ zeatin	237

LIST OF PLATES

	Page
PLATE 1.1. The female gametophyte six weeks after fertilization	64
PLATE 1.2. The embryo sporophyte twelve weeks after fertilization	66
PLATE 1.3. The female gametophyte twelve weeks after fertilization	68
PLATE 1.4. The female gametophyte eighteen weeks after fertilization	73
PLATE 1.5. The embryo sporophyte eighteen weeks after fertilization	76
PLATE 1.6. The female gametophyte from mature seeds	79
PLATE 1.7. The embryo sporophyte from mature seeds	82
PLATE 4.1. The seed ultrastructure after six weeks storage at 4°C	162
PLATE 4.2. The seed ultrastructure after three days of incubation	166
PLATE 4.3. The seed ultrastructure after six days of incubation	169
PLATE 4.4. The seed ultrastructure after nine days of incubation	172
PLATE 4.5. The seed ultrastructure after twelve days of incubation.	174

INTRODUCTION

The Podocarpaceae, comprising seven genera, and about one hundred and fifty species has been described as one of the most important families of conifers in the Southern Hemisphere (STILES, 1912). This family occupies a place in the Southern Hemisphere comparable to that which the Pinaceae attains in the Northern Hemisphere. Members of the Podocarpaceae are found in South America, the West India Islands, Southern and Central Africa, Australia, Tasmania, New Zealand and the Australian and Malaysian Archipelagoes. Extensions into the Northern Hemisphere occur in China, Japan, the Malay Peninsula, Burma and India. This family is of great interest as it displays a wide range of morphological characteristics. In some species the ovules are borne in cones, while in others the female cone is reduced to form a swollen fleshy receptacle with a single terminal ovule. In most species the male cone is usually a simple structure characteristic of all other conifers. The male cones of a few species however, form part of a compound structure which indicates a very low level of evolution (SPORNE, 1965). With regard to geographical distribution and numbers of species, the two most successful families of conifers of the present day are the Podocarpaceae and the Pinaceae (STILES, 1912).

There are approximately one hundred and ten species in the genus *Podocarpus*, only four of which are indigenous to South Africa, namely *Podocarpus falcatus* (Thunb.) R. Br. ex Mirb., *P. elongatus* (Ait.) L'Hérit. ex Pers., *P. henkelii*

Stapf. ex Dallim. & Jacks. and *P. latifolius* (Thunb.) R. Br. ex Mirb. (LEISTNER, 1966). Of these four species, *P. henkelii* and *P. falcatus* are straight trees 10-35 metres in height, whereas *P. latifolius* may be found either as a straight tree up to 33 metres in height, or in marginal regions as a stunted tree or shrub no more than two metres in height. *P. elongatus* grows as a rounded tree or spreading shrub usually 3-6 metres high but may attain heights of up to 20 metres. All four species are dioecious and display a wide provincial distribution in South Africa, where they are confined mainly to the montane and coastal forests. Of the four species, *P. latifolius* and *P. falcatus* are most widely distributed, occurring in the Cape Province, Natal and the Transvaal. *P. elongatus* is confined to the winter-rainfall region of the Western Cape, and *P. henkelii* to the moist regions of Natal and the North Eastern Cape. The densest stands of the latter species stretch across the southern slopes of the mountains, usually in the mist belts of East Griqualand. Other localities are regions of Natal, namely Ixopo, Bergville, Pietermaritzburg, Polela and Port Shepstone (LEISTNER, 1966; SCHOONRAAD, 1971). Podocarpus species are more often to be found amongst other forest types where they can be the most dominant and numerous species. They seldom form pure stands (EDWARDS, 1967).

The timber of *P. henkelii*, *P. latifolius* and *P. falcatus* is fine grained, non-resinous, light and moderately strong. This, combined with its aesthetic appearance and weight advantage over pine, makes it a potentially superior substitute for the latter, being an excellent wood for use in beams, rafters, flooring and ceiling, doors, planks and furniture. This has

resulted in natural stands being exploited heavily in the past (ADAMSON, 1956; ZON and SPARHAWK, 1923). Over exploitation of natural forests, poor regeneration and the long growth cycle has resulted in this genus being listed as protected species (Ordinance 15 of 1974, The Natal Parks Board). In addition to the value of the timber, *P. henkelii* has found favour as an extremely attractive landscape tree.

Despite a copious production of seeds, VON BREITENBACH (1965) observed that there was little natural regeneration of *Podocarpus henkelii*. This partially contributes to this species' comparatively limited distribution range, and suggests that there may be a problem associated with their germination or seedling establishment. The name of the genus *Podocarpus* by derivation from the Greek (podo meaning foot, and carpus meaning kernel), sets the seeds of this genus apart from other seeds with regard to gross morphology. The embryology and morphology of *P. henkelii* have been investigated thoroughly and extensively by COERTZE, SCHWEICKERDT and VAN DER SCHIJFF (1971), COERTZE, VAN DER SCHIJFF and SCHWEICKERDT (1971) and SCHOONRAAD (1977). In *P. henkelii* the female cone is solitary, on a short naked stalk (podocarpium). The receptacle is clavate, glaucous but never fleshy and is often clearly demarcated from the stalk. The length of the receptacle and stalk together ranges from 4-12 millimetres. The receptacle comprises two bracts of which only the terminal one is fertile. The seed is ovoid-ellipsoid in shape and narrows toward the base. It consists of outer tegumentary layers 2-3 millimetres thick, comprising the thick tough epimatium, which is fused with a thin integument except in the micropylar region. The epimatium

may be divided into three regions: the thick epidermis with its cuticle and epicuticular wax; a shallow chlorenchymatous layer; and a broad inner zone which is permeated by vascular strands and contains secretory cavities. Below the integument lies the thin nucellus which encloses a massive female gametophyte (endosperm) containing ample food reserves. In the centre of this tissue lies the relatively large axile embryo sporophyte (Fig. I).

In common with other gymnosperms, the seeds of *P. henkelii* take a long time to develop (SINGH and JOHRI, 1972). The male cones appear in July and the pollen is shed two months later, concurrent with the female cones becoming receptive. The female cones are borne on newly formed shoots towards the end of August (early spring). The pollen germinates immediately but fertilization only occurs approximately two months later. After fertilization, a series of cell divisions leads to the formation of a pro-embryo and a pro-suspensor. The latter pushes the pro-embryo deep into the female gametophyte tissue along the corrosion cavity. During the following two months there follows a period of intense morphological development of the embryo sporophyte, resulting in this organ becoming visible to the naked eye in dissected seeds. The female gametophyte tissue develops during this period beginning with no cellular detail, into an extensive, amorphous tissue with accumulating food reserves. Very few morphological changes occur in either the embryo sporophyte or female gametophyte in the ensuing three to four months. The seed matures in late April - early May (autumn) when it falls off the tree. At maturity, the embryo sporophyte varies in length from 13-18

Fig.1

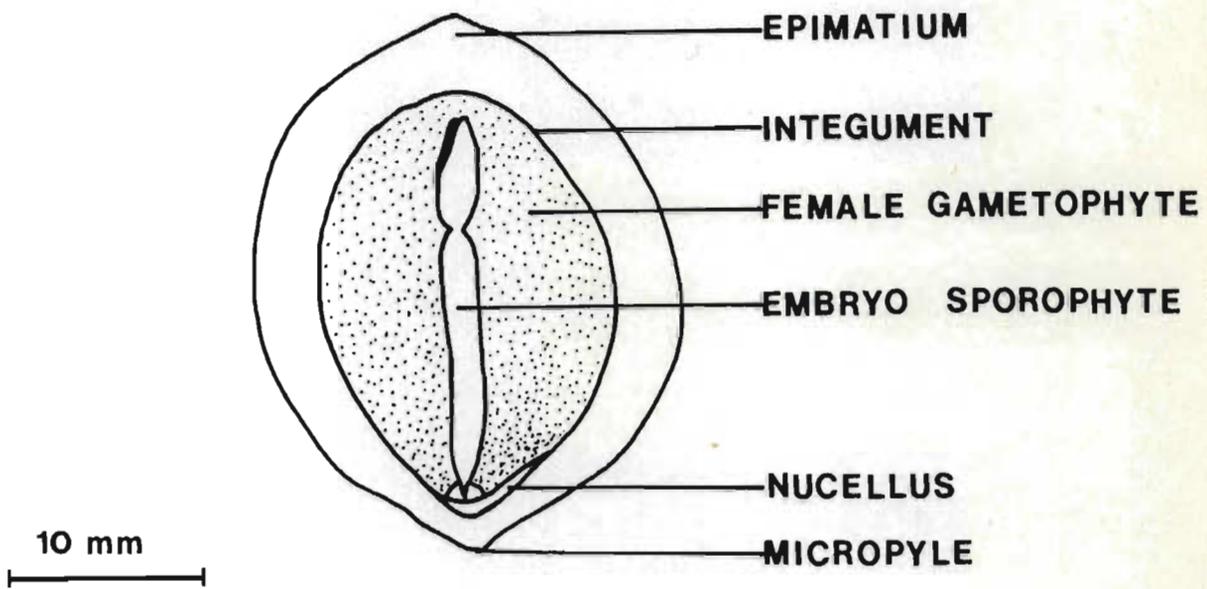


Figure 1. Line diagram of the seed components of the mature seed of *Podocarpus henkelii*.

millimetres, consisting of a firm hypocotyl of about 7-10 millimetres, a radicle with a conspicuous radicle cap about 3-5 millimetres long, and two cotyledons each about 3-5 millimetres in length. At this stage the embryo sporophyte lies deeply buried in the female gametophyte tissue (COERTZE, SCHWEICKERDT and VAN DER SCHIJFF, 1971; COERTZE, VAN DER SCHIJFF and SCHWEICKERDT, 1971; and SCHOONRAAD, 1977).

The development of many other species of gymnospermous seeds has been collated and well defined by SINGH and JOHRI (1972). A similar thorough review has been completed on the development of angiospermous seeds by BHATNAGAR and JOHRI (1972). There is however, from a plant physiologist's point of view, a great paucity in our knowledge of the molecular and ultrastructural aspects of seed development in all but a few economically and nutritionally important annuals. Examples of these are peas (*Pisum sativum* L.), beans (*Phaseolus vulgaris* L. and *Vicia faba* L.), soybeans (*Glycine max* L.), lupins (*Lupinus albus* L.) and cotton (*Gossypium hirsutum* L.). Amongst the monocotyledons the species which have been investigated extensively are maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and oats (*Avena fatua* L.) (BEWLEY and BLACK, 1978; DURE, 1975). No references could be found to any molecular studies on developing gymnosperms. There have however, been some studies on germinating pine seeds (CHING, 1966; CHING, 1972). The lack of developmental studies, even on the economically more important Conifers, is perhaps firstly due to the ever present problem of funding, and secondly to the very long embryogenesis of most gymnosperms.

The metabolic relationships between the components of seeds and their covering structures have received considerable attention over the years. Seeds of the garden pea (*Pisum sativum*) may be selected as a representative example of non-endospermic dicotyledonous seeds. Much of the early research on developing legume seeds focused upon establishing the gross levels of cellular components such as starch, sugars and protein (BISSON AND JONES, 1932). Later analyses focused on measurements of the size of the pods and seeds, cell numbers in the seed components and on increases in fresh and dry masses. FLINN and PATE (1968) found that the pea pod wall increases in length, width and thickness prior to attaining maximum fresh mass. After this activity the seeds contained inside the pod begin their deposition of food reserves. The seeds accumulate most of their carbon (85-90 per cent) in the form of sucrose between the fifth and the twenty eighth day after anthesis. The studies of FLINN and PATE (1968) showed that the upper leaflets act as a major source of fixed carbon to the seeds during their early development. Their importance wanes as the pod becomes the main source of carbon from about 16 days after anthesis. The pods not only provide fixed carbon, but also can mobilize previously accumulated dry matter in the pod and translocate it to the seed. The carbohydrate reserves are stored as starch. This can occupy 34 per cent of the dry mass in wrinkled seed varieties and up to 45 per cent in smooth seeded varieties (CROCKER and BARTON, 1953). According to BAIN and MERCER (1966a), the deposition of carbohydrate reserves begins before the deposition of protein. The metabolic pathway for the formation of starch has been

elucidated by TURNER (1969). All synthesis begins with sucrose which is converted to fructose and to uridine diphosphoglucose. In the next stage fructose is converted into glucose-1-phosphate which is then added to a small glucose primer, thereby increasing the chain length. This continues until the starch molecule is complete. In the embryo, starch deposition occurs in the stroma of chloroplasts ten days after anthesis. As the quality and quantity of light entering the pods decreases with ageing, photosynthesis is limited. Consequently, later starch accumulation can only come from sucrose stored within the cotyledons and from that which is transported to the seed from the vegetative plant (BAIN and MERCER, 1966a).

The other storage reserve in seeds of *Pisum sativum* is protein. This can account for 25 per cent of the dry mass of the mature cotyledons (BEEVERS, 1976). The bulk of the synthesis of this reserve occurs during the cell expansion phase of growth, which is accompanied also by an increase in both DNA and RNA (PATE, 1975). The nitrogenous components in the form of protein and amino acids had been studied earlier by FLINN and PATE (1968). They showed that the gain in nitrogenous components by the embryo during embryogenesis is much greater than that lost by the pod, testa and endosperm combined. Thus although nutrients build up in the ovarian and maternal tissue of the seed itself, a continuous flow into the seed from the vegetative plant must take place until the seed matures. LEWIS and PATE (1973) reported that the principal amino acids derived from photosynthesis in the pea

leaves were serine, glycine and alanine, and these together with the amides asparagine and glutamine made up the bulk of the amino acids transported to the seed during development. The important amino acids of the stored proteins in the cotyledons were found to be tyrosine, arginine, lysine, leucine, phenylalanine and histidine. As these were not shown to be present in the phloem sap, it can only be concluded that the latter group of amino acids must have been synthesized within the seeds themselves. Support for this comes from the work of ATKINS, PATE and SHARKEY (1975), who showed that in *Lupinus albus* fruits and seeds, asparagine accounts for 50-70 per cent of the nitrogen carried in the phloem sap. An asparaginase increases greatly in activity some four to five weeks after anthesis and its appearance coincides with the early stages of protein synthesis. It is thought that this enzyme deaminates the excess asparagine. The free amido group may then be utilized for the synthesis of those amino acids necessary for protein synthesis. The development of the embryo thus appears to be dependent upon the translocation of nutrients initially from the endosperm and then later from the drying pod and testa (PATE, SHARKEY and ATKINS, 1977). The different events in the development of seeds of *Pisum sativum* can be summarized as follows:

Maximum pod length	- cell division	10	Days after anthesis
Maximum endosperm content			
Endosperm reserves depleted			
Maximum pod fresh mass	- cell enlargement	30	
Dry matter accumulation			
RNA accumulation			
Starch accumulation			
Protein accumulation			
Pods dry out		65	
Seeds mature			

Monocotyledonous seeds vary in structure from those of the dicotyledons and as such, they warrant a separate discussion. The monocotyledonous seed comprises a single cotyledon which is highly modified to form the scutellum. This organ usually stores oil, whereas the endosperm stores starch. Protein is found in the testa/pericarp layer known as the aleurone layer. According to DURE (1975), the process of seed development in this group takes place in the same way. The major cellular events leading up to the formation of the endosperm and aleurone layers have been followed carefully by EVERS (1970) in *Triticum aestivum*. The timing of food reserve deposition is very similar to that of maize which will be considered in detail later. The endosperm of wheat arises from free division of the triploid nucleus about two days after anthesis. By day four, cell walls begin to form and the outer meristematic layer can be observed. This layer undergoes tangential division to produce cells inwards, and then radial divisions to increase the surface area. By day 14, the outer meristematic layer ceases cell division and becomes the aleurone layer. The deposition of storage reserves in monocotyledonous seed formation has been well documented by BERNSTEIN (1943). *Zea mays* is selected as a representative example to illustrate biochemical development of seeds in this group. INGLE, BEITZ and HAGEMAN (1965) in their comprehensive study of the development of maize kernels, showed that the development period lasts 46 days from anthesis. Cell division in the endosperm ceases roughly 28 days from anthesis by which time the concentration of the free sugars reaches a maximum. Starch accumulation then begins at the expense of these sugars, reaching its final level about 46 days after anthesis. Protein

levels increase in the endosperm during the cell formation period, and this probably reflects an increase in enzymes and structural proteins for endosperm growth and metabolism. A second phase of protein accumulation was detected about 40 days after anthesis when protein synthesizing capacity as well as amino acid levels were declining. This was coincidental with the deposition of reserve protein in the aleurone layer. A major portion of the storage protein is sequestered within protein bodies in the endosperm. The embryo which is still developing between 25 to 40 days after anthesis has been shown to act as a protein synthesizing region, and takes over as the major source of protein when the endosperm starts to dehydrate. This event occurs about 35 days after anthesis. By day 50, neither the starchy endosperm nor the embryo can synthesize protein. The former, because the metabolic machinery is crushed by the deposited starch, and the latter due to the increasing water stress imposed by desiccation of the kernel. Apart from the oil reserves deposited in the scutellum, very little reserve deposition occurs within the embryo itself.

The biosynthetic pathway of starch accumulation has been the subject of numerous investigations and is well documented (BAXTER and DUFFUS, 1973; DICKINSON and PREISS, 1969). Sucrose from photosynthesis is the starting point for starch synthesis and the pathway is the same as that already outlined for *Pisum sativum* (TURNER, 1969). The DNA content of the embryo continues to increase during development, whereas the level in the endosperm starts to fall 40 days after anthesis when cell division ceases. The RNA content of the embryo increases throughout the development period reflecting increased

metabolism in this region. The RNA necessary for protein synthesis during germination is stored in the dry embryo (JENNINGS and MORTON, 1963).

The results of the studies with both *Pisum sativum* and *Zea mays*, as examples of dicotyledons and monocotyledons respectively, show that the biochemistry of their development is fairly well understood. The origin of the food reserves, the timing of their deposition and the regions within the seed where they are deposited are well documented and serve as good general examples of seed development. There is however, no current literature on the development of any gymnosperm seeds, despite the many interesting and varied seed types to be found in this sub-division of plants.

Some of the events in seed development are thought to be regulated by various phytohormones. The evidence for this stems from the fact that developing seeds are often rich sources of auxins, gibberellins and cytokinins. These high levels of hormones drop markedly as the seed matures and often levels of abscisic acid increase at this time. Indole acetic acid was first identified in seeds of *Zea mays* by HAAGENS MIT, DANDLIKER, WITTEW and MURNEEK (1946). The highest levels of auxin in the developing kernels of *Triticum aestivum* were associated with the time of intense accumulation of dry matter (WHEELER, 1972). Gibberellin-like activity was first found in immature seeds of *Phaseolus vulgaris* by MITCHELL, SKAGGS and ANDERSON, (1951). In developing kernels of *Hordeum vulgare*, two peaks of gibberellin activity were recorded before the levels fell as maturity approached (MOUNLA and MICHAEL, 1973).

Cytokinins were first isolated from developing maize kernels by LETHAM (1963). This group of hormones has also been shown to be involved in seed development. For example, in *Zea mays* kernels, a peak of cytokinin is recorded 11 days after pollination and this declines considerably over the following ten days (MILLER, 1967). The presence of abscisic acid has been reported in many species of seeds (MILBORROW, 1967) and the levels have been shown to increase in the seeds of *Pisum sativum* as maturity approached (EEUWENS and SCHWABE, 1975).

The physiological role that these hormones play is not yet clear. There appear to be two possible functions: (i) the control of fruit growth and development, and (ii) the control of growth and development of the seeds themselves. Fine examples of the first concept are the work of EEUWENS and SCHWABE, (1975) and DAVEY (1978) with *Pisum sativum* and *Lupinus albus* respectively. In the first case the levels of auxin, gibberellin and abscisic acid were studied in relation to seed and pod wall development. These authors showed that the growth rate of the ovary wall closely relates to changes in the hormone contents of the seeds. Similar results were presented for the levels of cytokinins in the developing fruits of the white lupin. For the second concept, the work of BURROWS and CARR, (1970) with the developing seeds of *Pisum arvense* L. is a good example. They found that growth of the pea seeds followed a double sigmoid curve, two periods of rapid growth being separated by a lag phase with a slower growth rate. Changes in growth rates of these seeds were associated closely with levels of extractable cytokinins. The peaks of cytokinin-

like activity coincided with times of maximal development of the endosperm, and the two periods of rapid growth of the embryo. In *Pisum sativum* there is a close correlation between maximum growth of the seed and the levels of auxin and gibberellin in the endosperm and embryo (EEUWENS and SCHWABE, 1975). All these data point to the very close association between the plant hormones and the metabolic and morphological events that occur during seed development.

One group of hormones which has received particular attention in relation to seed development is the cytokinins. The main interest in this group of hormones stemmed from their known ability to regulate cell division (MILLER, 1961). This process is an absolute prerequisite for embryogenesis, and related processes during seed development. A cytokinin may be defined as a N⁶-substituted adenine which can be extracted from plant tissues, which promotes growth in cultured callus cells, or is responsible for particular biological responses within plant tissues.

Immature kernels of *Zea mays* were the first plant tissues reported to contain a cytokinin. The active compound was identified as zeatin (LETHAM, 1963). Subsequent investigations have shown that many other cytokinins may be present in seed tissue. Zeatin, ribosylzeatin and zeatin ribonucleotide were isolated from immature maize kernels (MILLER, 1967) and in developing seeds of apple fruitlets (LETHAM and WILLIAMS, 1969). Another cytokinin-like compound which displays polar properties in paper chromatography has been identified in immature *Zea mays* kernels (LETHAM, 1973), in the liquid endosperm

of *Cocos nucifera* L. (VAN STADEN, 1976b), and in developing lupin fruits (DAVEY and VAN STADEN, 1977). This compound was identified in *Cocos nucifera* L. by VAN STADEN (1976b) as glucosylzeatin. In conjunction with the identification of the cytokinins present in developing seeds work was done to establish the location of cytokinins within the different seed components. This was done to find explanations as to what role these hormones played in seed development.

Investigations of the cytokinin contents in the components of the seed of avocado (*Persea americana* Mill.) revealed that the levels changed during development (BLUMENFELD and GAZIT, 1970). Cytokinin levels were found to be very high in the endosperm as long as this tissue was present in the seed. In the seed coat, cytokinin levels were initially high, but decreased as maturity was attained. In the embryo, high levels were recorded at early stages of development, but these decreased as the seed matured. In addition, bound cytokinins were found in the mesocarp of the avocado fruit and it appeared that a decrease in fruit growth was paralleled by a reduction in bound cytokinin activity. Similar studies on the immature seeds of pumpkin (*Cucurbita pepo* L.) (GUPTA and MAHESHWARI, 1970), and watermelon (*Citrullus lanatus* (Thunb.) Hans.) (PRAKASH and MAHESHWARI, 1970) showed that cytokinin activity changed with development. In the pumpkin the cytokinin activity was at its lowest in very young and mature seeds. In watermelon seeds the maximum cytokinin level was achieved 11 days after pollination, whereas the maximum increase in fresh and dry masses of the seeds occurred between days four and nine after pollination. These results suggest that the growth of

the seed does not result from increased cytokinin levels, but rather that the increased cytokinin content was an accompaniment of growth in the seed.

The relationship between cytokinins in the developing fruits and those in the seeds of some species has also received some attention. GAZIT and BLUMENFELD, (1970) showed that in the maturing fruit tissues of avocado, the cytokinin levels decreased. A similar pattern of high cytokinin-like activity falling with fruit maturity has been demonstrated in tomato (*Lycopersicon esculentum* L.) (DAVEY and VAN STADEN, 1978a; DESAI and CHISM, 1978). Investigations of the cytokinin-like activity of developing fruits and seeds of *Lupinus albus* by DAVEY and VAN STADEN (1977, 1979) show that the highest cytokinin-like activity was to be found in the seeds of the 14 week old plants. This activity was ten times the level found in the pods of the same age. The highest levels of cytokinin-like activity in the seed were found in the endosperm four to six weeks after anthesis, after which time the levels decreased and the testa contributed the highest levels of cytokinin-like activity. The embryo showed low levels of activity during the developmental study. The high levels of activity in the seeds as a whole were found to decrease as maturity approached. These data conform with original citations where cytokinin levels were low in the mature seeds.

The source of cytokinins in the developing seeds has been the subject of much debate. There could be two sources: (i) translocation from other parts of the plant or (ii) synthesis within the seeds themselves. There is some evidence to

support the first possibility from work of BEEVER and WOOLHOUSE (1973), who suggest that cytokinins from the roots may be supplied to the developing seed. There is however, stronger evidence for the second possibility. LETHAM and WILLIAMS (1969) suggest that in the developing fruitlets of apple (*Malus sylvestris* (L.) Mill.) the seeds are the sites of active cytokinin synthesis. More recently VARGA and BRUINSMA (1974) have suggested that the seeds of *Lycopersicon esculentum* are the sites of synthesis in developing fruit as are the seeds of *Pisum sativum* (HAHN, de ZACKS and KENDE, 1974). After investigating the biosynthesis of zeatin and its derivatives in maize endosperm, MIURA and HALL (1973), proposed that a latent capacity for the synthesis of cytokinin is present in all tissues, and that the site of synthesis may alter during development. Another possibility was that the relative contributions of the potential sites of cytokinin synthesis may also change during the course of development. BLUMENFELD and GAZIT (1970, 1971) also concluded that the seed of *Persea americana*, and more specifically the embryo, is a site of synthesis. They based their statement on the fact that the embryo contained high levels of cytokinin, and that callus derived from the cotyledons produced these compounds. There is thus much evidence to suggest that cytokinins may be synthesized within seeds.

The cytokinin levels in mature seeds have been the subject of some investigation. Most results indicate that in the species investigated, low levels of cytokinins are present (THIMANN, SHIBAOKA and MARTIN, 1970; VAN STADEN and BROWN, 1973). These data conform with the generally accepted view that

low levels of cytokinins are indicative of low metabolic activity, and this is what one would expect in a mature seed. The apparent decrease in activity could be due to firstly the breakdown of cytokinins, or secondly the conversion of active cytokinins into biologically inactive ribonucleotides. The occurrence of the latter phenomenon has been demonstrated in fruits of *Lycopersicon esculentum* by ABDEL-RAHMAN, THOMAS, DOSS and HOWELL (1975). It seems possible that during development, active cytokinins may be converted into inactive ribonucleotides which are stored until required for germination. All these data show that cytokinins may be synthesized in developing seeds, but their function in many cases is still to be elucidated.

There is also information to suggest that the other phytohormones are also synthesized within seeds. It has been shown that in *Triticum aestivum* kernels the build up of gibberellin does not coincide with increased export from other parts of the plant (WHEELER, 1972). It has been shown that seeds of wild cucumber (*Echinocystis macrocarpa* Greene) convert mevalonic acid into gibberellin through various intermediates of the suspected biosynthetic pathway (MOORE and ECKLUND, 1975). The embryo and endosperm of *Triticum aestivum* can synthesize abscisic acid from supplied mevalonate (MILBORROW and ROBINSON, 1973). There is however, little further evidence for the synthesis of abscisic acid within seeds and it is thought that in most cases it may be derived from the mother plant (MILBORROW, 1974). There have been many reports of high levels of auxins in developing seeds, but no work appears to have been done on whether or not this hormone is synthesized within seeds

(SCHNEIDER and WIGHTMAN, 1978).

The germination of seeds is perhaps the one botanical phenomenon which has captured the interest of more researchers in the field of plant physiology, than any other. Consequently, not only are the scientific publications numerous, but there have also been many review articles on the subject (MAYER and SHAIN, 1974; TAYLORSON and HENDRICKS, 1977). In addition many books have been compiled with germination as the main theme. Some of the more notable examples are books written or edited by BEWLEY and BLACK, 1978; KHAN, 1977; KOZLOWSKI, 1972 and Mayer and POLJAKOFF-MAYBER, 1975. Thus the literature on this subject is voluminous. Despite this fact, there are some serious gaps in our knowledge of some of the steps in this fascinating process.

Germination may be defined as the transformation of the embryo into a seedling. This in physiological terms, means the resumption of metabolism and growth which were earlier limited or prevented, and the reactivation of the 'switched off' genome. These events can only be achieved if the seed is placed under favourable environmental conditions of temperature and moisture. The latter factor is essential as almost all seeds are highly desiccated during the final stages of maturation. The desiccated state of the seed contributes to the characteristically low metabolic rate (ROBERTS, 1972). Once the seed starts to imbibe water, the metabolic machinery is slowly hydrated. One of the first metabolic events after hydration is the increase in respiratory rate from a value close to zero to an appreciable level. The increase in respiration is accompanied by hydrolysis and oxidation of reserve

materials in the seeds, resulting in a net loss of dry mass. This event is only made possible by the reactivation of previously stored protein and the synthesis of new protein. Thus, all these aforementioned processes may be described as amphibolic (CHING, 1972), as both catabolism and anabolism occur. The former involves the breakdown of reserve material to provide energy and raw materials for seedling development. The latter process provides new metabolic enzymes for the synthesis of new organelles and cellular material. The initial processes of germination may thus be listed as follows:

- (i) Rehydration of desiccated tissue (JIRGENSONS, 1962).
- (ii) Reactivation of pre-existing macromolecules and organelles, both formed during maturation (MARRÉ, 1967).
- (iii) Increase in respiration rate (MAYER and POLJAKOFF-MAYBER, 1975).
- (iv) The breakdown of reserve materials within the seed.
- (v) The transport of these materials from one organ to another within the seed, in particular from the endosperm or cotyledons to the embryo.
- (vi) The synthesis of new material from these breakdown products.

There have been numerous studies executed on aspects of seed metabolism during the germination process. Some of the more thorough and notable efforts are those on rice (*Oryza sativum* L.) (OTA, FUJII and OSAWA, 1953), on *Zea mays* (INGLE, BEEVERS and HAGEMAN, 1964), and on Douglas fir (*Pseudotsuga menziesii* Franco) (CHING, 1966). A similar state of affairs

exists in the germination literature as was found in the developing seed literature. The most intensive and thorough investigations have been those on seeds of economic and agronomic importance, these being confined largely to the Gramineae and Leguminosae.

The stages of germination as outlined previously will be discussed in further detail to illustrate the important aspects of each one.

- (i) **Imbibition:** The first step in the germination process. The amount and rate of water uptake are naturally dependent upon the nature of the seed coat, the chemical composition of the seed and its size. The water content of the seed rises from somewhere between five and ten per cent, to between 40 and 60 per cent. This involves the rapid uptake of water by bicolloids in dry seeds, which is followed in turn by the build up of multiple layers of water-enveloping cellular constituents (JIRGENSONS, 1962). The uptake of water by the seeds usually follows a triphasic pattern: PHASE 1. Rapid water uptake which appears to be independent of metabolic activity, as it occurs in both living and dead tissues. PHASE 2. A plateau phase of varying length which is characterized by a rapid metabolic rate preparing the seed for germination. PHASE 3. A further rapid increase in the amount of water imbibed. This phase is only associated with germination, and it is during this time that the food reserves are mobilized (BEWLEY and BLACK, 1978). The rates of water uptake appear to be determined by the main type of stored food

reserves, starchy seeds imbibing slowly, whereas proteinaceous and fatty seeds imbibe rapidly (ALLERUP, 1958).

Once the seed has imbibed, the biochemical processes that were restricted at maturation are reactivated. One of the first noticeable events during the second phase of water uptake by seeds is the reactivation of organelles (ABDUL-BAKI and BAKER, 1973). Protein and ribosome synthesis starts in decoated cotton seed within 16 hours after the commencement of incubation (WATERS and DURE, 1966). These authors used inhibitors to study the incorporation of ^{14}C -labelled amino acids into the protein. From their studies, they concluded that protein synthesis in the early stages of imbibition was directed by stable mRNA which had been preformed in the maturing seed. CHEN, SARID and KATCHALSKI (1968) studied the quantities of new mRNA formed over the stages of germination in wheat embryos. They discovered that there was a steady flow of new mRNA, amounting to three per cent of other DNA bases formed on days two and three of germination. Similar investigations in the germinating seeds of Red pine (*Pinus resinosa* Ait.) by SASAKI and BROWN (1969) revealed that few changes in the quality of sRNA, rRNA and DNA were observed in the megagametophyte, whereas in the embryo a continuous increase in all these fractions occurred over the 14 days of germination. Thus, there is evidence to show that new RNA species are produced in the seed tissues during germination. Current

views are that new mRNA's are necessary for the production of new proteins, which will control food reserve breakdown, and the resynthesis of these catabolites into new material.

- (ii) Respiration: The following is a generalized account, as some exceptions have been noted, one example of which follows this discussion. In most seeds respiration follows a similar pattern to that of the water uptake except that there is sometimes a fourth phase, which is characterized by a marked lowering of the respiration rate. KOLLÖFFEL (1967) and KOLLÖFFEL and SLUYS (1970) demonstrate this very clearly for peas.
- PHASE I. This is characterized by a rapid rise in respiration activity, which is attributed to activation and hydration of the mitochondrial enzymes associated with the citric acid cycle and the electron transport chain. The respiratory quotients (RQ) at this time are usually above one, indicating that highly oxidized components such as organic acids are being respired (MAYER and POLJAKOFF-MAYBER, 1975).
- PHASE 2. A plateau phase in which there is a lag in respiratory activity and RQ values of as high as three have been reported, indicating anaerobic respiration (SPRAGG and YEMM, 1959). It is evident that when this lag occurs, it is due to lack of oxygen penetration to the deeper tissues of the seed.
- PHASE 3. An increase in respiratory activity is recorded with RQ values in the region of one, indicating the respiration of carbohydrates.

In *Pisum sativum* this phase is accompanied by the protrusion of the radicle through the testa (KOLLÖFFEL, 1967). PHASE 4. Characterized by a lowered respiration rate and not present in all seeds. As previously mentioned, there are exceptions to the abovementioned pattern.

In seeds of oats (*Avena fatua*), the respiration rate rises rapidly to a certain level which is maintained almost constantly throughout the germination period (CHEN and VARNER, 1970). The respiration during the early stages of imbibition prior to the hydrolysis of stored reserves appears to be dependent upon readily available substrate such as sucrose. Analyses of many dry monocotyledonous and dicotyledonous seeds have shown the presence of sucrose (DUPERON, 1955). This disaccharide was shown to be depleted rapidly in the embryo of germinating barley (*Hordeum vulgare*) (JAMES, 1940). The aforementioned phases are very arbitrarily drawn, as the length and timing may vary greatly between species. There does however, appear to be some consistency in the metabolic events which occur in each of these phases (BEWLEY and BLACK, 1978).

- (iii) Mobilization of reserves: The germination process requires a considerable amount of biological energy. Some of this is utilized for the synthesis of new proteins, resulting in the production of enzymes. These are utilized to degrade and convert the stored reserves for both respiration and the biogenesis of new cellular

components in the expanding embryo. The utilization of food reserves is one aspect of seed germination which has received detailed attention in certain species of plants. The grain of *Hordeum vulgare* has received particular consideration because of its importance to the brewing industry. Consequently, the mobilization of food reserves in this seed is well understood. Grains of barley commence germination within 18 hours of being placed in water. Starch begins to accumulate in the scutellum within 24 hours. This accumulation appears to be at the expense of lipid reserves in this tissue (JAMES, 1940). Sucrose and raffinose are depleted in the embryo within 24 hours of rehydration. By the 72nd hour the sucrose levels are supplemented by reserves from the mobilized starch in the endosperm. The control of mobilization of reserves from the endosperm lies with the embryo. This control is via the hormone gibberellin which is synthesized within the embryo prior to the mobilization of the endosperm reserves (MACLEOD and PALMER, 1966, 1968; RADLEY, 1969). Gibberellin is transported to the aleurone via the scutellum. Once in the aleurone, the gibberellin stimulates the production of hydrolytic enzymes such as α -amylase (BRIGGS, 1972). These enzymes hydrolyze the starch grains and the cell walls of the endosperm (FINCHER and STONE, 1974). The scutellum then absorbs the products of starch digestion (mainly α - and β -maltose) converts them to sucrose, and then transports this to the growing embryo. Here the sucrose is utilized for respiration requirements and the biogenesis

associated with the growth of the embryo. All these events have been very succinctly described by MACLEOD (1969), who writes, "The reserve material of cereal grain can be divided into two categories; those which are present in the seed and are immediately available for use by the growing seedling; and those which are stored in an insoluble form in the endosperm and require to be hydrolyzed and translocated through the scutellum before they can be utilized. Reserves which are used by the seedling during its first 24 hours of growth include sucrose and raffinose (which together account for 20 per cent of the dry mass of the embryo), lipids and amino acids. The reserves of endosperm include the hemicelluloses which form the cell walls, starch and protein. In *Hordeum vulgare* their hydrolysis is carried out sequentially by β -glucanases and xylanases, amylases and peptidases. Apart from β -amylase, which is present in the endosperm of ungerminated grain, the hydrolytic enzymes originate in the aleurone layer. They are synthesized in response to gibberellins which migrate from the embryo, possibly via the upper half of the scutellum to reach the aleurone normally between twelve and twenty hours after the grain has been moistened."

The pattern of reserve breakdown and utilization is quite different in fatty seeds such as Douglas fir (*Pseudotsuga menziesii*) (CHING, 1966). In a thorough investigation CHING showed that lipids made up 48 and 55 per cent of the dry masses of the megagametophyte and embryos respectively. During stratification there

was a small decrease in the gametophytic lipids but no change was recorded in the embryonic lipid. These seeds took five days to germinate after introduction to water, and by day 14, all the gametophytic reserves had been used up. Upon germination, the lipids in the gametophyte dropped rapidly, whilst those in the embryo decreased slightly but rose again by ten days after germination. There was a slow accumulation of sugars and starch during stratification, followed by a plateau just prior to radicle emergence and then a rapid increase from day seven. This rapid increase was confined only to the embryo and the levels in the gametophyte decreased after day seven of germination, indicating that sugars are a means of food transfer from the storage tissue to the embryo during germination. A similar pattern of mobilization of total nitrogen, soluble nitrogen and amino acids was recorded commencing on day five of germination. Of the total nitrogen compounds in the seedling 50 per cent were soluble, and of this amount only 15 per cent were free amino acids and amides. These results are comparable to those reported to occur in germinating castor beans (*Ricinus communis* L.) (KREIDEMANN and BEEVERS, 1967; STEWART and BEEVERS, 1967) but differ from those found in germinating *Picea abies* L., where protein digestion occurs more rapidly than lipolysis (SIMOLA, 1976). Investigations of the changes in the major lipid classes during germination of Douglas fir by CHING (1966) indicate that triglycerides are the main lipids utilized, and that phospholipids are synthesized. The latter are presumed to be

included in the synthesis of cellular and organelar membranes in the growing embryo.

The other major food reserve not discussed so far is protein. Almost all seeds contain some protein, varying from about ten per cent of the dry mass of starchy seeds such as maize, to about 35 to 40 per cent in oily seeds such as *Gossypium hirsutum*. In the so-called proteinaceous seeds such as *Pisum sativum* and *Phaseolus vulgaris* 20 to 40 per cent of their dry mass is protein, and 40 to 60 per cent carbohydrate (CROCKER and BARTON, 1953). The breakdown of protein is by proteinases and peptidases. The former release amino acids and peptide units and the latter hydrolyze endogenous and hydrostatically-produced peptides to amino acid units. In dry seeds of *Phaseolus vulgaris* there is a low activity of proteinases until five days after the start of imbibition, when there is an increase accompanied by a decrease in protein nitrogen (YOMO and SRINIVASEN, 1973). In the cotyledons of *Phaseolus vulgaris*, the dry matter reserves were depleted within nine days of imbibition. The maximum rate of transfer out of the cotyledons occurred on day two. The protein reserves were mobilized slightly faster than the dry matter components (METIVIER and PAULILO, 1980). The digestion of protein bodies has been shown to start in the centre of the cotyledons and move towards the periphery (SMITH, 1974). The liberated amino acids are transferred from the cotyledons to the growing embryo via the phloem. In *Pisum*

sativum, this movement commences about two to three days after imbibition and continues until the cotyledons collapse (LARSON and BEEVERS, 1965). These authors found that in the pea, the major transport form of amino acids was homoserine. In other plant seeds such as *Ricinus communis*, it was glutamine (STEWART and BEEVERS, 1967). Once in the growing embryo the amino acids are either respired or converted into other amino acids for further protein synthesis.

The preceding discussion has shown that although a great deal of effort has been extended in the study of food reserve mobilization in seeds upon germination, there are very few comprehensive studies on one particular seed. The two main exceptions to this are *Zea mays* (INGLE, BEEVERS and HAGEMAN, 1964), and Douglas fir (*Pseudotsuga menziesii*) (CHING, 1966), which have already been cited. However, few researchers have attempted to study with a particular seed, aspects of germination such as water uptake, respiration, food reserve mobilization and the possible control of these processes by endogenous factors. Our current knowledge is built up on the synthesis of data from many different seeds. Often in the case of identical species different cultivars or varied germination conditions have been used.

The actions and interactions of the plant hormones in their control of dormancy in seeds is another field which has been thoroughly studied and reviewed (KHAN, 1977). The endogenous factors which regulate certain processes in germinating seeds have already been shown in some cases to be plant

hormones. One example of this is the release of α -amylase in the aleurone in response to gibberellin produced by the embryo (CHEN and PARK, 1973). Gibberellin has also been shown to increase the activity of isocitrate lyase and hydroxylacyl co-enzyme A dehydrogenase in *Ricinus communis* endosperm (MARRIOTT and NORTHCOTE, 1975). Other studies with gibberellin have shown that its effect may be at the level of the endoplasmic reticulum (PYLIOTIS, ASHFORD, WHITECROSS and JACOBSEN, 1979), and that it may be involved in polysome formation (JONES, 1969).

Little is known about the metabolic effect of abscisic acid. There is some evidence that it may interfere with nucleic acid metabolism. VILLIERS (1968), using autoradiographic techniques showed that abscisic acid inhibits the inclusion of ^3H labelled uridine and thymidine into embryos of *Fraxinus excelsior* L.. Protein synthesis itself was however, not directly inhibited in these studies. In excised *Gossypium hirsutum* embryos abscisic acid prevented translation of pre-existing mRNA which coded for known enzymes. This effect was dependent upon the continued presence of abscisic acid and was inhibited by the simultaneous addition of actinomycin D, a known inhibitor of RNA synthesis. The actinomycin D alone had no effect on the synthesis of the enzymes studied (IHLE and DURE, 1972). In addition to this, IHLE and DURE (1972) showed that the effects of abscisic acid were reversed by both kinetin and gibberellin in *Pisum sativum* embryos. Thus, there is strong evidence for the intimate involvement of abscisic acid in the germination process of seeds. The recent review by WALTON (1981) highlights the role of this phytohormone in germination.

Since their discovery, the cytokinins have been implicated in germination. Their ability to overcome dormancy is well documented, and KHAN (1975) suggests that they play a permissive role, interacting with inhibitors in the germination process. However, other studies have indicated that cytokinins may be involved in other biochemical processes central to germination. When imbibed at 30 °C, the seeds of pecan nuts (*Carya illinoensis* (Wang.) K. Koch) showed a rise in the endogenous cytokinins prior to germination (DIMALLA and VAN STADEN, 1977). The levels of cytokinin increase in stratified seeds of *Acer saccharum* Marsh (VAN STADEN, WEBB and WAREING, 1972) and *Protea compacta* R. Br. (BROWN and VAN STADEN, 1973). All these results suggest that certain levels of endogenous cytokinins are necessary in some seeds for germination to take place. The role this hormone plays in the early stages of germination is not yet clear but it has been suggested that its primary function is related to energy mobilization (DIMALLA and VAN STADEN, 1977). For this to occur, there has to be an initial step in the biochemical pathways to set the machinery in motion. Cytokinins could be involved in mediating this step. BURROWS (1975) has suggested three ways in which they may act: control at the gene level, control at the translation level and regulation of protein synthesis. There is little experimental evidence to support the first suggestion. The second possibility would involve the incorporation of cytokinins into tRNA. Four cytokinins have been isolated from wheat germ tRNA (BURROWS, ARMSTRONG, KAMINEK, SKOOG, BOCK, HECHT, DAMMAN, LEONARD and OCCOLOWITZ, 1970). For these to function *in vivo* there should be considerable incorporation of cytokinin into tRNA. There is little evidence in support of this theory

and the work of ARMSTRONG, MURAI, TALLER and SKOOG (1976) and SKOOG (1973) show that incorporation is at such a low level that this theory is unlikely. The third suggestion that cytokinins may regulate protein synthesis is more feasible. There is evidence to show that in the seeds of *Cucurbita maxima* Duchensne and *Arachis hypogea* L. cytokinins, but not gibberellin can partially substitute for the embryo in promoting the formation of isocitrate lyase, an enzyme of the glyoxylate cycle (PENNER and ASHTON, 1967a). In *Triticum aestivum* catabolism of part of the triglyceride reserves is induced by cytokinin (TAVENER and LAIDMAN, 1972). The increase in glyoxysomes and breakdown of lipids in *Protea compacta* appear to be correlated with increasing cytokinin and gibberellin levels (VAN STADEN, DAVEY and DU PLESSIS, 1976). Benzyladenine has been shown to promote cotyledon expansion and accelerate lipid digestion in *Cucurbita pepo* (LONGO, LONGO, ROSSI and SERVETTAZ, 1976). It has been demonstrated that kinetin enhances extractable α -amylase activity in *Phaseolus vulgaris* cotyledons and appears to promote sugar formation in *Helianthus annuus* L. cotyledons (GILAD, ILAN and REINHOLD, 1970), albeit to a very limited extent. The cytokinins thus appear to have a regulatory effect on the catabolism of stored reserves, through some of the enzyme systems.

A further aspect of seed germination involving cytokinins is the 'regulating' influence the embryo has over some aspects of the reserve catabolism in the storage tissues. LOCKER and ILAN (1975) demonstrated that a combination of ribosylzeatin and gibberellic acid could completely replace the effect of the axis in the seeds of *Pisum sativum*. Studies on

seeds with non-endospermic reserves have shown that the embryo in some cases exhibits a positive influence over enzymes in storage tissues. In stratified *Pinus ponderosa* Hartw. (which germinates within two days after the addition of water) the levels of isocitrate lyase were found to rise steadily during the first four days of germination. When the embryo was removed at the start of imbibition, the level of this enzyme decreased by 60 per cent. However, the removal of the embryo after two days had no effect on the enzyme level (BILDERBACK, 1974). In *Pinus sylvestris* L. the level of α -amylase in the gametophyte tissue was greater when the embryo was present than when it was absent (NYMAN, 1971). Although in some of the cited results the authors did not try and substitute cytokinin for the embryo effect, there is evidence from work on isolated maize embryos which demonstrates that the 'factor' is cytokinin. In this case however, the embryos are dependent upon cytokinin from the endosperm in order to germinate fully (SMITH, 1977). Embryos without this supply of cytokinins display reduced germination, as well as reduced radicle lengths and radicle dry masses. Similar observations have been made in two gymnosperms, *Pinus lambertiana* Dougl. (BERLYN and MIKSCHE, 1965) and *Podocarpus henkelii* (NOEL and VAN STADEN, 1976) where it was shown that excised embryos developed more slowly and in many cases not as well in which the female gametophyte tissue was retained. These results suggest that the mature gymnosperm embryo was partially dependent upon the female gametophyte at the time of germination.

Embryos of *Pseudotsuga menziesii* (CHING, 1966) and *Pinus resinosa* (SASAKI and KOZLOWSKI, 1969) have been shown to

contain adequate reserve material. Since the constituents of both the embryo sporophyte and female gametophyte change only after radicle emergence, it seems that some substance other than food reserves is required for the initiation of radicle growth. PINFIELD and STOBART (1972) suggest that in the embryos of *Acer pseudoplatanus* L. endogenous cytokinins may be necessary for the initiation of radicle growth and development. There is sound evidence to suggest that cytokinins serve a very important function in the early stages of germination in many seeds.

This general discussion has brought to light the paucity in our current knowledge of both the development and germination of gymnospermous seeds. With the exception of the comprehensive biochemical studies of CHING (1966), the only other thorough investigations were purely ultrastructural. These were detailed studies of germinating *Welwitschia mirabilis* Hooker Fil. seeds by BUTLER (1975), and experiments by SIMOLA (1974, 1976) on germinating *Pinus sylvestris* and *Picea abies*. The seed of *Podocarpus henkelii* provides a valuable vehicle for research for several reasons. The seed structure is different from that found in the most commonly studied gymnospermous seeds. No reports have been noted on the biochemical development of any gymnospermous seeds. Seeds of *Podocarpus henkelii* were found to have an extremely high water content at maturity which dropped rapidly under natural conditions. As most seeds dehydrate at maturity to enhance viability (ROBERTS, 1972), the significance of this high moisture content to the viability of *P. henkelii* seeds was of interest.

This project details the studies on developing and germinating *Podocarpus henkelii* seeds with special reference to their food reserves, cytokinins, and the ultrastructure of the female gametophyte and embryo sporophyte during these developmental processes.

MATERIALS AND METHODS

1.0 General.

Seeds for the developmental and germination studies of *Podocarpus henkelii* were collected from trees in the Natal Botanical Gardens in Pietermaritzburg. These gardens lie within the natural geographical and climatic range of the species. The studies were conducted over two growing seasons, 1978 to 1979 and 1979 to 1980. For the developmental study, seeds were randomly collected from four trees within the gardens on four occasions. The first sampling was taken in early December when the cellular detail was just forming in the female gametophyte. At this stage the seed was separated into the female gametophyte and epimatium only. The second sample was collected 46 days later in late January. At this time the embryo sporophyte was sufficiently developed to enable the seeds to be divided into three components; the embryo sporophyte, female gametophyte and epimatium respectively. The third sample was taken 48 days later in mid-March and the fourth when the seeds were mature and had fallen to the ground approximately 45 days later at the end of April. At each sampling the seed was placed in a cold box at $\pm 4^{\circ}\text{C}$ and transported to the laboratory.

1.1 Developmental study.

A sample of seed (50 seeds per sampling) was dissected for measurements of the seed components and the recording of their fresh masses. This material was dried for 24 hours at 100°C in a ventilated oven for estimation of the dry mass. A

small sample was prepared at the same time for electron microscopy. The remaining seed material was dissected, massed, and the three seed components placed into separate flasks immersed in liquid air. This material was then freeze-dried, massed and stored in sealed glass containers at -20°C until required for biochemical and hormonal extraction.

1.2 Germination study.

A large sample of mature seed (off the ground) from the fourth sampling was used for germination and viability studies. This seed was placed in sealed polythene bags in the dark at $\pm 4^{\circ}\text{C}$ for storage until needed. In order to germinate the seeds, they were scarified by carefully removing a four millimetre square of the epimatium tissue around the micropyle (Fig. 2). The seeds were then placed scarified side downwards in trays of vermiculite, moistened with distilled water and incubated in the dark at 25°C . A high relative humidity was maintained by covering the trays with polythene. The criterion for germination was the protrusion of the radicle through the covering structures. In all cases, four replicates of 25 seeds each were used for each germination experiment. To assess the effect of dehydration on germination, seeds were placed on open trays in an incubator at 25°C for varying lengths of time. For biochemical, hormonal and ultrastructural studies of these seeds, samples were taken on days 0, 3, 6 and 9 of incubation, measured, massed and then frozen as described in Section 1.1.

Fig. 2

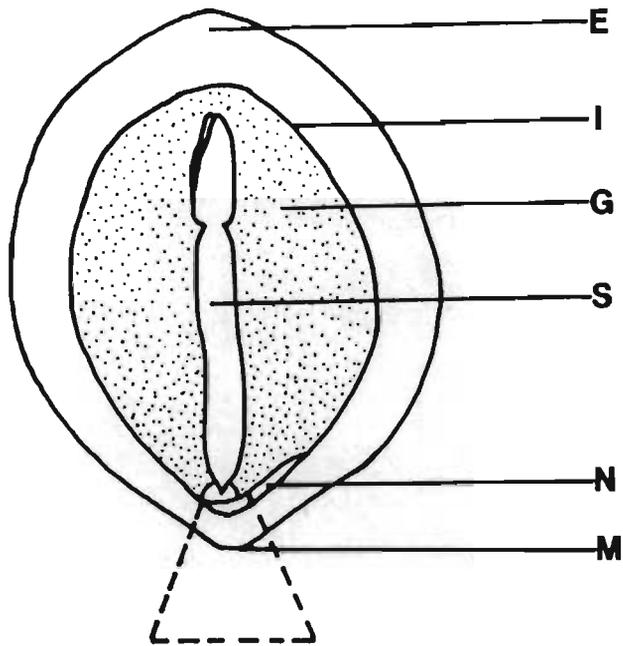


Figure 2. Line diagram of the mature seed of *Podocarpus henkelii*. The broken line indicates the region of scarification. E = epimatium, I = integument, G = female gametophyte, S = embryo sporophyte, N = nucellus and M = micropyle.

2.0 Microscopy techniques.

At each of the developmental stages and during the germination study, three millimetre squares of female gametophyte and embryo sporophyte tissue were dissected from the regions shown in Figure 3. These were fixed for 24 hours at 4°C in six per cent gluteraldehyde, buffered at pH 7,2 with 0,05 Molar sodium cacodylate. The fixed material was washed three times for periods of 30 minutes each in 0,05 Molar sodium cacodylate buffer. It was then post-fixed in two per cent osmium tetroxide buffered as above, for two hours. After washing three times in 0,05 Molar sodium cacodylate buffer the material was dehydrated in an alcohol series. The samples were washed in propylene oxide, then propylene oxide plus resin before being embedded in pure Araldite resin. Polymerization lasted 48 hours at 70°C. Sections for both light- and electron microscopy were cut with glass and diamond knives respectively. The sections for electron microscopy were stained with uranyl acetate and lead citrate as described by REYNOLDS (1963). These sections were examined using a Hitachi HU 11E electron microscope at an accelerating voltage of 50 Kv and photographed. Monitor sections of each developmental and germination stage were cut for light microscopy. These sections were stained using one per cent toluidene blue in one per cent borax: one per cent pyronin Y (1 : 1 v/v).

3.0 Biochemical analyses.

The biochemical analyses were conducted on freeze-dried material which had been ground in a Thomas-Wiley mill using a 40 mesh strainer. The analyses were performed according to the flow diagram in Figure 4.

Fig.3

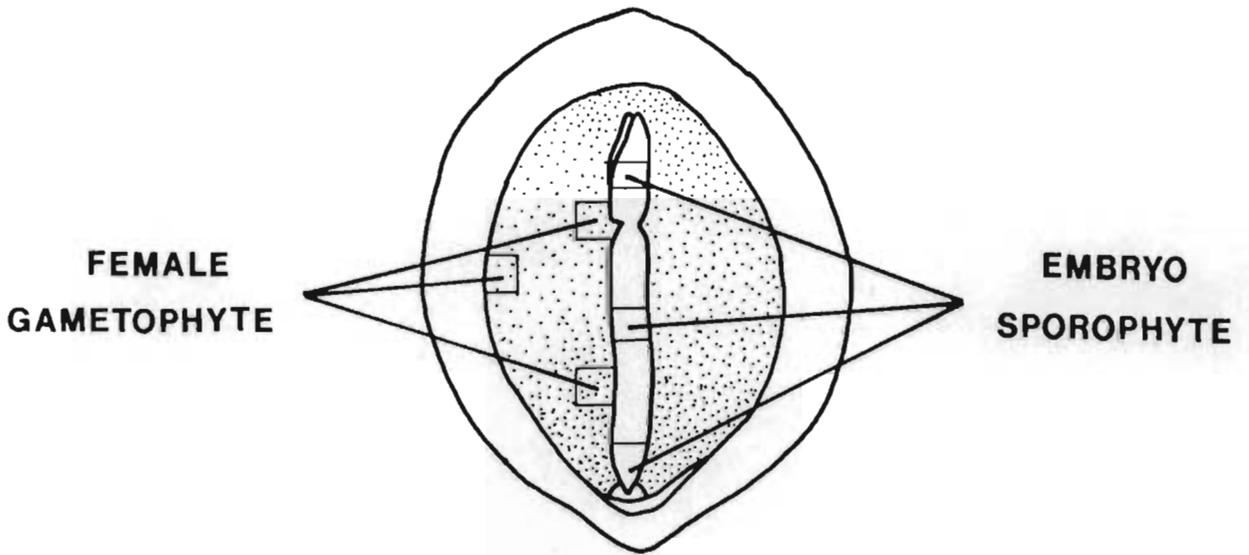
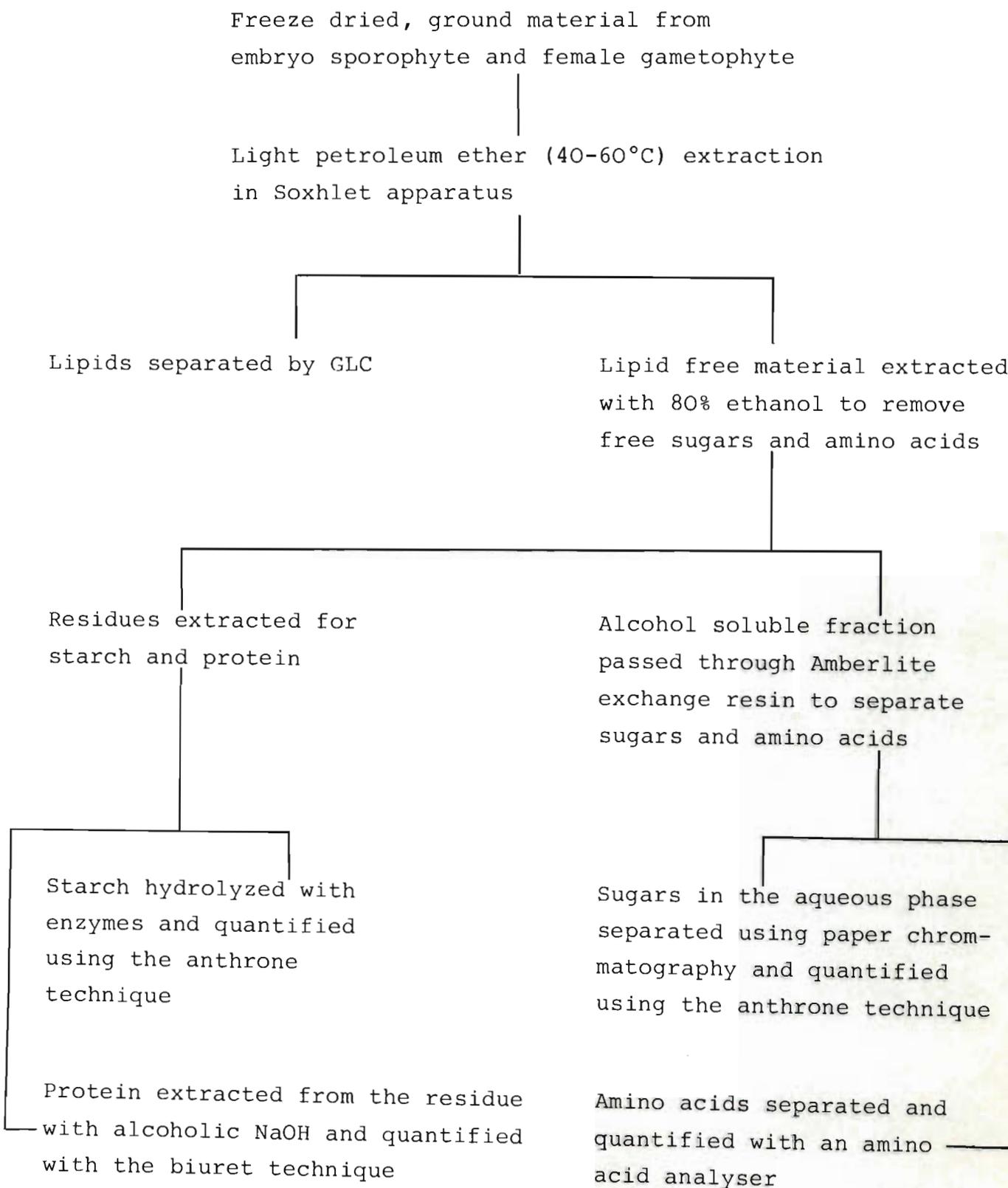


Figure 3. Line diagram of the mature seed of *Podocarpus henkelii* showing the regions of embryo sporophyte and female gametophyte which were sampled for electron microscope studies.

Fig. 4. Flow diagram for the extraction of lipids, carbohydrates, amino acid, protein and starch from the seed components of *Podocarpus henkelii* seeds.



3.1 Extraction and identification of lipids.

Lipids were extracted from the seed material using a method adapted from MEARA (1955). Ten grammes of ground female gametophyte and 2,5 grammes of embryo sporophyte material were massed to an accuracy of one milligramme and placed in previously washed and massed Whatman cellulose extraction thimbles. The thimbles were then placed in a Soxhlet apparatus and the lipid extracted with light petroleum ether (boiling point 40-60°C) by percolation for eight hours. Subsequently, thimbles were removed from the Soxhlet extractor, air-dried in a desiccator and remassed. The mass lost from each thimble represented the mass of the petroleum ether soluble fraction. Each estimate is a mean of three extractions. The lipids remaining in the petroleum ether in the Soxhlet apparatus were taken to dryness in a rotary evaporator at 30°C. The lipids were dissolved in five millilitres of petroleum ether placed in glass vials and the petroleum ether evaporated off under a stream of nitrogen. When all the petroleum ether was removed, the vials were sealed and sent to the C.S.I.R. in Pretoria for analysis of the constituent lipids. The fatty acid composition of the neutral lipids was determined by saponifying approximately 0,3 grammes of the oil with five per cent alcoholic potassium hydroxide. The soaps were then immediately converted to methyl esters by boiling with BF_3 -methanol (METCALFE, SCHMITZ and PELKA, 1966). The methyl esters were dissolved in hexane and analysed with a Varian 3700 gas chromatograph. The instrument was fitted with a glass-capillary column coated with 0,4 per cent FFAP, and programmed to run between 160-180°C at 12°/minute. The results are presented

as the individual fatty acid composition expressed as a percentage of the total fatty acid content.

3.2 Extraction of soluble sugars and free amino acids.

Lipid freed material was used for the analyses. The material (2,5 grammes) was placed into flasks containing 100 millilitres of 80 per cent ethanol. The flasks were agitated for 24 hours at room temperature. After this time, the suspensions were filtered through premassed Whatman's No. 42 filter paper into weighed beakers. The filter papers containing the residues were air-dried and remassed. The loss in mass of each residue was recorded. The ethanolic extracts were evaporated to dryness and the beakers remassed. The value of loss from the residues and the mass of the ethanolic extracts were averaged. This average mass was used to estimate the percentage of ethanol soluble fraction on a dry mass basis.

The dried extracts were dissolved to 100 millilitres of distilled water and the sugars and amino acids separated using a cation exchange resin. A column containing 30 grammes (Amberlite IR-120) was prepared in the H^+ form by washing the resin slowly with 200 millilitres of 5N HCl. This was then washed repeatedly with distilled water until all the chloride ions had been removed. The dried ethanolic extract was re-suspended in distilled water and passed slowly through the column. The column was then washed with 100 millilitres of distilled water and the aqueous phase containing the free sugars was evaporated to dryness. The dried extract was re-suspended in ten per cent *iso*-propanol for storage and subsequent experimentation.

The amino acids were eluted from the exchange resin with 200 millilitres of 1N ammonium hydroxide. The extracts were reduced to dryness, and resuspended in five millilitres of ten per cent *iso*-propanol and stored at 4°C for further experimentation.

3.3 Chromatographic separation and quantification of sugars.

The free sugar extracts were further purified by dissolving each sample in three millilitres of redistilled pyridine. This was heated at 100°C for three minutes, filtered to remove the inorganic salts, and the pyridine subsequently evaporated off. The dried samples were then resuspended in one millilitre of ten per cent *iso*-propanol. Aliquots of the extracts and standard sugars were spotted onto Whatman's No. 1 chromatography paper and developed descendingly for 72 hours with *n*-butanol: ethanol: water (45: 5: 50 v/v). After drying, the positions of the authentic sugars were ascertained by spraying with 0,3 per cent *p*-aminohippuric acid and three per cent phthalic acid dissolved in ethanol. The chromatograms were heated in an oven at 140°C for eight minutes to develop the spots. The regions which corresponded to the known markers were then taken and eluted with five millilitres of distilled water. The quantity of each sugar present was estimated using the anthrone reaction of BELL (1955). One millilitre of the extract eluted from the chromatogram was placed in each acid washed oven-dried test tube with ten millilitres of anthrone reagent (0,05 grammes anthrone in 100 millilitres of 70 per cent sulphuric acid). These were heated in a boiling water bath for 15 minutes after which they were removed and cooled rapidly. The

percentage transmittance of each sample was then recorded using a Varian DMS 90 UV-Vis spectrophotometer at 610 nm. The quantity of each sugar present was estimated from a standard curve of that sugar. The results are presented as milligrammes of sugar per gramme dry mass. The figures presented are the means of three extractions.

3.4 Qualitative and quantitative estimation of free amino acids.

The extracts eluted from the cation exchange resin with ammonium hydroxide were analysed for acidic, basic and neutral amino acids. This was achieved on a Beckman 116 Amino Acid Analyser housed in the Chemistry Department, University of Natal, Pietermaritzburg. The acidic and neutral amino acids were separated on a 55 centimetre column packed with M72 resin and run at 50°C. The basic amino acids were analysed on a five centimetre column containing PA35 resin also at 50°C. The results were plotted on a chart recorder. The qualitative and quantitative estimates of the amino acids present were achieved by comparison with a Beckman calibration mixture and are expressed as micromoles of amino acid per gramme dry mass.

3.5 Extraction and quantification of protein.

Protein was extracted from the lipid, sugar and amino acid free material using the technique of DEACON (1972). Dried material weighing 0,2 grammes was placed into flasks and moistened with two millilitres of *iso*-propanol. One millilitre of copper sulphate solution (25 grammes of copper sulphate in 100 millilitres of water) and 100 millilitres of alcoholic

sodium hydroxide (20 grammes sodium hydroxide in 300 millilitres of *iso*-propanol made up to one litre with water) was added. This mixture was heated on a magnetic stirrer to 70°C in two minutes and then stirred for a further two minutes without heat. The mixtures were filtered through a fibre-glass filter and the transmittance of the solution measured at 500 nm on a Varian DMS 90 spectrophotometer. The quantity of protein present was estimated using a standard curve prepared from Bovine serum albumin (BSA). The results which are expressed in milligrammes of BSA per gramme dry mass are the means of three extractions.

3.6 Extraction and quantification of starch.

Starch was extracted from the lipid, sugar and amino acid free material according to the method of ADAMS, RINNE and FJERSTAD (1980). The material was oven-dried and 0,2 grammes weighed out. This mass was added to five millilitres of water and 25 millilitres of hot 80 per cent ethanol. After ten minutes the sample was centrifuged at 1700 g for ten minutes and the supernatant removed. Two subsequent extractions were carried out using 30 millilitres of hot 80 per cent ethanol. The supernatant fractions were combined and taken down to a small volume on a rotary evaporator. This fraction was diluted to ten millilitres and analysed for residual free sugars with the aforementioned anthrone reaction (Section 3.3). The insoluble residue was resuspended in 15 millilitres of water and autoclaved at 120°C for 60 minutes. The gelatinized starch was subsequently hydrolyzed by adding five millilitres of 0,1M citrate buffer pH 5,0, containing five milligrammes amyloglucos-

idase and one milligramme α -amylase. The mixture was diluted to 30 millilitres and incubated for 4,5 hours at 30°C. An aliquot of the reaction mixture was centrifuged at 5 000 g for five minutes and the glucose in the supernatant determined with the anthrone reagent. The amount of starch present was estimated from a standard curve of glucose. The results, presented as milligrammes of starch per gramme dry mass of seed component, are the means of three extractions.

4.0 Extraction and purification of seed material for cytokinins.

The cytokinins were extracted from epimatium, female gametophyte and embryo sporophyte material by homogenizing the appropriate quantities of material (on a dry mass basis) in 80 per cent ethanol (ten millilitres of ethanol per gramme of material). This was allowed to stand overnight at 4°C. These extracts were then filtered through Whatman's No. 1 filter paper and concentrated to dryness under vacuum at 40°C. The residues were dissolved in 50 millilitres of 80 per cent ethanol and the pH of these extracts adjusted to 2,5 with dilute hydrochloric acid. The acidified extracts were passed through Dowex 50W-X8 cation exchange resin (J.T. Baker Chemical Co. Phillipsburg, N.J.; H⁺ form, 20-50 mesh, 2,5 x 25 centimetres) at a flow rate of 20 millilitres per hour. The columns were washed with 200 millilitres of 80 per cent ethanol. These eluates were stored for further analysis. The cytokinins were then eluted from the column with 200 millilitres of 5N ammonium hydroxide. The ammonia eluates were concentrated to dryness under vacuum and the residues taken up in 5 millilitres of 80

per cent alcohol. This was then strip-loaded onto Whatman's No. 1 chromatography paper.

The aforementioned technique utilizing cation exchange resins to purify ethanolic extracts of cytokinins has been widely used (ENGELBRECHT, 1971; HEWETT and WAREING, 1973; LORENZI, HORGAN and WAREING, 1975; VAN STADEN, 1976a; WANG, THOMPSON and HORGAN, 1977). Endogenous cytokinin glucosides have been extracted from plant materials by this technique (VAN STADEN, 1976b), and they appear to be heat stable as autoclaving did not result in any loss of activity (VAN STADEN and PAPAPHILIPPOU, 1977).

4.1 Paper chromatography of cytokinins.

Extracts were applied as a one centimetre strip to sheets of Whatman's No 1 chromatography paper. The chromatograms were then developed with *iso*-propanol: 25 per cent ammonium hydroxide: water (10:1:1 v/v) (PAW) in a descending manner until the solvent front was approximately 30 centimetres from the origin. The chromatograms were then dried in a drying oven at 30°C for 24 hours. The chromatograms were divided into ten equal R_f zones and stored at -20°C until further analysis. For estimation of cytokinin activity, the strip of chromatography paper corresponding to each R_f zone was placed into an ehrlenmeyer flask. This was subsequently assayed for cell division-promoting activity using the soybean callus bioassay of MILLER (1965).

4.2 Column chromatography.

Column chromatography was used as an aid in the determination of the possible kinds of cytokinins present in seed extracts. The technique used was based on that of ARMSTRONG, BURROWS, EVANS and SKOOG (1969). The columns (90 x 2,5 centimetres) were packed with Sephadex LH-20 which had been swollen in 35 per cent ethanol. They were eluted with 35 per cent ethanol at a flow rate of 15 millilitres per hour. Forty millilitre fractions were collected. Samples of authentic zeatin (Z) (mixed isomers) and ribosylzeatin (ZR) (mixed isomers), both Calbiochem A grade, were eluted through the columns and assayed for cell division activity in order to obtain the elution volumes of these compounds in each individual system. The zone marked glucosylzeatin (ZG) on the histograms indicates the elution volume of the O-glucoside of zeatin described by VAN STADEN and PAPAPHILIPPOU (1977). Some of the extracts on paper chromatograms had shown the presence of cell division-promoting compounds in certain of the R_f zones. Some of these extracts were prepared for column chromatography by dividing the chromatograms into two fractions. Fraction A, R_f 0,1-0,5 and fraction B, R_f 0,6-0,9. These fractions were eluted from the paper using 50 millilitres of 10, 20, 35, 50 and 80 per cent ethanol in successive elutions, all of which were filtered and combined. This combined extract was concentrated to dryness and taken up in one millilitre of 35 per cent ethanol, and then applied to the columns.

After elution through the column, 40 millilitre fractions were transferred to the ehrlenmeyer flasks and dried on

a hotplate set at 30°C in a stream of air. They were subsequently assayed for cell division activity using the soybean callus bioassay.

4.3 Soybean callus bioassay.

The fractions obtained as a result of paper and column chromatography were assayed for cytokinin activity using the soybean callus bioassay of MILLER (1963; 1965). This bioassay was chosen as the range of concentration over which a linear response is obtained is greater in this assay than in the tobacco pith bioassay (FOX, 1969; SKOOG and ARMSTRONG, 1970). Another consideration is that this assay is superior to the chlorophyll retention bioassay (KENDE, 1971). Callus was obtained from the cotyledons of *Glycine max* L. var. Acme according to the procedures described by MILLER (1963; 1965) and was maintained by three weekly subculture.

Four stock solutions were made and the nutrient medium prepared from these (Table 1). Twenty-five millilitre flasks were prepared with 15 millilitres of medium and 0,15 grammes of agar for small samples. Fifty millilitre flasks containing 20 or 30 millilitres of medium and 0,2 or 0,3 grammes of agar respectively, were prepared for samples extracted from larger amounts of seed material. The flasks were stoppered with non-absorbant cotton wool bungs, which were then covered with aluminium foil. The flasks were autoclaved at a pressure of 1,05 bars for 20 minutes before being transferred to a 'sterile transfer' chamber. Once the agar had solidified, three pieces of soybean callus of approximately ten milligrammes each were placed on the basal medium. The flasks were then incubated in

TABLE 1
 BASAL MEDIUM FOR SOYBEAN CALLUS BIOASSAY
 (adapted from MILLER, 1963; 1965)

Stock Solution	Chemical	g/l ⁻¹ Stock Solution	Millitres of stock solution per litre of medium
Stock 1	KH ₂ PO ₄	3,0	100
	KNO ₃	10,0	
	NH ₄ NO ₃	10,0	
	Ca(NO ₃) ₂ ·4H ₂ O	5,0	
	MgSO ₄ ·7H ₂ O	0,715	
	KCl	0,65	
	MnSO ₄ ·4H ₂ O	0,14	
Stock 2	NaFeEDTA	1,32	10
	ZnSO ₄ ·7H ₂ O	0,38	
	H ₃ BO ₃	0,16	
	KI	0,08	
	Cu(NO ₃) ₂ ·3H ₂ O	0,035	
	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0,01	
Stock 3	Myo-inositol	10,0	10
	Niacine	0,2	
	pyridoxine HCl	0,08	
	Thiamine HCL	0,08	
Stock 4	NAA	0,02	10
Additional	Sucrose		30g/l ⁻¹ medium
	Agar		10g/l ⁻¹ medium

pH adjusted to 5,8 with NaOH

a growth room where conditions of constant temperature ($26^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and continuous low intensity light were maintained. After 28 days the three pieces of callus in each flask were massed simultaneously. The amount of callus growth in each fraction was plotted on a histogram relative to the control value. The significant limit at 0,01 per cent level was calculated and is indicated on the histograms as a dotted line. Standards of zeatin were included with each bioassay. In order to estimate gross levels of cytokinin activity at a given time, the results from bioassays were expressed in zeatin equivalents. However, the inherent difficulties in making such an estimate were recognized.

5.0 Estimation of carbon dioxide evolution in germinating *Podocarpus henkelii* seeds.

In order to establish how soon after scarification and incubation the seeds began to actively respire, measurements of the amount of carbon dioxide evolved by the seeds were recorded. Whole and scarified seeds from cold storage were placed in separate open flasks (five in each) with moist vermiculite and incubated at 25°C in the dark. The seeds were equilibrated for 24 hours before samples were taken. This was achieved every 12 hours by placing bungs in the mouths of the flasks to prevent gaseous exchange with the atmosphere. Fifteen minutes later a five millilitre sample of air was withdrawn from each of the flasks and injected into separate evacuated test tubes. These tubes were stored for a short while until the carbon dioxide concentrations could be estimated using a gas chromatograph. A Perkin Elmer F11 instrument

fitted with a 6 metre x 3 millimetre poropak column connected to a hot wire detector was used. The column and the detector were both run at room temperature. The results obtained are expressed as percentage increase in carbon dioxide concentration over the unscarified control.

6.0 Radiochemical studies.

^{14}C Labelled compounds were used in order to study three aspects of metabolism of *P. henkelii* seeds. Firstly, to examine whether or not the seeds could fix carbon. Secondly, to trace the movement of labelled sucrose and linoleic acid in mature scarified and unscarified seeds, and thirdly to study the transport and metabolism of labelled zeatin in germinating seeds.

6.1 Determination of ability of *Podocarpus henkelii* seeds to fix carbon.

To establish whether or not the seeds of *P. henkelii* (which are green in colour) have the capacity to fix carbon, they were exposed to $^{14}\text{CO}_2$. This was generated from $\text{Ba}^{14}\text{CO}_3$ (specific activity 58,3 mCi/mmol). The seeds were placed in sealed flasks of known volume in CO_2 free air. Sufficient $^{14}\text{CO}_2$ was then injected into these flasks to provide a final concentration of 0,03 per cent CO_2 . These flasks were incubated under continuous light at 25°C for 1,2 and 3 days respectively. At each sampling the seeds were separated into their component parts (epimatium, female gametophyte and embryo sporophyte). These tissues were dried in an oven at 80°C, ground to homogeneous powders, and three sub-samples (\pm ten

milligrammes) from each placed in glass scintillation vials with 0,5 millilitres of digestion fluid. This consisted of hydrogen peroxide: perchloric acid (1:1 v/v). Digestion was accomplished by incubation at 60°C for three hours. After cooling, ten millilitres of 'Redy-Solv' EP scintillation fluid were added to each vial (BREVEDAN and HODGES, 1978). The vials were left for 12 hours whereafter radioactivity was assessed by counting in a Tri-Carb spectrometer (Model 3380). Correction for quenching was achieved by the channels ratio method. To establish which free sugars incorporated the ^{14}C into the seed tissue, the sugars were extracted in the following manner: One gramme of epimatium and female gametophyte and 0,1 gramme of embryo sporophyte were extracted with five millilitres and one millilitre of 80 per cent ethanol respectively. The samples were filtered, taken to dryness and resuspended in 0,5 millilitres of 80 per cent ethanol. The component sugars were separated from these samples with paper chromatography as previously detailed (Section 3.3). The regions of the chromatograms which corresponded to known markers were taken and placed in scintillation vials. One millilitre of methanol followed by ten millilitres of scintillation cocktail containing four grammes per litre PPO and 0,2 grammes per litre POPOP in toluene, were added to each (HENSON, 1978), whereafter the radioactivity was counted as above.

The photosynthetic pigments were extracted from 20 grammes of leaf and epimatium material by homogenizing the tissues in 150 millilitres of acetone. Five grammes of anhydrous sodium sulphate were added to remove excess water and the extracts filtered. The absorption spectra of the crude extracts

were measured in a Varian DMS 90 UV-Vis spectrophotometer between 350 and 800 nm. The extracts were then dried in a rotary evaporator at 40°C, resuspended in chloroform, and streaked on to silica gel thin layer chromatography plates. These were developed in an ascending manner in hexane : diethyl ether : acetone (60:30:20 v/v), for 20 minutes. The Rf values of the pigments were recorded and the pigments scraped off the plates and eluted from the silica gel with acetone. The absorption spectra of the individual pigments were recorded as described above.

6.2 Translocation of ^{14}C labelled sucrose in unscarified *Podocarpus henkelii* seeds.

To ascertain whether or not there was translocation of food reserves between the seed components in unscarified 'dormant' seeds, ^{14}C labelled sucrose was used. This was applied to whole seeds in three positions; 1, a cut on the epimatium; 2, injected into the female gametophyte and 3, injected into the embryo sporophyte (Fig. 5). In each treatment ten seeds received five microlitres of ^{14}C sucrose (specific activity 434,6 mCi/mmol), and the cut or injection holes were covered with lanolin. The seeds were then placed in separate flasks under conditions of continuous light and constant $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The air in the flasks was continually changed by pumping in small equal volumes of fresh air. The exhaust air was passed through a carbon dioxide trap containing ten millilitres of methanol : ethanolamine : 'Redy-Solv' EP (6:1:7 v/v) (DUCASSE and VAN STADEN, 1981). These traps were replaced with fresh solutions every 24 hours. The mixture from these traps was

Fig.5

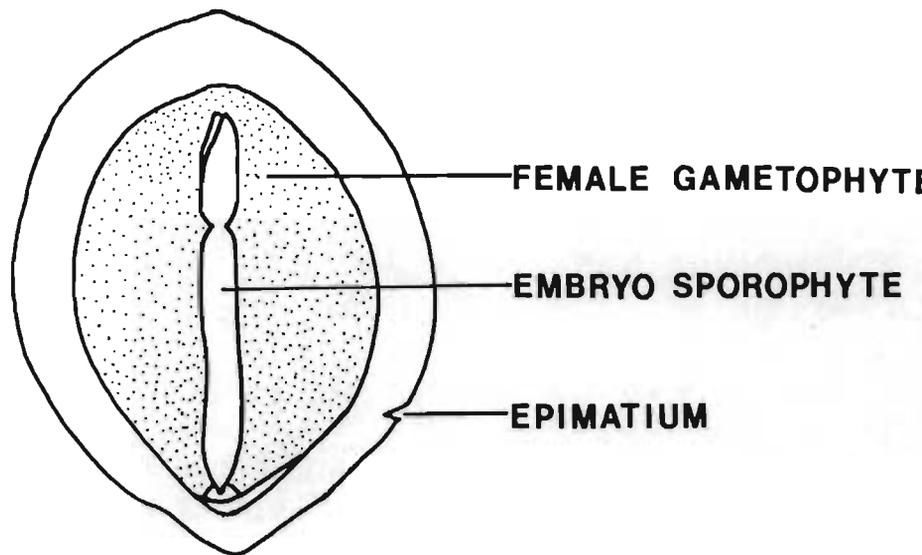


Figure 5. Line diagram of the mature seed of *Podocarpus henkelii* showing the regions where ^{14}C labelled sucrose, linoleic acid and zeatin were applied.

then placed in glass scintillation vials and the radioactivity counted as previously stated (Section 6.1). After 24 and 48 hours the seeds were removed from the flasks and dissected into their component parts. Precautions were taken to prevent cross contamination. The separated seed components were massed and then dried in an oven at 80°C. These components were then assayed for radioactivity (Section 6.1). The results are expressed as dpm per gramme dry mass or percentage radioactivity in each component.

6.3 Translocation of ^{14}C labelled sucrose and linoleic acid in scarified *Podocarpus henkelii* seeds.

To determine whether or not free sugars and lipids are translocated between the seed components of *Podocarpus henkelii* seeds during germination, use was made of ^{14}C labelled sucrose and linoleic acid (specific activity 52 mCi/mmol). Five microlitres of each were applied in separate experiments to the epimatium and female gametophyte (ten seeds each) of scarified seeds as described in the previous section. These seeds were then incubated with moist vermiculite at 25°C for two, four and six days respectively. At these time intervals seeds were sampled, dissected into their component parts and assayed for radioactivity (Section 6.1).

6.4 Metabolism of ^{14}C zeatin in germinating *Podocarpus henkelii* seeds.

Three microlitres of ^{14}C labelled zeatin (specific activity 11,7 mCi/mmol) were applied to the epimatium and female gametophyte of scarified seeds (Section 6.2). Ten seeds

were used in each treatment and three samplings were taken after three, six and nine days of incubation. At sampling, the seed was separated into the three component parts, massed and extracted immediately for cytokinins (Section 4.0). The purified extracts were strip-loaded onto Whatman's No. 1 chromatography paper and developed with PAW. After drying, the chromatograms were divided into ten Rf zones and one fifth of each zone was removed. This was placed in a scintillation vial with one millilitre of methanol followed by ten millilitres of scintillation cocktail (Section 6.1). The radioactivity was determined with a Packard Tri-Carb spectrometer (Model 3380).

In order to further purify the metabolites of zeatin, certain fractions were subjected to column chromatography. This technique differed from that previously explained in that the Sephadex LH-20 column was eluted with ten per cent methanol (HUTTON and VAN STADEN, 1981). The paper chromatograms were divided into three regions; A corresponding to Rf's 0,1-0,2, B corresponding to Rf's 0,3-0,5 and C corresponding to Rf's 0,6-0,9 respectively. These were eluted separately with increasing concentrations of ethanol, the combined extracts were taken to dryness in a rotary evaporator and the extracts resuspended in ten per cent methanol. The extracts were eluted through the column at a flow rate of 15 millilitres per hour. Forty millilitre fractions were collected and placed in 50 millilitre flasks. One millilitre of each fraction was removed and placed in separate scintillation vials before drying on a hot plate set at 30°C. These one millilitre fractions were dried under a stream of air after which one millilitre of methanol followed by ten millilitres of scintillation cocktail were

added (Section 6.1). The radioactivity was counted as previously described. When the fractions in the 50 millilitre flasks were dry they were prepared for the soybean callus bioassay (Section 4.3) to assess biological activity.

CHAPTER I

THE ULTRASTRUCTURE AND BIOCHEMISTRY OF DEVELOPING
PODOCARPUS HENKELII SEEDS.

The biochemical development of gymnospermous seeds is a field of study that has been neglected by research workers to date. This probably stems from the two problems mentioned earlier; firstly, the very long embryogenesis of gymnospermous seeds which does not present an attractive and easy research proposition and secondly, the probable lack of funding for such a project. Consequently, there is a large gap in our knowledge of the physiological development of the seeds in this sub-order, some members of which display diverse and interesting characteristics. The structure of the mature seed of *Podocarpus henkelii* is very different from that of *Pinus sylvestris* (SIMOLA, 1974) and also of *Welwitschia mirabilis* (BUTLER, 1975), both of which have been studied extensively.

An ultrastructural and biochemical study of the developing seed of *Podocarpus henkelii* was thus undertaken. The seeds were sampled on four occasions during the nine month long period of embryogenesis. Although the seeds first appear on the female trees in late August, fertilization does not take place until sometime in October. The first cell wall formation in the female gametophyte takes place in late November, at which time the embryo sporophyte consists of only a few cells and a long suspensor (COERTZE, VAN DER SCHIJFF and SCHWEICKERDT, 1971). Consequently the first sample was collected during early December when there was some cellular detail

observable in the female gametophyte. This sample was divided into the epimatium and female gametophyte only. The second and subsequent samples were taken at 46 day intervals and divided into the three component parts; epimatium, female gametophyte and embryo sporophyte. Before freeze-drying the dissected material for later biochemical analyses, measurements of length, breadth and fresh and dry mass of the individual seed components were recorded. The results of these studies are presented in Tables 1.1, 1.2 and 1.3. No electron microscopic studies were undertaken on the epimatium at any stage as this material proved exceedingly troublesome to fix and embed due to the presence of resins and phenolics.

The first sample in which cellular detail could be recorded in the female gametophyte was in early December, approximately six weeks after fertilization. At this time, the moisture content of this seed component on a dry mass basis, was 97.15 per cent (Table 1.1). This indicates the very low dry matter content of these cells. The fine structure of a typical female gametophyte cell at this stage of development is shown in Plate 1.1 a. The cells were relatively large, 13-16 micrometres in diameter and consisted almost entirely of a large central vacuole. The peripheral cytoplasm contained a small nucleus in which dense heterochromatin areas may be seen interspersed in the more electron-transparent nucleoplasm. Ribosomes, polyribosomes, mitochondria, smooth endoplasmic reticulum and active dictyosomes with associated dictyosomal vesicles were also present, (Plate 1.1 b). The mitochondria however, do not have large or well defined cristae suggesting that the metabolic activity of this tissue is slow. The first

TABLE 1.1. Measurements of size, mass and moisture content of developing *Podocarpus henkelii* seeds. Each sample is the mean of fifty seeds and the figures in parentheses represent the standard errors.

SEED COMPONENT		DATE OF SAMPLE			
		DECEMBER 12	JANUARY 28	MARCH 15	APRIL 30
WHOLE SEED	FRESH MASS GMS/SEED ⁻¹	2,50 ± 0,73	4,45 ± 0,68	5,36 ± 0,34	7,0 ± 1,51
	% MOISTURE OF SEED ⁻¹	81,28	74,69	66,76	61,77
EPIMATIUM	DIAMETER MILLIMETRES	15,3 ± 0,94	19,9 ± 1,3	21,0 ± 0,86	23,2 ± 1,46
	LENGTH MILLIMETRES	19,3 ± 1,24	23,1 ± 1,37	23,6 ± 1,2	24,8 ± 1,16
	FRESH MASS GRAMMES	1,87 ± 0,49	3,1 ± 0,38	3,36 ± 0,10	3,99 ± 0,16
	DRY MASS GRAMMES	0,45	1,04	1,20	1,62
	% MOISTURE	75,94	66,46	60,72	59,40
FEMALE GAMETOPHYTE	DIAMETER MILLIMETRES	9,6 ± 0,47	14,1 ± 1,86	14,8 ± 0,4	17,0 ± 1,09
	LENGTH MILLIMETRES	13,3 ± 0,49	16,5 ± 2,06	17,6 ± 1,2	20,4 ± 0,8
	FRESH MASS GRAMMES	0,63 ± 0,2	1,35 ± 0,31	1,96 ± 0,2	2,95 ± 0,31
	DRY MASS GRAMMES	0,018	0,14	0,56	1,09
	% MOISTURE	97,15	89,63	71,43	63,06
EMBRYO SPOROPHYTE	DIAMETER MILLIMETRES	-	1,0 ± 0,6	2,6 ± 0,6	3,0 ± 0,51
	LENGTH MILLIMETRES	-	5,0 ± 1,2	13,8 ± 3,6	17,6 ± 2,1
	FRESH MASS GRAMMES	-	0,002 ± 0,0009	0,04 ± 0,02	0,06 ± 0,027
	DRY MASS GRAMMES	-	0,0004	0,01	0,015
	% MOISTURE	-	80,0	75,0	75,0

Table 1.2. The contribution that each seed component makes to the developing seed of *Podocarpus henkelii* on a percentage fresh mass basis.

Seed Component	Date of Sample			
	December 12	January 28	March 15	April 30
Epimatium	74,80	70,99	63,11	57,44
Female Gametophyte	25,20	28,95	36,82	42,47
Embryo Sporophyte	-	0,04	0,075	0,086

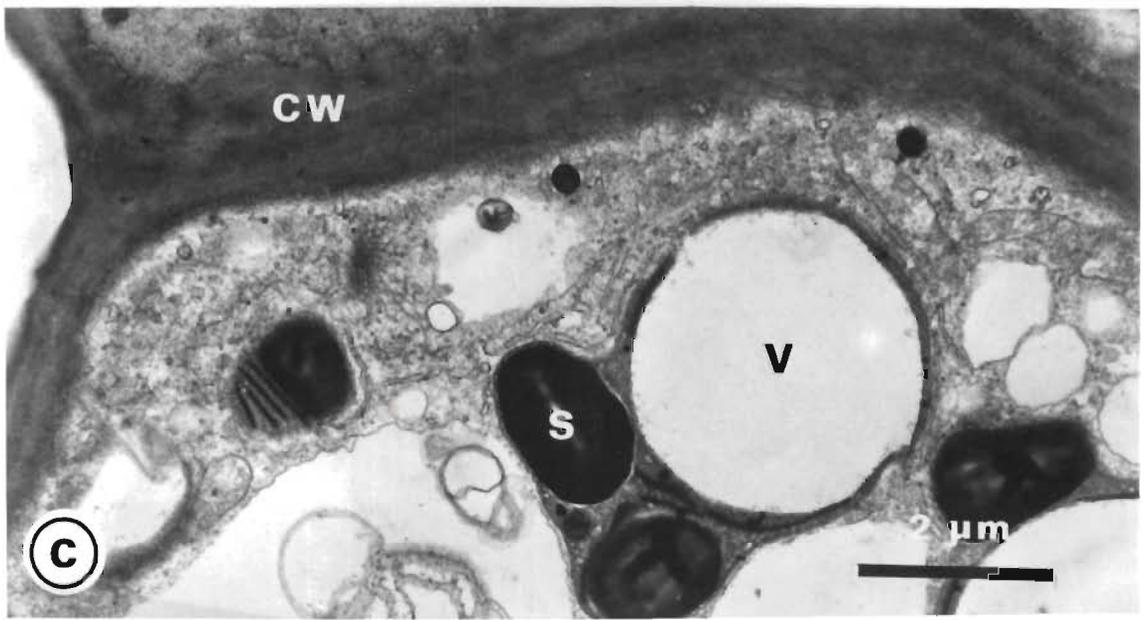
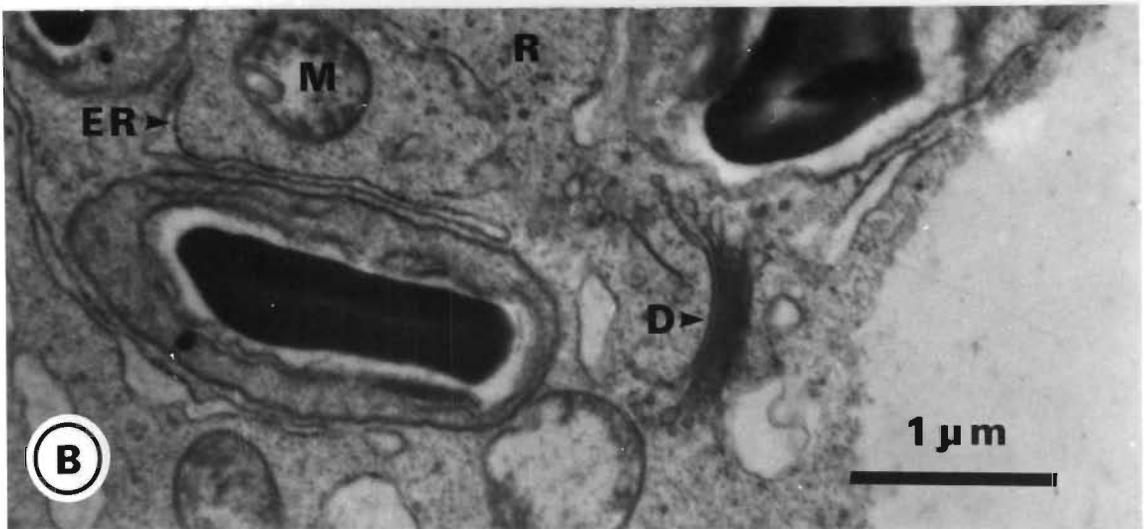
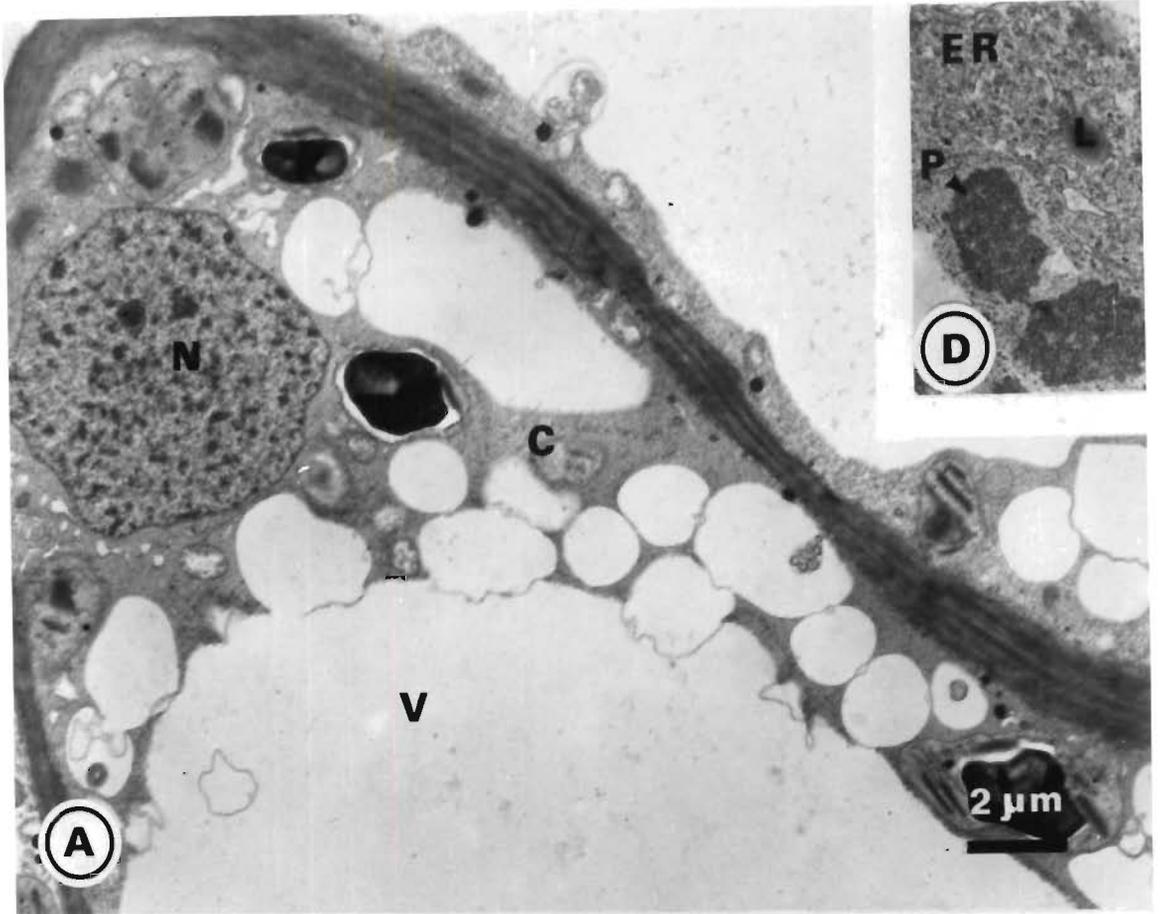
Table 1.3. The contribution that each seed component makes to the developing seed of *Podocarpus henkelii* on a percentage dry mass basis.

Seed Component	Date of Sample			
	December 12	January 28	March 15	April 30
Epimatium	96,15	88,10	67,79	59,44
Female Gametophyte	3,85	11,86	31,63	40,00
Embryo Sporophyte	-	0,03	0,56	0,56

PLATE 1.1

THE ULTRASTRUCTURE OF THE FEMALE GAMETOPHYTE ON THE 12TH DECEMBER, APPROXIMATELY SIX WEEKS AFTER FERTILIZATION

- A: The cells are large, consisting of thin cell walls, a sparse cytoplasm (C), and a large central vacuole (V).
- B: The cytoplasm in the cells is densely staining, consisting of many ribosomes (R), some polyribosomes, endoplasmic reticulum (ER) and mitochondria (M). The dictyosomes are very active with many associated bodies.
- C: Some cell walls (CW) display an irregular thickening thought to be the deposition of reserve material. The starch grains are often found to be associated with membrane bound vacuoles (V).
- D: Non-membrane bound protein granules (P) are found associated with regions of active cytoplasm. Lipid bodies (L) are also present. Magnification is the same as for Plate 1.1C.



storage reserves in the form of starch were already in evidence in plastids scattered throughout the cytoplasm. These plastids contained the usual pro-lamellar bodies (Plate 1.1 c) and are similar to those described by BADENHUIZEN (1969). Some were however unusual, as they featured a large associated vacuole. It is suggested that this is a characteristic of large but immature plastids which have not yet swollen to their full extent. Consequently areas of their periphery become depressed and thus in cross section appear to be vacuoles, particularly as the contents of these vacuoles resemble very closely the contents in the main cell vacuoles.

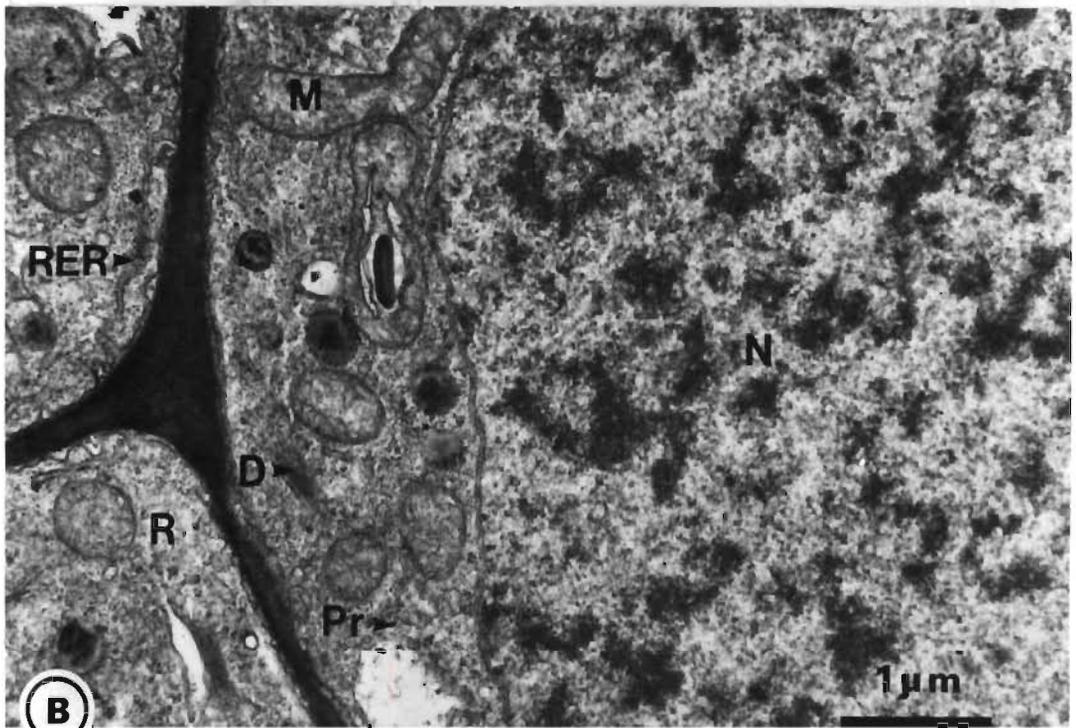
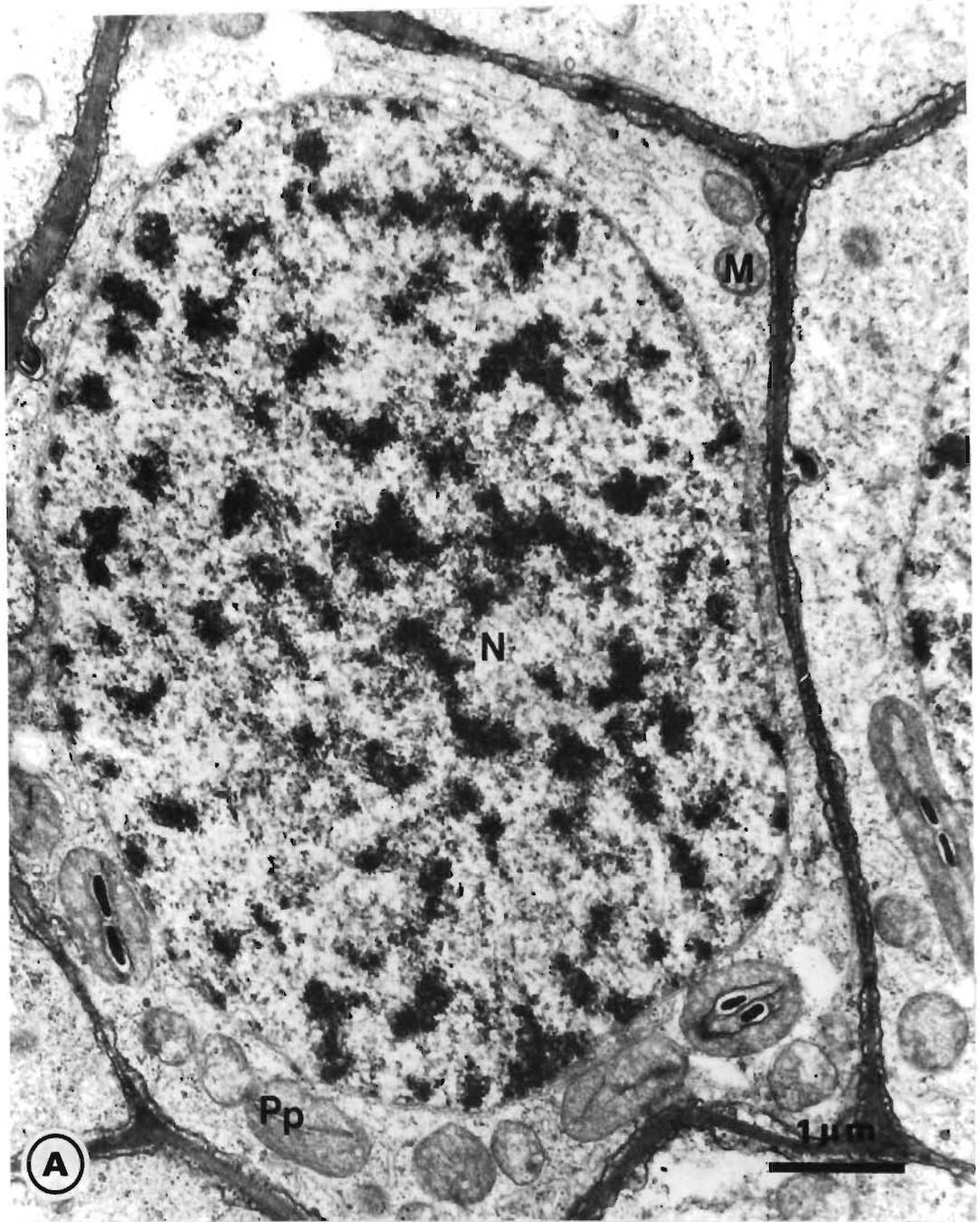
Some regions of dense cytoplasm contain aggregations of protein granules (Plate 1.1 d). These were not limited by a membrane and resemble those reported in the developing seed of *Lupinus albus* (DAVEY and VAN STADEN, 1978b). The cytoplasm in the vicinity of the protein granules contained a high density of polyribosomes, free ribosomes and endoplasmic reticulum indicative of protein synthesis. A few lipid bodies were also associated with the dense cytoplasm. In some cases the cell walls show very irregular thickening, even at this early stage of development. This thickness is often associated with active dictyosomes surrounded by dictyosomal vesicles (Plate 1.1 c). These cell wall proliferations are similar to those reported in a number of plant species (BOWES, 1979); FASSEAS and BOWES, 1980) and may be associated with storage of celluloses.

The second sample of seed was collected in late January, approximately 12 weeks after fertilization. The whole seed had increased in fresh mass from 2,5 grammes to 4,7 grammes

PLATE 1.2

THE ULTRASTRUCTURE OF THE EMBRYO SPOROPHYTE SAMPLED ON THE 28TH OF JANUARY APPROXIMATELY TWELVE WEEKS AFTER FERTILIZATION.

- A: A hypocotyl cell which has recently divided and contains a large nucleus (N), active mitochondria (M) and immature plastids (Pp) containing starch grains.
- B: A typical cell from the cortical zone of the root tip. The cell is active with a large densely staining nucleus (N) and active mitochondria (M). The cytoplasm also contains rough endoplasmic reticulum (RER), dictyosomes (D), many ribosomes (R) and polyribosomes (Pr).



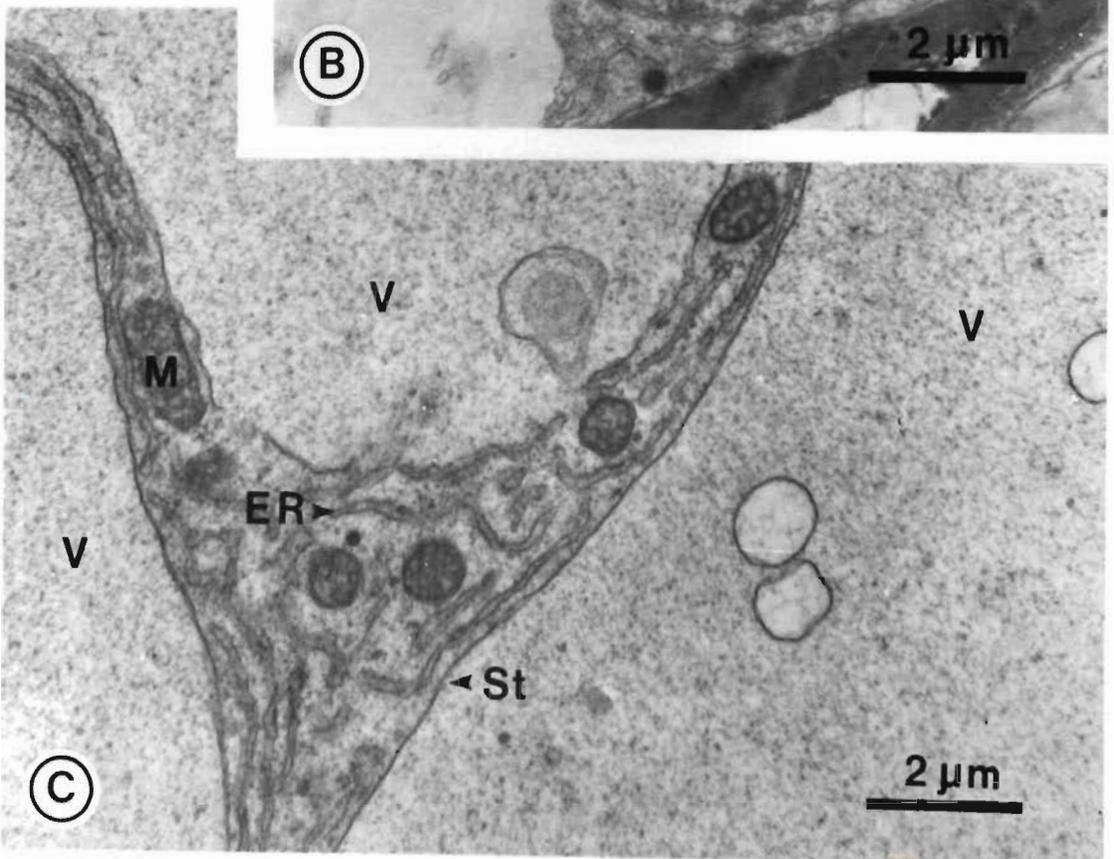
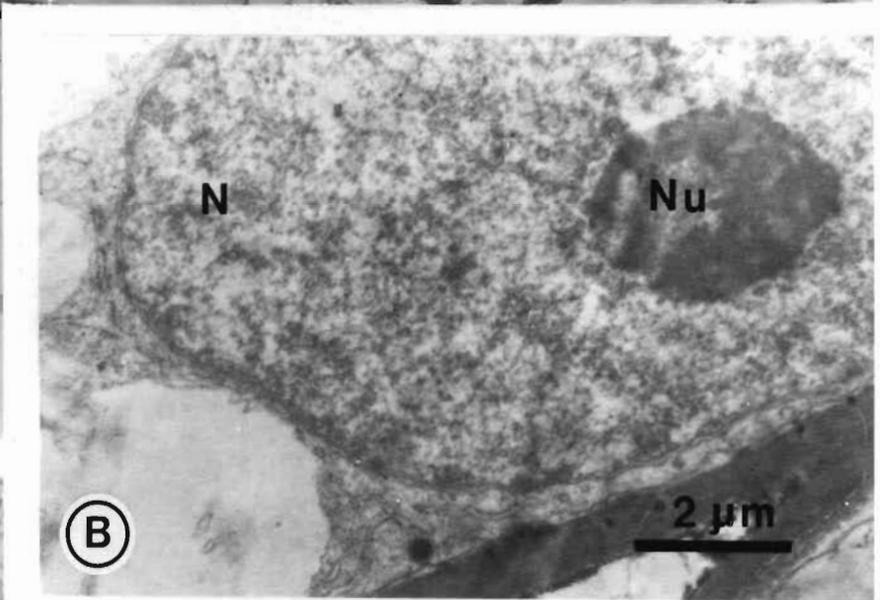
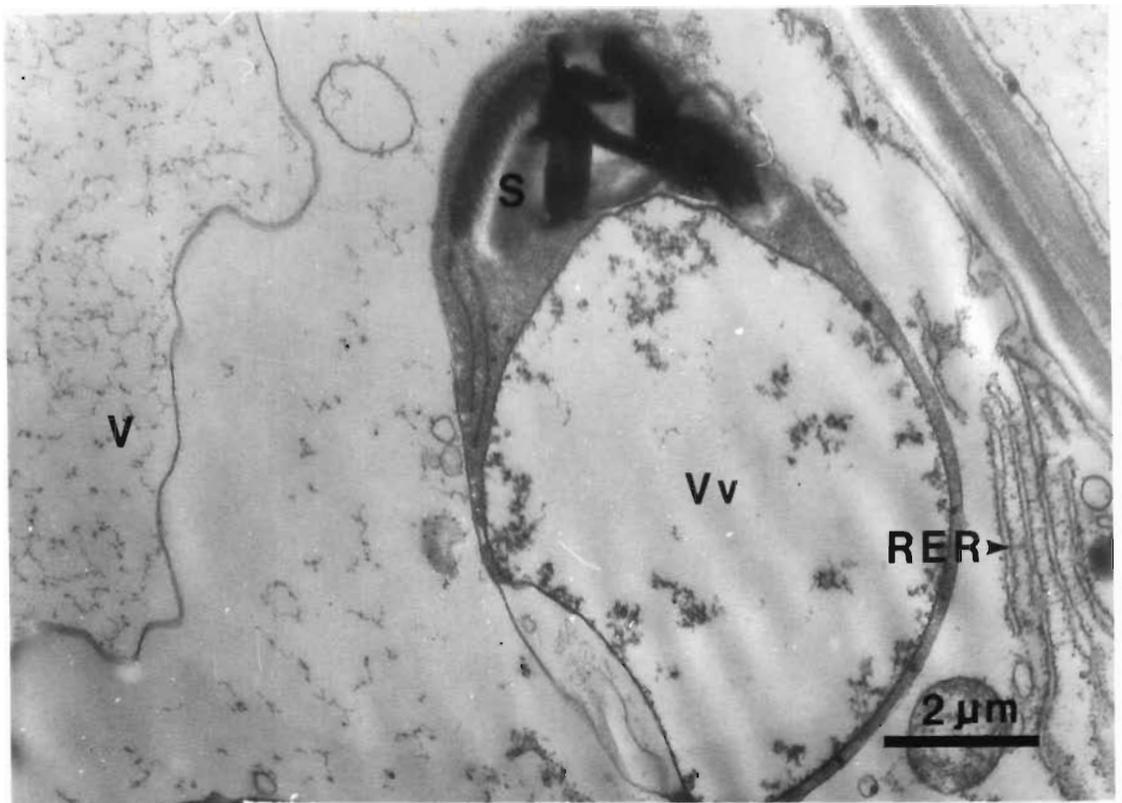
during this period (Table 1.1). The epimatium contributed the largest proportion of this mass (Tables 1.2 and 1.3), with the female gametophyte increasing in contribution on a dry mass basis from 3,9 to 11,9 per cent. Despite its small size the embryo sporophyte had grown rapidly during the preceding 46 days and was relatively well developed, (5 millimetres x 1,0 millimetres). The cotyledons, hypocotyl and radicle were easily distinguishable from one another even though the fine structure revealed little differentiation at this time (Plate 1.2 a and b). There was evidence of recent cell division as indicated by the presence of immature cells, thin cell walls and large nuclei with smooth outlines. The nucleoplasm in both radicle and cotyledonary cells was fairly electron-dense and interspersed with dense areas of heterochromatin. These features are indicative of cells in transition from the meristematic to the expanding state (JORDAN, 1971). The cell size in both these tissues ranged between 6-8 micrometres in diameter. Young plastids were present in hypocotyl, cotyledonary and root tip cells. All cells contained increasing amounts of starch. The high moisture content of 80 per cent and low dry mass of this seed component indicates the immaturity of the embryo sporophyte tissues.

In contrast to the rapid development of the embryo sporophyte the female gametophyte showed relatively little development during the preceding six weeks. An interesting feature of the tissue in this seed component is the uniformity of the cells. Samples from regions close to the corrosion cavity, integument, or deep within the female gametophyte all displayed the same characteristics. The increase in both fresh

PLATE 1.3

THE FEMALE GAMETOPHYTE CELLS APPROXIMATELY TWELVE WEEKS AFTER FERTILIZATION SAMPLED ON THE 28TH OF JANUARY.

- A: The cells still appear relatively inactive with a large central vacuole (V) and very electron-transparent cytoplasm. Rough endoplasmic (RER) is present in some regions and starch grains often contain large vacuoles (Vv).
- B: The nucleus (N) in all cells is small in relation to cell size, relatively smooth in outline and contains a very electron-dense nucleolus (Nu).
- C: In some cells sheets of cytoplasm permeate and compartmentalize the central vacuole (V). These sheets contain mitochondria (M) and endoplasmic reticulum (ER).



and dry mass of the female gametophyte is manifested in the increase of cell wall thickness from 1,5 to 2 micrometres and the accumulation of some starch (Plate 1.3 a). The cells also increased in size to a diameter of \pm 30 micrometres thus accounting for the increased volume of this seed component. The nucleus in all cells was small, \pm six micrometres in diameter, and regularly shaped (Plate 1.3 b). The nucleolus was very electron-dense and probably consisted of mainly fibrillar material with a few granular components. These nucleolar features are all indicative of a low level of cellular, and thus metabolic activity (HYDE, 1967 and JORDAN, 1971). The lack of polyribosomes in the cytoplasm (Plate 1.3 a) supports this suggestion.

The large central vacuole in some cells was separated by sheets of cytoplasm that extended from the cell periphery (Plate 1.3 c). These confined regions contained active mitochondria and endoplasmic reticulum. The function of these sheets or the compartmentalization of the vacuole which results is not clear. The starch synthesizing plastids had increased in numbers and larger starch grains were present. This tissue gives the impression of having a low metabolic rate, possibly indicative of the long embryogenesis or perhaps a consequence thereof.

During the following six weeks the development of the seed was not as rapid in terms of increase in fresh and dry mass (Table 1.1, Fig. 1.1). The mass of the whole seed increased by only 0,6 grammes with the greatest dry mass increment occurring in the female gametophyte (0,14 to 0.56 grammes).

Figure 1.1. Change in fresh mass of the whole seed \otimes - \otimes , epimatium \bullet - \bullet , female gametophyte X-X and embryo sporophyte O-O of *Podocarpus henkelii* seeds during development. Bars represent the standard error.

Figure 1.2. Change in dry mass of the whole seed \otimes - \otimes , epimatium \bullet - \bullet , female gametophyte X-X and embryo sporophyte O-O of *Podocarpus henkelii* seeds during development.

Fig.1.1

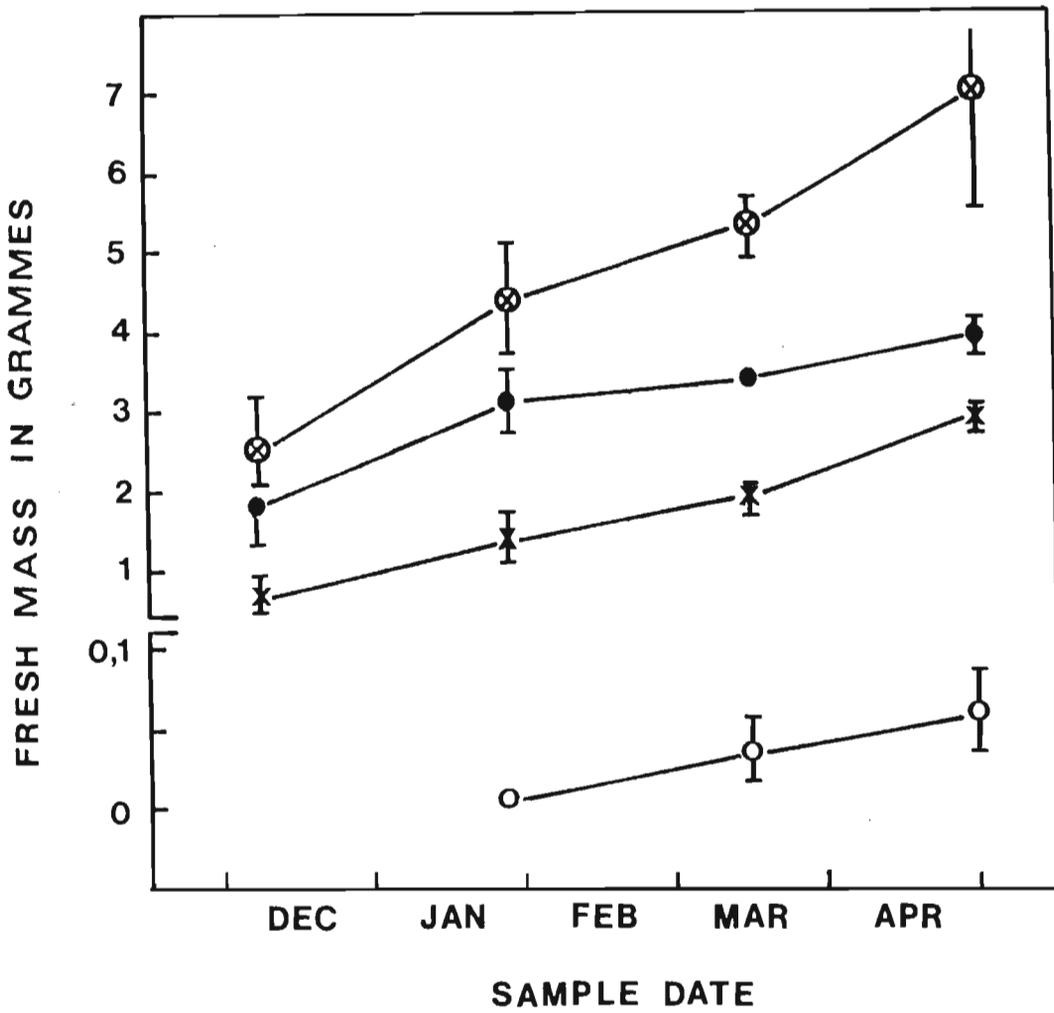
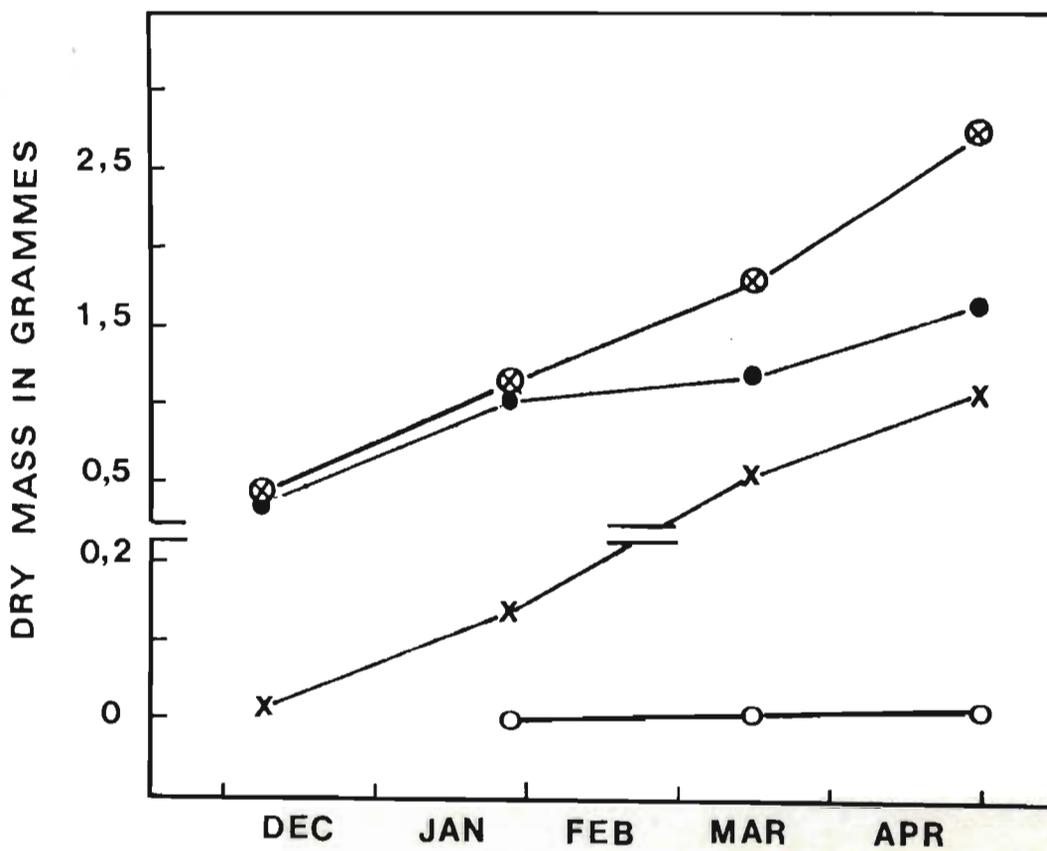


Fig.1.2



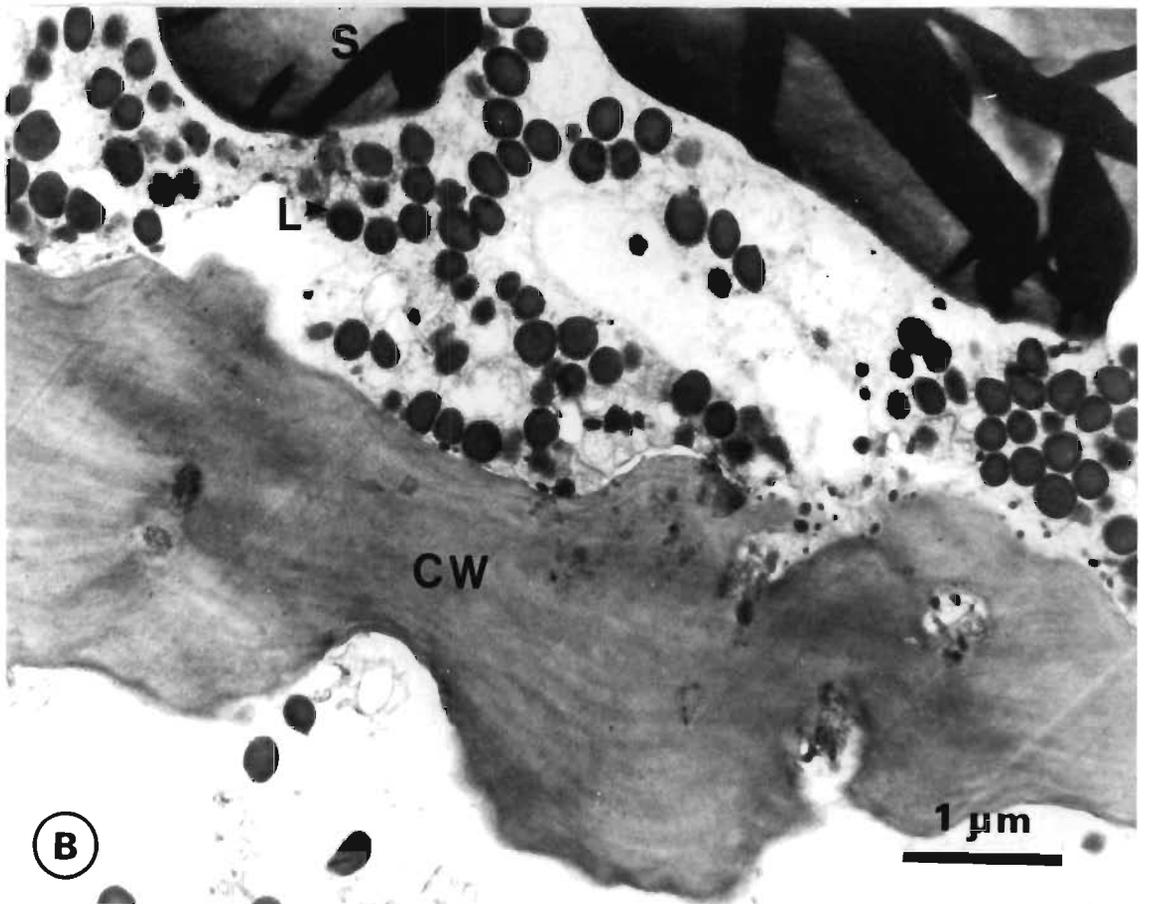
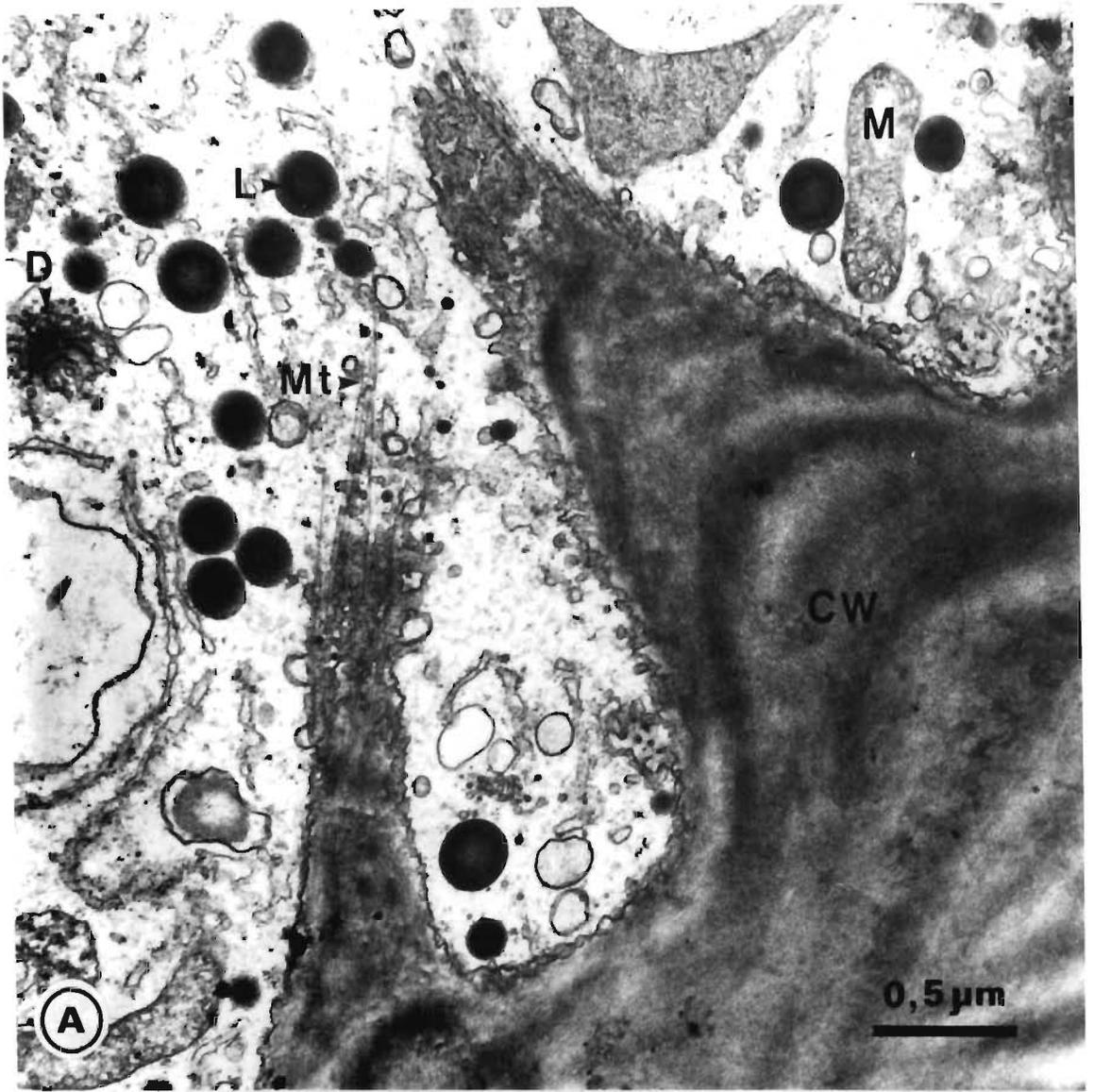
Fine structural observations revealed that this growth was due to an increase in the cell wall thickness from 0,65 micrometres to 1,1 micrometres. Lipid bodies were to be found in restricted regions of the cytoplasm for the first time. These bodies were spherical and smooth in outline with diameters of 0,4 to 0,6 micrometres. They were homogeneous in structure, osmiophilic and appeared to be bound by a thin peripheral electron-dense membrane (FREY-WYSSLING, GRIESHABER and MÜHLETHALER, 1963). Some starch grains had increased in size and they completely filled the original plastid envelope.

In most cells of the female gametophyte one wall continued to thicken. These thickenings showed many varied shapes and were often found in association with very distinct microtubules (Plate 1.4 a and b). The cytoplasm in close proximity to these thickened areas was usually very active containing ribosomes, polyribosomes and active dictyosomes with numerous dictyosomal vesicles (Plate 1.4 a). The microtubules are thought to be involved not only in microfibril deposition in cell walls, but also in the orientation of the cell walls (GUNNING and HARDHAM, 1979; HEPLER and PALEVITZ, 1974; HOGETSU and SHIBAOKA, 1978; PALEVITZ and HEPLER, 1976). In addition, the thickened cell walls give the appearance of being spongy rather than hard. Under these circumstances the microtubules may act as skeletons to support the plasmalemma and prevent collapse of the cell walls (MARCHANT and HINES, 1979). The function of these cell wall thickenings is not clear. Their spongy appearance does not give the impression of strengthening and clearly there is no need for

PLATE 1.4

THE FEMALE GAMETOPHYTE CELLS APPROXIMATELY EIGHTEEN WEEKS AFTER FERTILIZATION SAMPLED ON THE 15TH OF MARCH

- A: Some cells have irregularly thickened cell walls (CW) which are often associated with clear microtubules (Mt). The cytoplasm contains active dictyosomes (D), mitochondria (M) and lipid bodies (L).
- B: The lipid bodies stain darkly and are surrounded by a thin electron-dense layer. The starch grains (S) have increased in size and the cell wall (CW) shows the typical irregular thickening.



strengthening. The thick tough epimatium provides sufficient protection to both female gametophyte and embryo sporophyte. The thickenings could be hemicelluloses laid down as a storage reserve for future mobilization. MITCHELL (1930) reports the presence of cell wall thickening in the endosperm of *Phoenix dactylifera* L. and *Asparagus sprengerii* Rigel. Histochemical analyses revealed that the thickenings were comprised of hemicelluloses. Similar thickenings of hemicellulose have also been reported in the female gametophyte cells of *Welwitschia mirabilis* (BUTLER, 1975).

The embryo sporophyte had increased markedly in terms of size and both fresh and dry mass. It still however, constituted only 0,56 per cent of the seed on a dry mass basis. The cell walls in all regions of the embryo sporophyte increased in size and there was evidence of differentiation (Plate 1.5 a, b and c). In the radicle cells meristematic activity continued. This accounts for the increase in length of the embryo to 13,8 millimetres. Relatively immature radicle cells undergoing vacuolation and differentiation are shown in Plate 1.5 d. Food reserve deposition was taking place in many cells at this stage. In the cotyledons the number of plastids had increased and consequently the amount of starch present in the cells (Plate 1.5 a). The size of the plastids had also increased from 1,4 micrometres in long axis to \pm 2 micrometres. Similar increases were observed in the hypocotyl but little starch storage was recorded in the differentiating radicle cells (Plate 1.5 b and d). This increase in plastid number and size accounts for the increase in starch content of the embryo sporophyte from 267 milligrammes to 381

milligrammes per gramme dry mass (Fig. 1.8). No plastids could be found in the embryo sporophyte tissues that resembled those observed in the female gametophyte.

Lipid was being accumulated rapidly in cells in the cotyledons, hypocotyl and radicle. This reserve was stored in bodies which were roughly spherical in shape and 0,8 to 1,0 micrometres in diameter. In most instances the bodies were limited by a distinct boundary. The lipid accumulation was always associated with dense cytoplasm containing numerous ribosomes, polyribosomes and smooth endoplasmic reticulum (Plate 1.5 c). The origin of these lipid bodies is not clear. They do not appear to be formed from swellings of endoplasmic reticulum as reported in plants containing large quantities of lipid such as *Brassica napus* L. or *Cucumis sativus* L. (WANNER, FORMANEK and THEIMER, 1981). As some of the lipid bodies appear not to be limited by a membrane, it is suggested that they are synthesized *in situ* in the cytoplasm (Plate 1.5 c).

One very noticeable feature was the different staining characteristics of the lipid bodies in the embryo sporophyte when compared with those in the female gametophyte. The lipid bodies in the latter seed component were far more osmiophilic than those found in the embryo sporophyte. Table 1.4 lists the more important fatty acids extracted from the mature female gametophyte and embryo sporophyte. The different staining characteristics may be accounted for by the different ratios of the fatty acids present in the lipid bodies of the two seed components.

PLATE 1.5

THE EMBRYO SPOROPHYTE SAMPLED APPROXIMATELY EIGHTEEN WEEKS AFTER FERTILIZATION ON THE 15TH MARCH.

- A: The cotyledons have accumulated starch (S) and lipid (L) reserves. The nuclei (N) are now reduced in size.
- B: The hypocotyl cells are also accumulating starch (S) and lipid (L). The nucleus contains a nucleolus with a nucleolar vacuole (Nu) indicative of lowered metabolic rate.
- C: Some lipid bodies (L) are bound by an electron-dense layer, whilst others (LL) appear to be free in the cytoplasm.
- D: The differentiating radicle cells in which the nucleus (N) is decreasing in size, and plastids (P) and vacuoles (V) are developing.

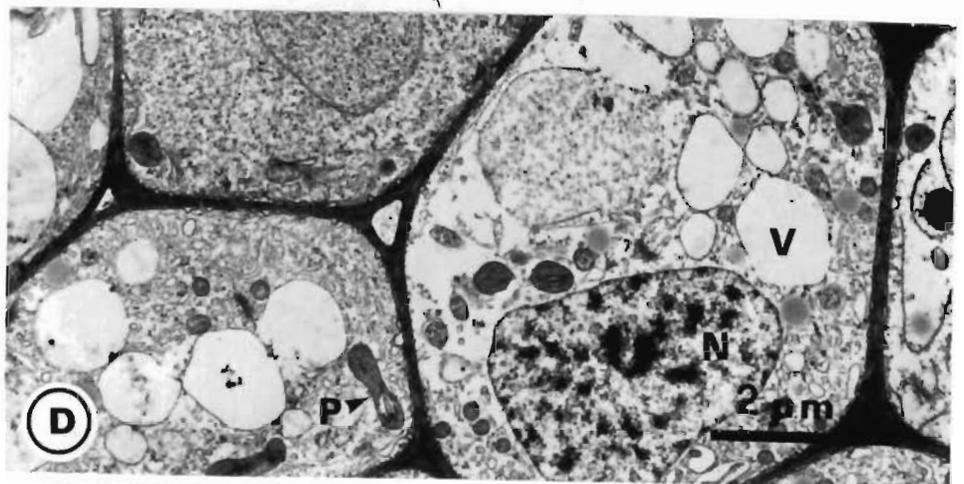
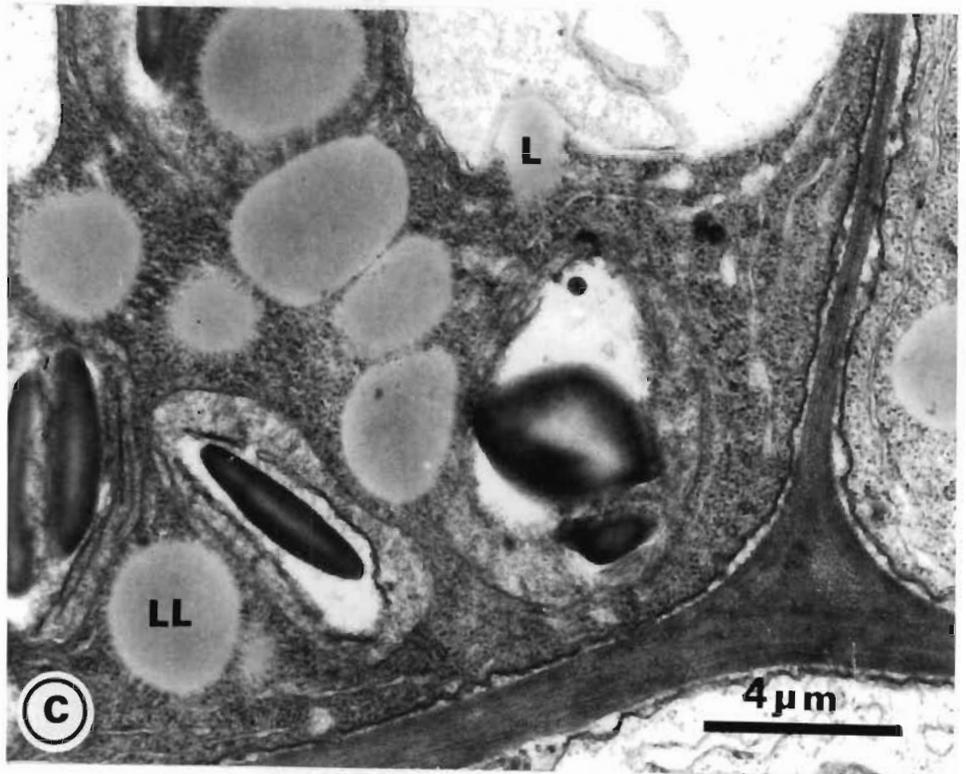
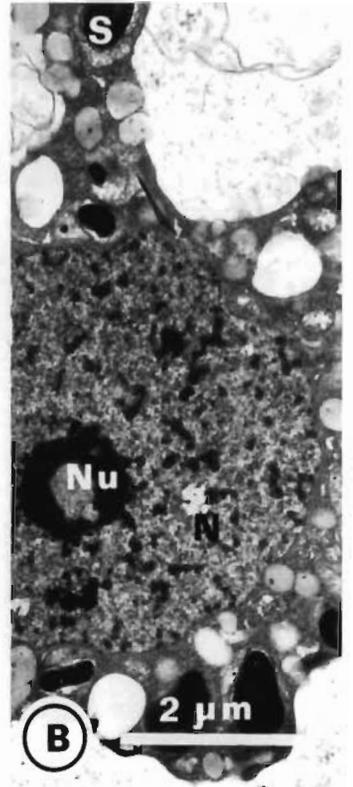
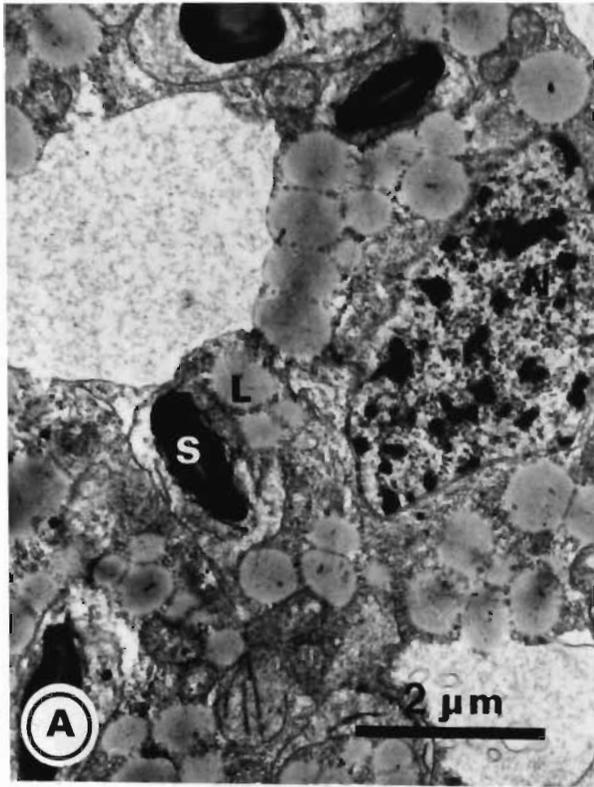


Table 1.4. The main fatty acids detected in the lipid component extracted from the female gametophyte and embryo sporophyte of the mature seed of *Podocarpus henkelii*. The figures are expressed as percentages of the total fatty acids present.

Fatty Acid	Seed Component	
	Female Gametophyte	Embryo Sporophyte
Palmitic	6,7	9,3
Oleic	21,4	19,9
Linoleic	31,0	44,5
Linolenic	3,7	10,1
Icos- adienoic	12,7	5,0
Others	24,5	11,2

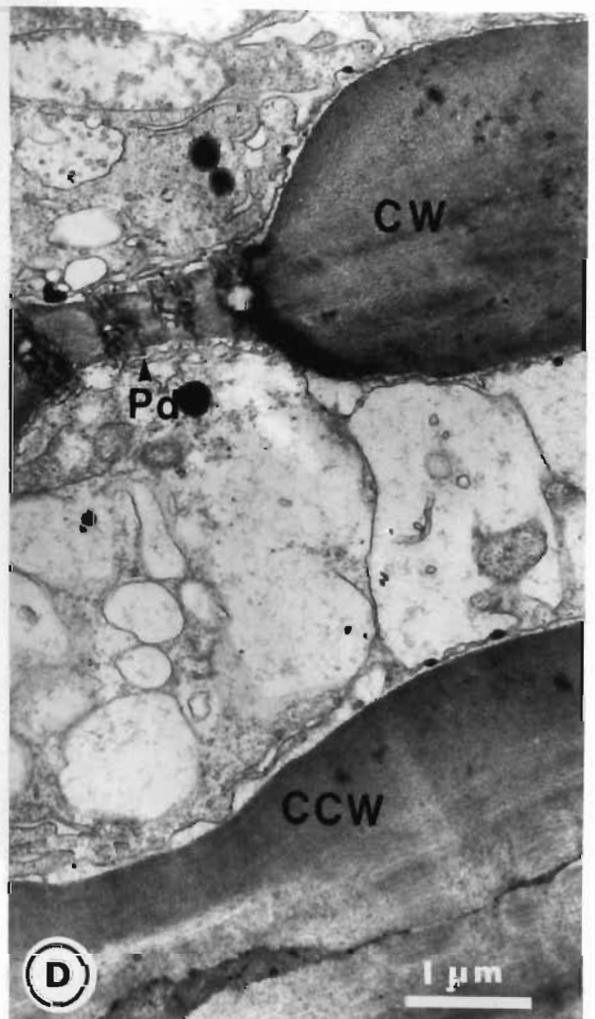
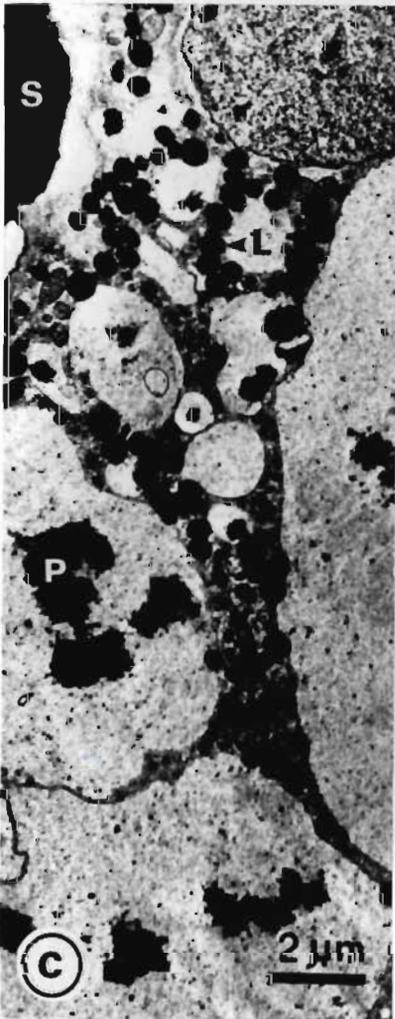
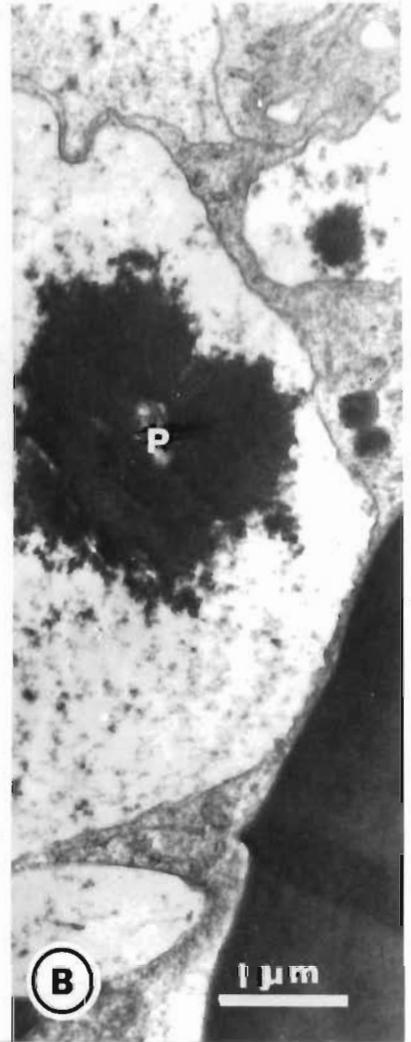
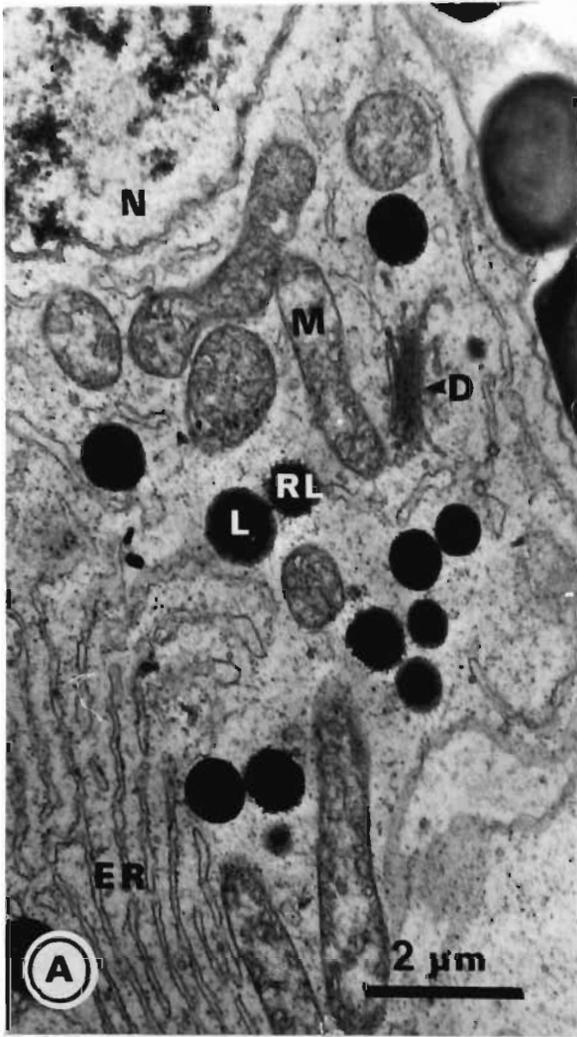
The final sample of the developmental study was collected at the end of April. The criterion for maturity was if the seed had fallen off the tree. During the preceding six weeks the seeds had increased in fresh mass from 5,3 to 6,9 grammes (Fig. 1.1). Most of this increase occurred in the female gametophyte tissue which increased in dry mass from 0,56 to 1,09 grammes. At this stage this seed component contributed 40 per cent of the dry mass of the seed. The increase in dry mass is reflected in the accumulation of more starch and lipid. Protein in the form of non-membrane bound aggregations occurred in vacuoles of the female gametophyte tissue. These vacuoles were always closely associated with the peripheral cytoplasm (Plate 1.6 b and c). The formation of storage protein in seeds was reported to occur in one of two ways. The successive filling of larger vacuoles with reserve proteins, possibly by dictyosome-derived vesicles, or the dilation and vesiculation of endoplasmic reticulum cisternae with protein (WEBER and NEUMANN, 1980). A good example of the former method is illustrated by BAIN and MERCER (1966a) in developing *Pisum sativum* seeds. The latter method is described in developing *Oryza sativa* kernels (HARRIS and JULIANO, 1977). In the seeds of *Podocarpus henkelii* only small areas of cytoplasm appear to be involved in protein synthesis. These areas contain a little rough endoplasmic reticulum and a few polyribosomes. Active mitochondria with well defined cristae and dictyosomes were also present. The subsequently synthesized protein was sequestered into vacuoles.

These features contrast markedly with the fine structure in developing *Lupinus albus* seeds (DAVEY and VAN STADEN,

PLATE 1.6

FEMALE GAMETOPHYTE CELLS FROM THE MATURE SEED COLLECTED ON THE 30TH OF APRIL.

- A: Cells contain a small nucleus (N), smooth endoplasmic reticulum (ER) and mitochondria (M). Lipid bodies with smooth (L) and rough (RL) outlines are present along with active dictyosomes (D).
- B: At this time protein (P) is deposited in vacuoles.
- C: Cell showing the maximum amount of reserve material recorded consisting of starch (S), lipid (L) and protein (P).
- D: The cells adjacent to the corrosion cavity become crushed and cell walls compressed together (CCW). The secondary thickening in cell walls (CW) is evident and plasmodesmata (Pd) are present.



1978b). In these seeds maximum protein accumulation is accompanied by sheets of rough endoplasmic reticulum and numerous polysomes in the cytoplasm. The nuclei became enlarged and amoeboid in shape. These features have also been reported to accompany protein storage in other seed tissues (BAIN and MERCER, 1966a and HARRIS and BOULTER, 1976). As the amount of protein present in the female gametophyte cells is small (25,0 milligrammes per gramme dry mass), little synthesizing machinery would be required. Thus synthesis could occur in the areas of dense cytoplasm adjacent to the protein vacuoles (Plate 1.6 b).

The full extent of the food reserves in the female gametophyte tissue is shown in Plate 1.6 c. The very large vacuoles dominate the fine structure of the cells. The female gametophyte cells adjacent to the corrosion cavity were crushed by the swelling embryo sporophyte. The crushed cell walls and cellular debris create a thick layer between these two seed components. In many seeds transfer tissue occurs in the interface regions between embryo and the tissues which store food reserves (PATE and GUNNING, 1972). Transfer-like tissue has been reported in the interface between feeder and female gametophyte cells of *Welwitschia mirabilis* seeds (BUTLER, 1975). No evidence of transfer tissue could be found in the interface cells of the female gametophyte or on the epidermal cells of the embryo sporophyte of *Podocarpus henkelii*. Thus the pathway of food reserves from the female gametophyte to the embryo sporophyte whilst the seed remains 'dormant' is not clear. The crushed cell walls and cellular debris interspersed between these two seed components does present a barrier to food reserve translocation. That food reserves are readily trans-

locatable between female gametophyte and embryo sporophyte during dormancy is clearly demonstrated in Chapter four with ^{14}C labelled sucrose.

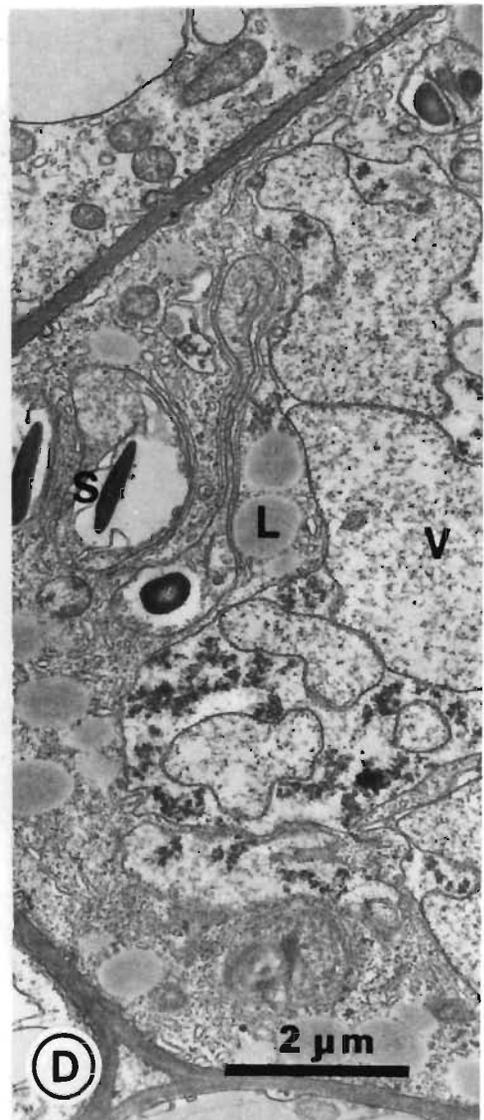
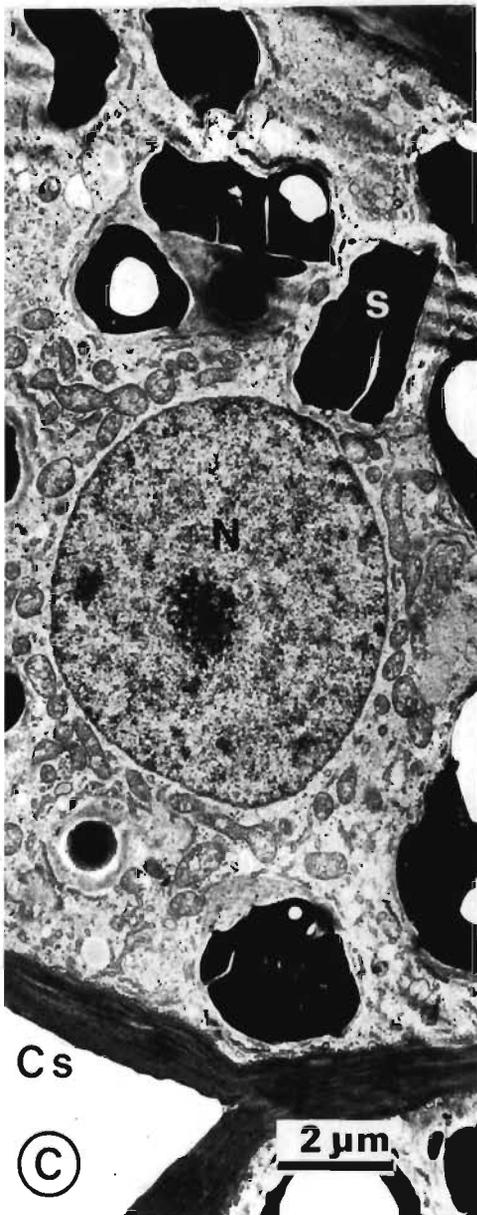
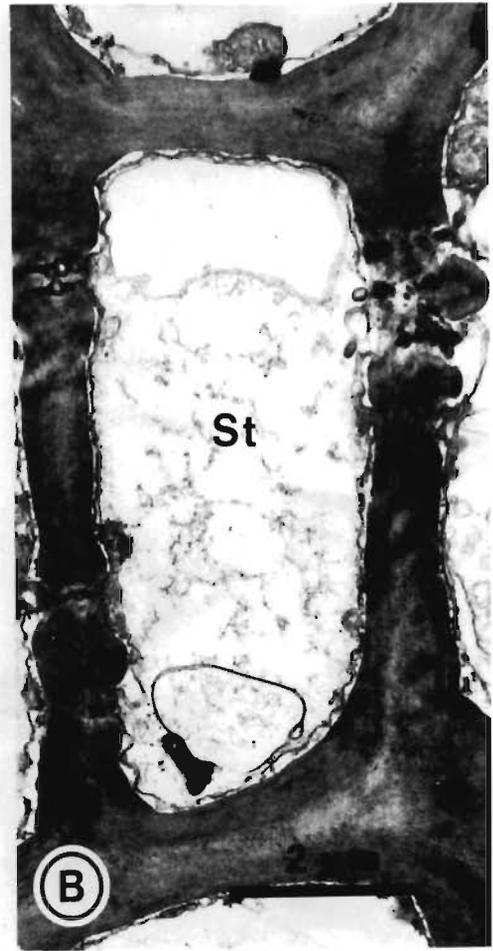
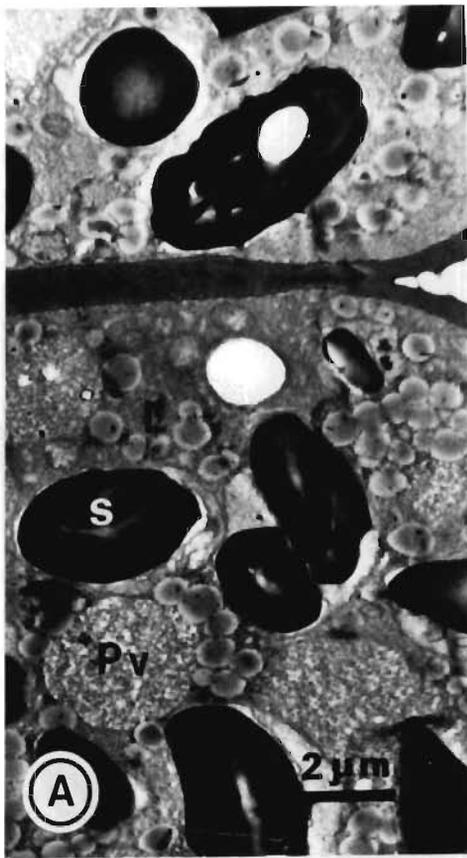
The embryo sporophyte increased in size and dry mass up to its being shed from the tree. The differentiation of the component tissues continued during this period with the vascular system developing (Plate 1.7 b). The meristem of the root tip remained active, thus contributing to the increase in length of the embryo sporophyte. Many of the cortical cells of the radicle were still differentiating with increased vacuolation and the accumulation of starch and lipid (Plate 1.7 d). The root cap comprises three to four millimetres of the length of the radicle. This tissue is composed of relatively large cells with thick cell walls and interspersed with large intercellular spaces (Plate 1.7 c). This tissue acts as a storage repository for starch. The cells along the periphery of the tissue which were in contact with the female gametophyte continually slough off. These features suggest that this tissue acts as a cushion to the radicle cells. Upon germination the embryo sporophyte as a whole, and the radicle in particular, has to exert considerable pressure on the epimatium in order to rupture it. This is necessary for radicle protrusion and germination. The root cap cells could protect the radicle cells during this phase.

The full extent of the food reserves in the embryo sporophyte is shown in Plate 1.7 a. Protein vacuoles were present for the first time in the cotyledonary tissue. A noticeable feature of these storage reserves is that they do

PLATE 1.7

THE EMBRYO SPOROPHYTE FROM MATURE SEEDS COLLECTED ON
THE 30TH OF APRIL.

- A: The maximum amount of reserve material recorded in the cotyledonary cells with starch (S), lipid (L) and protein in the form of protein vacuoles (Pv).
- B: The vascular system of the embryo sporophyte is well developed. The micrograph shows a sieve tube (St) cell from the hypocotyl region.
- C: The radicle is covered by a large spongy root cap. The cells in this region have thick cell walls, large inter-cellular spaces (Cs) and appear to act as a cushion for the expanding radicle.
- D: The radicle cells are still differentiating with evidence of vacuolation (V) and the deposition of starch (S) and lipid (L).



not resemble the tightly packed reserve tissues of *Picea abies* (SIMOLA, 1976), *Pinus sylvestris* (SIMOLA, 1974) or *Welwitschia mirabilis* (BUTLER, 1975). In *Picea abies* and *Pinus sylvestris* both the embryo sporophyte and female gametophyte seed components are packed with mainly lipid bodies interspersed with a few protein bodies. There was very little accumulation of starch in these seeds. The food reserves of *Podocarpus henkelii* seed components appear sparse by comparison. However, when it is considered how large both the female gametophyte and embryo sporophyte are in comparison to the seeds of *Pinus sylvestris*, the apparent lack of reserve material is perhaps compensated for by the sheer volume of cells present. This is particularly so in the case of the female gametophyte.

One of the unusual features of the mature seed of *Podocarpus henkelii* is the very high moisture content of \pm 60 per cent when shed from the tree (Table 1.1). This phenomenon could account for the very different appearance of the fine structure of both female gametophyte and embryo sporophyte seed components, when compared with other mature dry gymnospermous seeds. The tightly packed food reserves of *Pinus sylvestris* (SIMOLA, 1974) and *Welwitschia mirabilis* (BUTLER, 1975) are probably a consequence of their low moisture contents (\pm six per cent). This results in the cells shrinking with desiccation and thus giving the appearance of tightly packed food reserves.

The biochemical and ultrastructural studies are complementary in that the sequence of food deposition can be studied with these two techniques. In addition, the ultrastructural study facilitates the identification of the tissues responsible

for the storage of the various forms of food reserves. The biochemical analyses of the developing *Podocarpus henkelii* seeds concur with the aforementioned ultrastructural observations. The lipid content of the embryo sporophyte and female gametophyte are shown in Fig. 1.3. The relatively large quantities of lipid in the first sample of the embryo sporophyte is unusual. These levels could be due to the large quantities of structural lipids required for the functional membranes present. As cell division had only recently ceased, large numbers of such membranes would still be present. TAN and MORRISON (1979a) made similar observations in developing *Zea mays* kernels. The structural lipids consist of phospholipids and glycolipids. The early synthesis of these two lipid fractions in developing *Oryza sativa* kernels (CHOU DHURY and JULIANO, 1980) is consistent with the early synthesis of cell walls and membrane-bound organelles (HARRIS and JULIANO, 1977).

The proportions of the fatty acids present in *Podocarpus henkelii* seed components changed in proportion to one another during development. At the first sampling of the female gametophyte the most important fatty acids were linoleic, oleic and palmitic acids. In the mature female gametophyte the levels of linoleic, oleic and palmitic acids decreased and the levels of the two forms of icosadienoic acids and icosatetraenoic acid increased. In the immature embryo sporophyte linoleic, oleic and palmitic acids were the most important fatty acids present, and the levels of these did not change much with maturity. Unlike the female gametophyte, the levels of icosadienoic and icosatetraenoic acids did not increase with

Figure 1.3. The lipid content, expressed as a per cent, of the embryo sporophyte O-O and X-X female gametophyte, of developing *Podocarpus henkelii* seeds. Lipids were extracted with light petroleum ether in a Soxhlet apparatus. Bars represent the standard errors.

Figure 1.4. The ethanol soluble fraction expressed as a per cent of the embryo sporophyte O-O and female sporophyte X-X of developing *Podocarpus henkelii* seeds. Bars represent the standard error.

Fig. 1.3

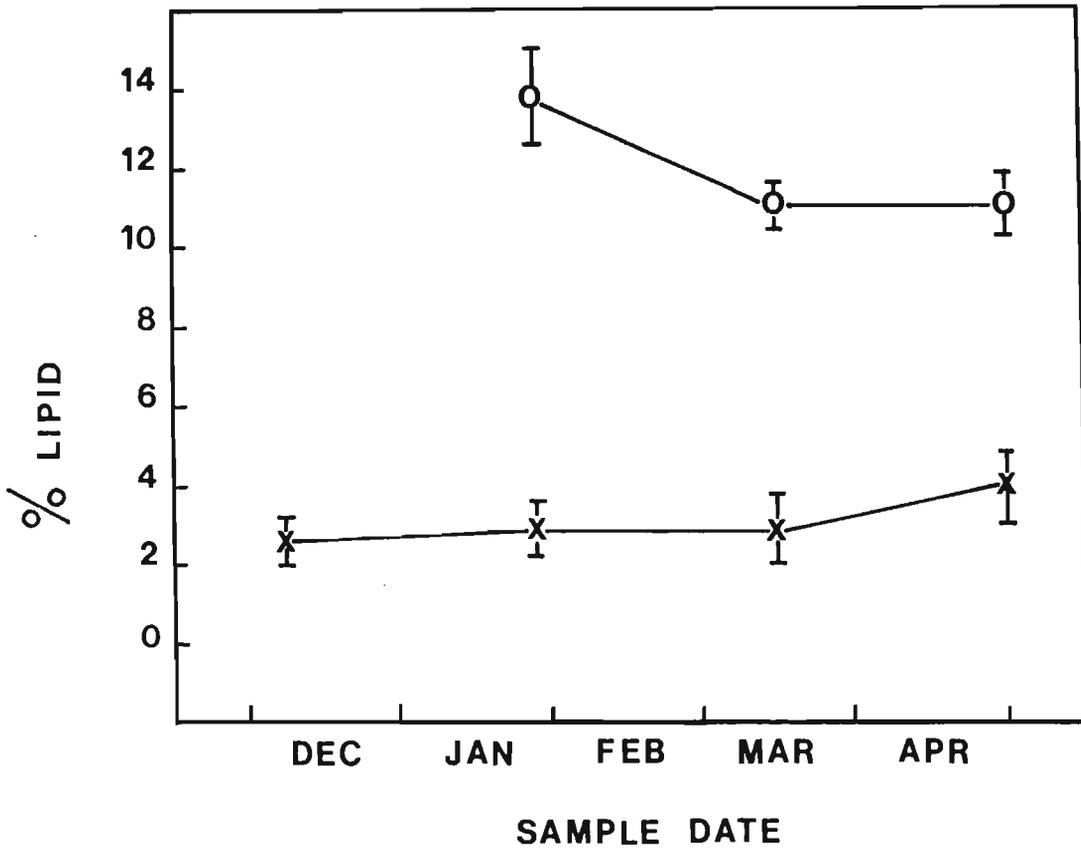


Fig. 1.4

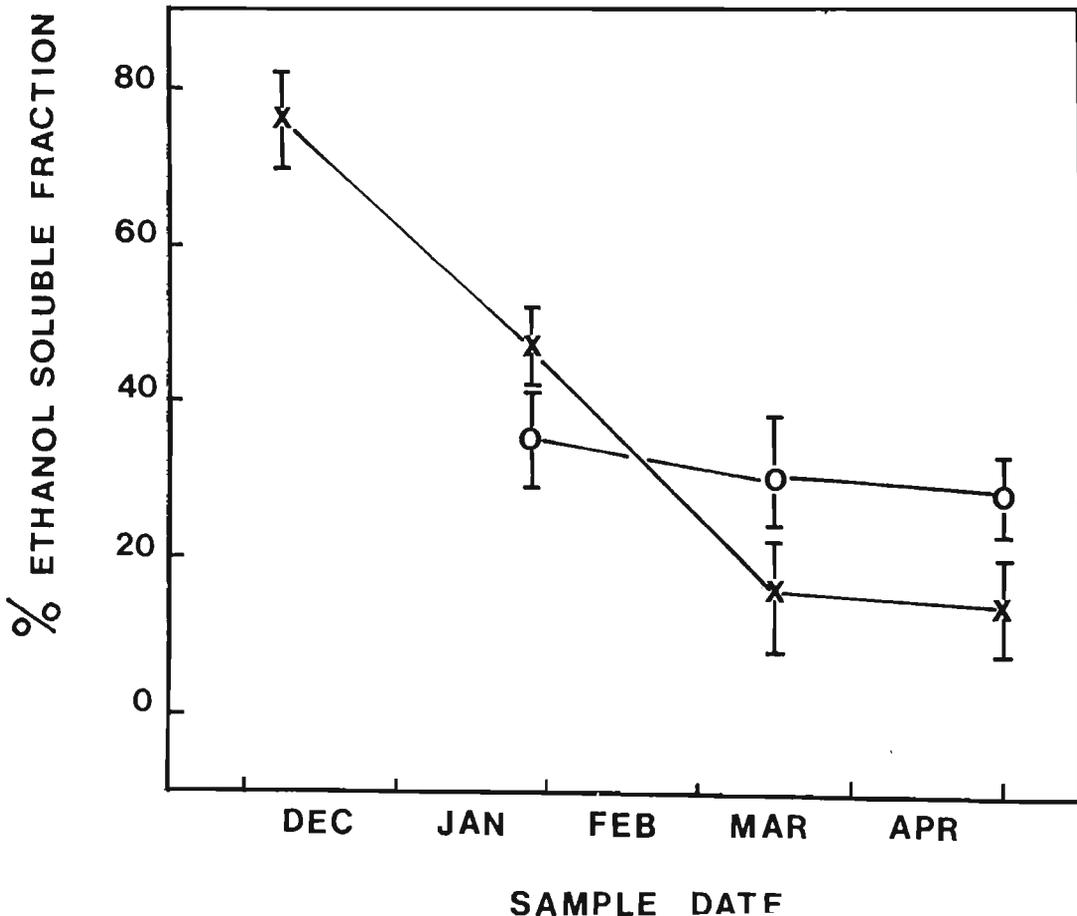


Table 1.5. Changes in the constituent fatty acids of *Podocarpus henkelii* seed components during development. Δ represents the position of the double bond in the fatty acid molecule. The results are expressed as percentages of the total fatty acid content.

Fatty Acid	Symbol	Female Gametophyte		Embryo Sporophyte	
		Date of Sample			
		December 12	April 30	January 28	April 30
Palmitic	16:0	13,2	6,7	10,0	9,3
Palmitoleic	16:1	0,7	0,2	0,4	0,4
Stearic	18:0	2,6	0,9	2,2	1,9
Oleic	18:1	28,3	21,4	20,0	19,9
Linoleic	18:2	35,1	31,0	43,0	44,5
Linolenic	18:3	4,9	3,7	7,2	10,1
Nonadecanoic	19:0	0,4	0,4	0,5	0,3
Nonadecadienoic	19:2	0,7	0,7	0,2	0,2
Arachidic	20:0	0,7	0,5	0,9	0,8
Gadoleic	20:1	0,8	1,5	0,4	0,3
Icosadienoic	Δ 8, 11 20:2	2,1	8,9	1,7	1,3
Icosadienoic	Δ 11, 14 20:2	2,0	12,7	5,5	5,0
Icosatrienoic	Δ 11, 14, 17 20:3	0,1	0,6	0,4	0,1
Icosatetraenoic	20:4	4,2	10,6	4,0	4,5
Behenic	22:0	1,4	0,2	0,3	0,3
Erucic	22:1	0,1	0,1	0,2	0,2

maturity suggesting that they were not storage forms in this seed component.

Detailed studies of the changes of fatty acid composition have been made on developing seeds of *Crambe abyssinica* (APPLEBY, GURR and NICHOLS, 1974). They found that the levels of the constituent fatty acids fluctuated throughout the period of development with the highest proportion being contributed by erucic acid (22:1) which peaked between 25 to 35 days after flowering. Similar changes in fatty acid composition with development have been recorded in *Oryza sativa* (CHOUDHURY and JULIANO, 1980). They found that linolenic acid decreased with maturity, and linoleic acid increased to become the most important fatty acid. In developing safflower seeds (*Carthamus tinctorius* L.) ICHIHARA and NODA (1980) showed that linoleic acid predominated in every lipid class during the period, and comprised 70 per cent of total fatty acids. Linolenic acid decreased with maturation and was not detected in mature seeds. The safflower seeds take 40 days to develop after flowering and maximum lipid biosynthesis was recorded in these seeds 15 to 18 days after flowering. The changes of fatty acid composition of developing *Zea mays* kernels have been described by TAN and MORRISON (1979a). They correlated the changes in free fatty acid composition with the known morphological developments in these kernels.

In mature kernels of *Zea mays*, INGLE, BEITZ and HAGEMAN (1965) report that the major storage of lipid occurred in the endosperm, with low levels present in the embryo. In contrast to this, PRICE and PARSONS (1979) report that in a

different cultivar of *Zea mays* 90 per cent of the oil was located within the embryo. Similarly TAN and MORRISON (1979b) report that in yet another cultivar of *Zea mays* 80 per cent of the lipid was stored in the embryo and the actual quantity of lipid present in this seed component was 43 per cent. In *Hordeum vulgare* and *Avena fatua* the stored lipids were located in the endosperm (PRICE and PARSONS, 1979). These data indicate that it is virtually impossible to draw any meaningful generalizations about the deposition of lipid storage reserves in seeds.

To consider the storage of lipid in a forest tree seed the acorns of White oak (*Quercus alba* L.) provide a good example. VOZZO (1978) reports that lipid is stored mainly in the cotyledons and embryo axis of acorns. Most of the gymnospermous seeds studied belong to forest trees although no reference could be found to the biochemical development of these seeds. Mature gymnospermous seeds are often referred to as 'fatty seeds' (KAO, 1973). Seeds of the Stone pine (*Pinus pinea* L.) contain 48 per cent lipid on a seed dry mass basis (BEWLEY and BLACK, 1978). The majority of this reserve is stored in the female gametophyte. Similarly in the seeds of Japanese Red pine (*Pinus densiflora* S. et Z.) contain 35 per cent lipid in the female gametophyte (HATANO, 1957). The seeds of Sugar pine (*Pinus lambertiana* Dougl.) and Jeffrey pine (*Pinus jeffreyi* Grew. and Balf.) also store lipid reserves in the female gametophyte (KAO, 1973). The mature seeds of Douglas fir (*Pseudotsuga menziesii*), Taiwan Red pine (*Pinus taiwanensis* Hay.) and Chinese fir (*Cunninghamia lanceolata* (Lamb) Hook.) contain 5, 3,7 and 3,5 milligrammes of lipid

per dry seed respectively (CHING, 1963a; KAO, 1973). The mature seeds of *Welwitschia mirabilis* contain ca. 28 per cent lipid, the majority of which is stored in the female gametophyte tissue.

The mature seeds of *Podocarpus henkelii* differ from the gymnospermous seeds mentioned above in that they contain only 4,25 per cent lipid which is divided between the female gametophyte and embryo sporophyte. The lastnamed seed component contains 12 per cent lipid on a dry mass basis at maturity. However, as this tissue contributes only ca. 0,5 per cent of the seed (Table 1.3), the majority of lipid storage occurs in the female gametophyte tissue.

The synthesis of fatty acids in seeds has been reviewed recently by STUMPF (1977). The precursor for fatty acid synthesis is sucrose which is converted in the cytoplasm to UDPG (uridine diphosphoglyceride) and fructose. The former compound is then thought to be translocated to the mitochondrion where conversion to pyruvate and thence acetyl CoA occurs. Acetyl CoA is then added to acetyl carrier protein (ACP) in the cytoplasm or, some workers suggest, proplastids. The ACP then combines with malonyl ACP to form palmityl ACP from which palmitic acid (16:0) is formed. The higher carbon number fatty acids are in turn synthesized from this base. The fatty acids are ultimately esterified with glycerol to form triglycerides. Thus sucrose occupies a very central role in the metabolism of developing seeds. This disaccharide provides the basic metabolic material for the synthesis of both fatty acids and starch.

The ethanol soluble fraction comprising free sugars and amino acids changed during the course of seed development, particularly in the female gametophyte (Fig. 1.4). The high levels in the early stages of development can be accounted for by the need for free sugars and amino acids for synthesis of structural carbohydrate and proteins. This correlates with the high moisture content and thus low dry mass of the female gametophyte at these early stages (Fig. 1.1). There is comparatively little change in the levels of the ethanol soluble fraction during the development of the embryo sporophyte. This could be accounted for by the fact that the embryo sporophyte was relatively well developed by the time of the first sample, thus the requirement for these reserves was lower.

The free sugars present in the seed components were analysed by paper chromatography and quantified using the anthrone reaction. The results of this study are shown in Table 1.6 and Fig. 1.5. The levels of the individual sugars analysed varied over the developmental period. Ribose is the most important sugar in the embryo sporophyte, whereas sucrose is the most important sugar in the female gametophyte. The highest level of sucrose in the female gametophyte coincides with the period of deposition of starch in this seed component (Fig. 1.8 and Plate 1.3). Another interesting feature of this study is the complete absence in some of the samples of fructose and in one case, glucose. This could be attributed to the demand for sucrose for starch and lipid synthesis. Thus high levels of sucrose synthetase may be present at this time to metabolize sucrose from the glucose and fructose present in the pool of free sugars. The increase of the free sugars

Table 1.6. Soluble sugars extracted from developing *Podocarpus henkelii* seed components. The individual sugars were separated by descending paper chromatography with butanol : ethanol : water (45:5:50) and quantified using the anthrone reagent. The free sugars are expressed as milligrammes **per** gramme seed component dry mass.

mg. g (seed comp)

Free Sugar Detected	Embryo Sporophyte				Female Gametophyte		
	January 28	March 15	April 30	December 12	January 28	March 15	April 30
Sucrose	51	2	45	40	120	8	8
Glucose	24	0	15	19	24	4	4
Fructose	0	0	7	6	100	0	0
Maltose	31	67	10	64	31	7	7
Ribose	105	43	53	11	7	13	6
TOTAL	211	115	130	140	282	32	25

Figure 1.5. The total free sugars extracted with ethanol from the embryo sporophyte O-O, and female gametophyte X-X of developing *Podocarpus henkelii* seeds. The results are expressed as milligrammes of sugar per gramme dry mass. The sugars were separated by paper chromatography and quantified using the Anthrone technique. Bars represent the standard errors.

Figure 1.6. The total free amino acids extracted from the embryo sporophyte O-O and female gametophyte X-X of developing *Podocarpus henkelii* seeds. The results are expressed as micromoles of amino acid per gramme dry mass. The amino acids were separated from the ethanol soluble fraction with exchange resin and separated and quantified on an amino acid analyser.

Fig.1.5

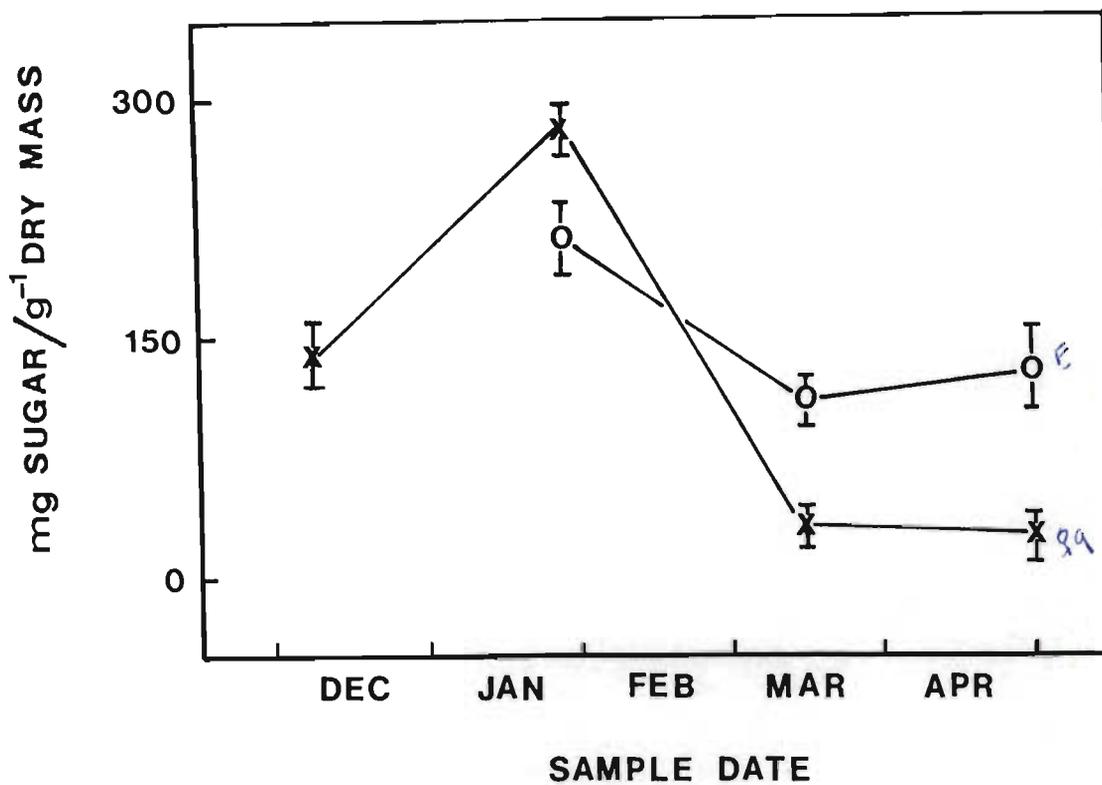
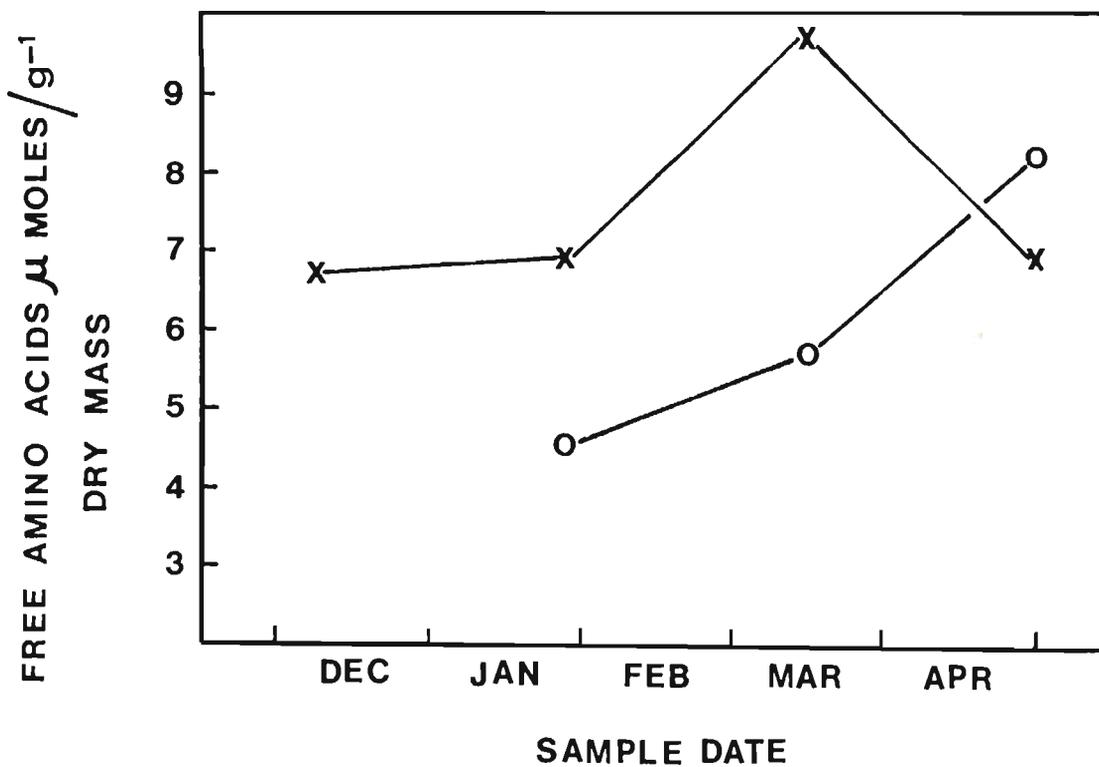


Fig.1.6



during seed development and the subsequent decrease with maturation concurs with the data of INGLE, BEITZ and HAGEMAN (1965) for the embryo of *Zea mays*. The free sugar levels in the endosperm however, were low throughout the developmental period. In contrast to this, DUFFUS and ROSIE (1975) report that the levels of reducing sugars in the developing embryo of *Hordeum distichum* L. were always low in comparison to the total carbohydrate content. In *Brassica napus* the levels of soluble carbohydrates increased to a peak about half way through the seed developmental period and then decreased to a low level at maturity (CHING, CRANE and STAMP, 1974). In developing Pigeonpea seeds (*Cajanus cajan* L.) SINGH, JAMBUNATHAN and NARAYANAN (1980) found that the soluble sugars increased up to 35 days after flowering and then decreased slightly until maturity at 42 days. They observed rapid starch accumulation between 14 and 28 days after flowering when soluble sugar levels were high. KONNO (1979) examined the free sugars in developing *Glycine max* seeds, and found that the very young seeds were rich in fructose and glucose. Small quantities of sucrose and stachyose were also present. At the time the cotyledons were developing the level of sucrose increased to comprise 90 per cent of the total sugars, and it remained the most important sugar up to and including maturity.

The developing seeds of *Podocarpus henkelii* conform in overall trends to the observations made in the abovementioned monocotyledonous and dicotyledonous seeds. The main difference appears to be that ribose is present in high levels compared to the other sugars in the embryo sporophyte. The significance of this could be associated with ribose acting

as a storage sugar in the pool of free sugars.

The free amino acids contained in the female gametophyte and embryo sporophyte of *Podocarpus henkelii* are shown in Table 1.7. The important amino acids of the female gametophyte are serine, lysine, histidine, glutamic acid, arginine and alanine. The levels of these amino acids change considerably throughout the developmental period. The most noticeable changes occur between the second (28th January) and third (15th March) samplings, with increases in the relative amounts of alanine, glutamic acid and a decrease of lysine. In developing *Triticum vulgare* kernels the free amino acids, aspartic acid and arginine increase in relative amount with maturation whilst lysine and proline decline. Most of the other amino acids including glutamic acid show relatively little change (JENNINGS and MORTON, 1963). These authors suggested that the change in amino acid composition of the wheat endosperm during development was a physiological process related to the nutrition of the embryo. A similar process may function in the developing seeds of *Podocarpus henkelii*. The levels of the total amino acids are shown in Fig. 1.6. These increase from their initial levels to a peak 18 weeks (15th March) after fertilization and then decrease as maturity approaches. These changes are concomitant with the changes in relative amounts of protein in the developing seeds (Fig. 1.7). The loss of storage protein (Plate 1.1 a) 12 weeks after fertilization is undoubtedly due to the catabolism of these reserves to release free amino acids into the amino acid pool. In the later stages of development the levels of the free amino acids drop at the same time as the quantities of protein in-

Table 1.7. Free amino acids detected in the female gametophyte and embryo sporophyte of developing *Podocarpus henkelii* seeds. The results are expressed in μ moles per gramme dry mass.

Amino Acid	FEMALE GAMETOPHYTE				EMBRYO SPOROPHYTE		
	DATE		OF		SAMPLE		
	December 12	January 28	March 15	April 30	January 28	March 15	April 30
Alanine	0,430	0,430	1,218	0,362	0,199	0,624	1,030
Arginine	1,102	1,186	1,186	1,178	1,180	1,190	1,200
Aspartic Acid	0,656	0,150	0,237	0,230	0,012	0,375	0,265
Cysteine	tr.	0,124	0,218	0,087	-	-	-
Glutamic Acid	0,637	0,812	1,190	1,125	0,531	0,499	0,500
Glycine	0,093	0,068	tr.	0,069	0,069	0,124	0,380
Histidine	1,116	1,148	1,181	1,170	0,437	0,780	0,780
Iso-Leucine	0,091	0,168	0,281	0,118	0,031	0,068	0,187
Leucine	0,056	0,091	0,262	0,106	0,037	0,043	0,362
Lysine	1,125	1,125	0,874	0,878	0,043	0,091	0,150
Methionine	tr.	0,031	0,081	0,024	tr.	-	-
Phenylalanine	tr.	tr.	0,099	0,037	tr.	tr.	0,181
Proline	tr.	tr.	0,081	0,062	0,443	0,324	1,280
Serine	1,012	1,150	1,220	1,101	1,281	1,270	1,230
Threonine	0,091	0,112	-	tr.	0,087	0,137	0,187
Tyrocine	tr.	tr.	0,018	0,018	tr.	tr.	0,156
Valine	tr.	0,062	0,031	0,037	0,049	0,056	0,218
Unknown 1	0,137	0,106	0,124	0,124	tr.	tr.	-
2	tr.	0,031	0,031	0,037	tr.	0,056	tr.
3	tr.	0,031	tr.	-	-	-	tr.
4	0,037	0,062	0,062	0,024	tr.	-	-
5	0,043	0,018	0,199	0,124	0,124	0,043	0,137
TOTAL	6,626	6,942	9,285	6,911	4,562	5,680	8,243

crease (Fig. 1.6 and Plate 1.6).

Similar observations of free amino acid levels decreasing with the increase in protein synthesis have been made in developing kernels of *Zea mays* (INGLE, BEITZ and HAGEMAN, 1965). This protein synthesis is a late occurrence. In developing *Cajanus cajan* seeds leucine, isoleucine, valine and glycine decreased, whereas phenylalanine increased with maturation (SINGH, JAMBUNATHAN and NARAYANAN, 1980). In these seeds the levels of free amino acids decreased 14 days after flowering, and at the same time the levels of protein nitrogen increased. In all cases it appears that the pool of free amino acids is utilized for the synthesis of storage protein. In the seeds of *Podocarpus henkelii* the same may apply. This seed is however, obviously still dependant upon a maternal source of nitrogen as the levels of protein increase in both the female gametophyte and embryo sporophyte at the same time (Fig. 1.7). Simultaneously, the levels of free amino acids increase (Fig. 1.6) in the embryo sporophyte throughout the period of development, even when protein is appearing in vacuoles (Plate 1.7 a). The relative amount of protein in both the female gametophyte and embryo sporophyte increased at the same time. Concurrently, the free amino acids increased in the embryo sporophyte. It can therefore be deduced that these nitrogenous compounds are continuously supplied by the maternal tissues. In developing *Oryza sativa* kernels CRUZ, CAGAMPANG and JULIANO (1970) reported that protein accumulated during the latter stage of maturation. In these kernels the amount of protein synthesized appeared to be related to the size of the amino acid pool, protein rich kernels thus having

a larger pool during the early stages of development.

There are clear differences in the free amino acids of the embryo sporophyte compared to those of the female gametophyte. In the former seed component, the important amino acids are serine, proline, glutamic acid, arginine and alanine. In the latter seed component serine, lysine, histidine, glutamic acid, aspartic acid, alanine and arginine are the important amino acids. These differences could be associated with the various amino acids necessary for the synthesis of enzymatic, structural and regulatory protein which will obviously differ between the two seed components (CHING, 1972). An interesting feature of the embryo sporophyte is the increase in the quantities of proline with maturation. This amino acid is present in very small amounts in the female gametophyte. It is thought that proline plays an important role in storage of nitrogen, as it has been shown to be the predominant component of storage protein in maize kernels (BARNARD and OAKS, 1970). Also of note are the very low levels or absence of the limiting amino acids cysteine and methionine at all stages of development in both the female gametophyte and embryo sporophyte. This could be due to one of two factors. In this seed the low levels could indicate a high rate of metabolism of these amino acids or it could simply indicate a different biochemical feature of the seed of *P. henkeli*.

In most plants nitrogen is transported to the developing seed in the phloem (PATE, 1980). The transport form does however vary between plants. In legumes the predominant nitrogen transport compound is asparagine and the storage form is

arginine (MIFLIN and LEA, 1977). In the mature seeds of *Malus sylvestris*, TROMP and OVAA (1973) have shown that asparagine is used for short distance transport. Arginine is utilized in long distance transport and as an overwintering storage compound in the form of polypeptide chains. In other plants such as Sugar beet (*Beta vulgaris* L.) (JOY and ANTCLIFF, 1966), and *Pisum arvense* (PATE, WALKER and WALLACE, 1965) asparagine is the predominant transport compound but glutamate and aspartate may also be utilized.

Studies with ^{15}N labelled asparagine and glutamine have shown that nitrogen of the amide grouping of these amides is utilized for the synthesis of a variety of amino acids of seed protein in *Pisum sativum*. Arginine, which is virtually absent from the phloem, comprises a large fraction of bound amino acid in seed proteins, and appeared to be the most successful recipient of this transfer of labelled nitrogen (LEWIS and BERRY, 1975; and LEWIS and PATE, 1973). In the female gametophyte of *Podocarpus henkelii* alanine, arginine, aspartic acid, glutamic acid and serine are the predominant amino acids. Which of these represents the transport form(s) could only be established by a careful study of the phloem sap. It is not clear from this study which amino acid is translocated from the female gametophyte to the embryo sporophyte. There is a decrease in the level of alanine in the female gametophyte between 18 (15th March) and 24 (30th April) weeks after fertilization. At the same time, the level of this compound increased in the embryo sporophyte. There are other interesting questions as to whether the amino acids are translocated *per se* within the seed or whether they are converted to amides

as appears to be the case in other seeds investigated (LEWIS and PATE, 1973).

The initial high levels of protein in the female gametophyte of *Podocarpus henkelii* appear to be an exceptional feature. In most other seeds studied such as *Pisum sativum*, the levels of protein are built up in the latter stages of the developmental period (MILLERD and SPENCER, 1974). Developing kernels of *Hordeum distichum* accumulate protein between 30 and 40 days post anthesis to a maximum of 0,5 milligrammes per embryo. This level does however decline to 0,3 milligrammes per embryo at maturity approximately 60 days after anthesis. In *Zea mays* kernels the levels of protein increase gradually from 15 days after pollination until maturity. The majority of protein in this seed is stored in the endosperm with low levels present in the embryo. The mature seeds of *Pinus taiwanensis* and *Cunninghamia lanceolata* display similar characteristics with low levels of storage protein in the embryo sporophytes and high levels in the female gametophytes (KAO, 1973). On a dry mass basis, mature seeds of *Pinus pinea* contain 35 per cent protein, most of which is located in the female gametophyte (BEWLEY and BLACK, 1978).

The deposition of protein reserves in the developing seeds of *Podocarpus henkelii* differs from the other seeds discussed. In the early developmental stages, relatively high levels of protein are present which are subsequently mobilized only to be reformed in the later stages of development. Substantial protein reserves are also present in the mature embryo sporophyte, (Fig. 1.7), a feature which is not apparent in other

Figure 1.7. The protein content of the embryo sporophyte O-O and female gametophyte X-X of developing seeds of *Podocarpus henkelii*. The results are expressed as milligrammes protein per gramme dry mass. The protein was extracted with alcoholic NaOH and quantified with the Biuret technique using B S A as a standard. Bars represent the standard errors.

Figure 1.8. The starch content of the embryo sporophyte O-O and female gametophyte X-X of developing seeds of *Podocarpus henkelii*. The results are expressed as milligrammes starch in glucose equivalents per gramme dry mass. The starch was converted to glucose with enzymes and quantified using the Anthrone technique. Bars represent the standard errors.

Fig. 1.7

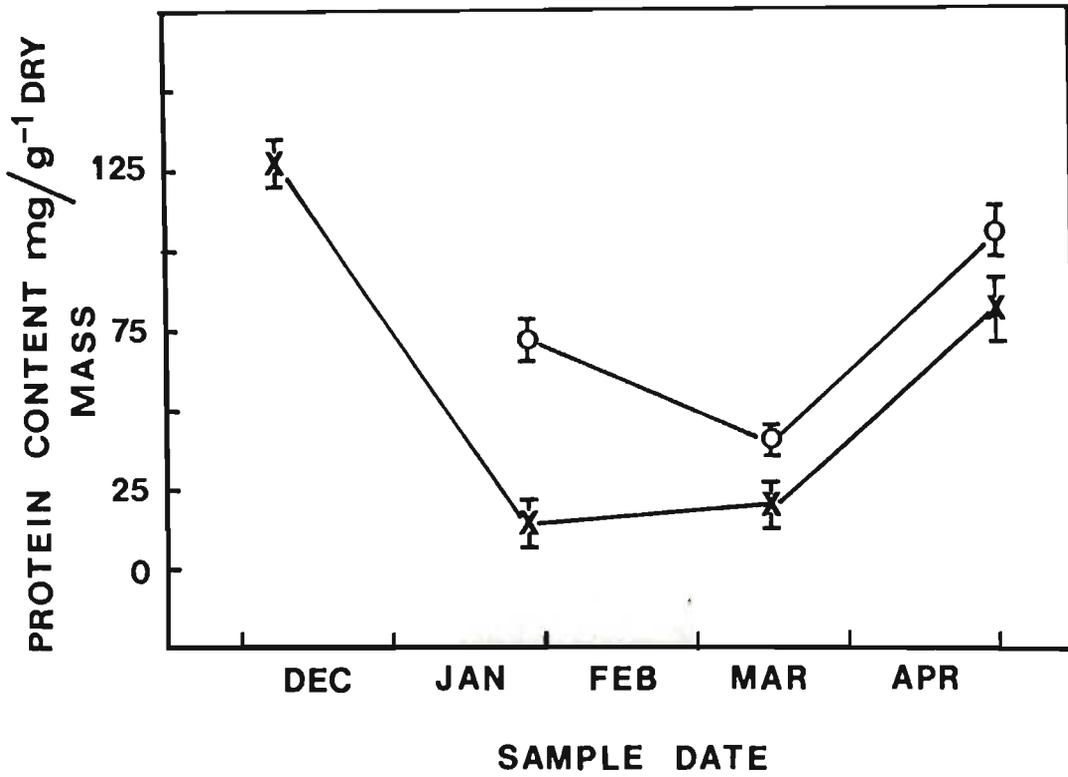
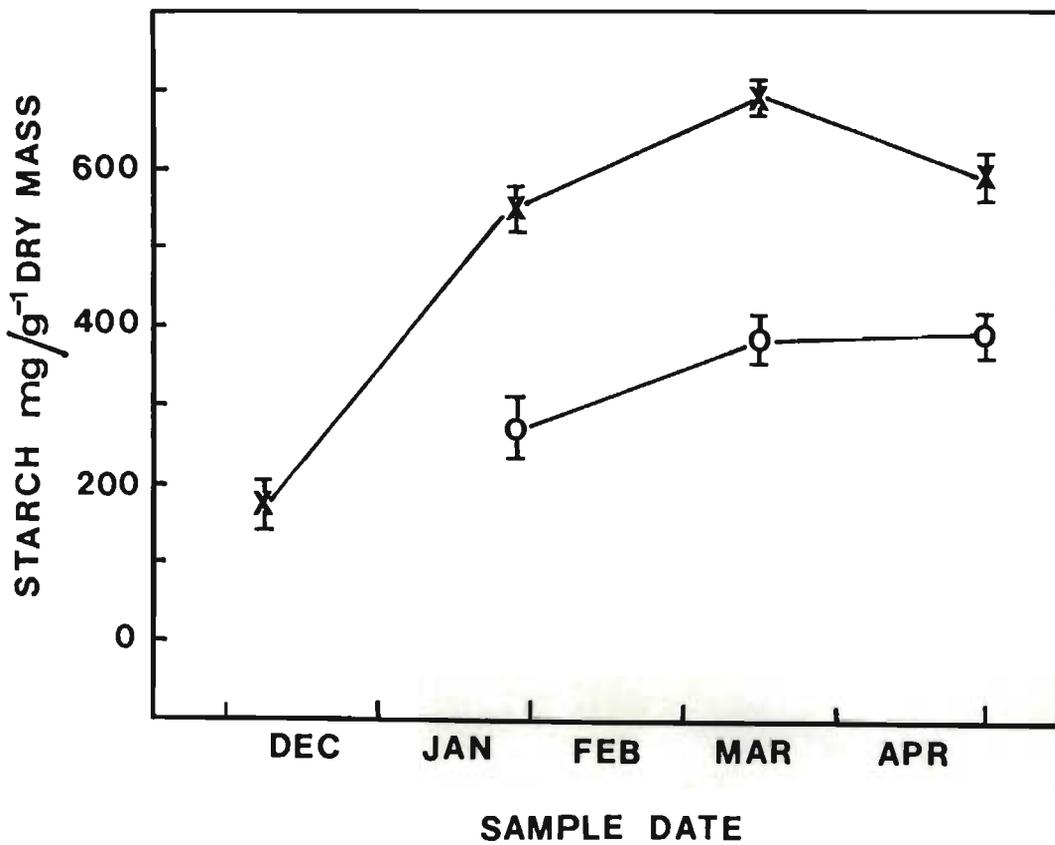


Fig. 1.8



gymnospermous seeds.

Starch is the most important food reserve present in the mature seeds of *Podocarpus henkelii* (Fig. 1.8). The build-up of this reserve in both the embryo sporophyte and female gametophyte occurs gradually throughout the developmental cycle. The levels of free sugars in both the embryo sporophyte and female gametophyte decreased at the same time (Table 1.6). These features are similar to the reports of starch deposition in other seeds such as *Pisum sativum* (MILLERD and SPENCER, 1974). In developing *Triticum aestivum* kernels the endosperm dry mass increases rapidly from about day 12 to day 35 after flowering. The reducing sugars, sucrose and starch make a significant contribution to the dry mass of the endosperm at this time. Upon the initiation of rapid starch synthesis on day 20 after flowering, the pool of sucrose, pentosans and reducing sugars is decreased but remains at a relatively constant level necessary for starch synthesis. The synthesis of starch in *Podocarpus henkelii* appears to follow the same pattern (Figs. 1.5 and 1.8). There is a slight decrease in the level of starch in the mature female gametophyte. This reserve could be used in a transitory manner, with hydrolyzing enzymes producing sucrose for metabolism or translocation to the embryo sporophyte. ADAMS, RINNE and FJERSTAD (1980) report that the initial high levels of starch in *Glycine max* cotyledons decrease with maturity. This is due to the starch being assimilated as transient reserve material, which is utilized later in development.

The development of the seeds of *Podocarpus henkelii* is a long process lasting ca. 40 weeks from when the female cones first appear in August until the mature seed falls from the tree in May. Fertilization takes place ca. ten weeks after the cones form. The first cellular detail in the female gametophyte only becomes apparent about six weeks later in early December. The female gametophyte develops slowly with few changes except the gradual accumulation of food reserves occurring in the following six weeks. An unusual feature is that the protein reserves which were present in the early stages of development are mobilized and are no longer apparent in late January. The embryo sporophyte which develops by rapid cell division from the pro-embryo cells, is microscopic at six weeks after fertilization. During the subsequent six weeks however, development is rapid. Externally, the cotyledons, radicle and hypocotyl are easily distinguished from each other. Fine structural examination reveals that there is little differentiation of the component tissues at this stage. During the subsequent 14 weeks to maturity the embryo sporophyte tissues differentiate and increase in size. At maturity the food reserves of the seed, expressed on a per cent dry mass basis, are as follows:

	Female Gametophyte	Embryo Sporophyte
Starch	59,0%	39,0%
Protein	8,0%	11,0%
Lipid	4,0%	10,0%
Free sugars	2,5%	12,5%

The mature seed of *Podocarpus henkelii* is extraordinary in that the seed as a whole has a very high moisture content of ca. 60 per cent. A further curious feature is the relatively advanced state of development of the embryo sporophyte which contains reserves of starch, protein and lipid.

CHAPTER TWO

CYTOKININS IN DEVELOPING *PODOCARPUS HENKELII* SEEDS

In the preceding chapter the development of the seeds of *Podocarpus henkelii* was detailed. The increase in fresh and dry mass of these seeds was accompanied by the laying down of food reserves in the female gametophyte and embryo sporophyte. The timing of these events appears to be very precisely controlled. There has been much speculation in the literature about the hormonal control of the development of seeds (BEWLEY and BLACK, 1978; DURE, 1975). Since their first discovery, cytokinins have been extracted from the developing fruits and seeds of many plants. In addition, the largest diversity of compounds displaying cytokinin-like properties has been extracted from seeds and fruits (DAVEY and VAN STADEN, 1977 and 1979; KOSHIMIZU, MATSUBARA, KUSAKI and MITSUI, 1976; LETHAM, 1963, 1973, 1974; VAN STADEN and DREWES, 1975).

The cytokinins have been implicated in the development of fruits and seeds for many reasons. Cytokinins appear to be involved in some systems in the process of nutrient mobilization (MOTHES, ENGELBRECHT and KULAJEWA, 1959; MOTHES, ENGELBRECHT and SCHÜTTE, 1961; MOTHES and ENGELBRECHT, 1961). It is also significant that plant growth substances including cytokinins have been linked with the nutrient status of developing fruits (LUCKWILL, 1977). All these considerations prompted a study of the cytokinin levels in the seed components of the developing seeds of *Podocarpus henkelii*. As the

seed size increased markedly during the period of study it was impossible to extract the same amount of material at each harvest. The cytokinins were thus extracted from varying amounts of seed material. The quantitative results were expressed in two ways. The convention is to express results in nanogramme equivalents of zeatin per gramme dry mass. (Table 2.1). The results have been presented as such, but as the embryo sporophyte is so relatively small at the early stages of development, it appears that the cytokinin levels are very high in this seed component. This is especially so when compared to the epimatium and female gametophyte. The data are therefore also presented as nanogramme zeatin equivalents per dry seed component (Table 2.2). In this case the results are more uniform. However, relative to the dry mass of each seed component, there are still large differences in the quantities of cytokinins present. The qualitative changes in the cytokinins were determined in two ways. The Rf zones displaying cytokinin activity in the soybean assays were compared with the Rf zones of authentic cytokinins on paper. To try and elucidate the cytokinins further, a column chromatographic system was employed. The cytokinins were first separated by paper chromatography. The paper chromatograms were then separated into slow- and fast-moving zones (0,1-0,5 and 0,6-0,9 Rf's respectively). The elution volumes displaying cell division properties were then compared with the elution volumes of authentic cytokinins.

Analyses of the epimatium and female gametophyte seed components in early December revealed that there were relatively low levels of cytokinins in the epimatium (6,23 nanogrammes

Table 2.1. The cytokinin activity detected in the three seed components of developing *Podocarpus henkelii* seeds. The callus yield, calculated from the bioassays of paper chromatograms is expressed as nanogramme zeatin equivalents per gramme dry mass of seed material.

Seed Component	Date of Sample			
	December 12	January 28	March 15	April 30
Epimatium	13,86	3,67	8,78	1,76
Female Gametophyte	91,06	155,12	9,92	5,40
Embryo Sporophyte	-	2 627,23	104,90	50,73
Whole Seed	104,92	2 786,02	123,60	57,89

Table 2.2. The cytokinin activity detected in the three seed components of developing *Podocarpus henkelii* seeds. The callus yield, calculated from the bioassays of paper chromatograms is expressed as nanogramme zeatin equivalents per individual dry seed organelle. (See text for explanation).

Seed Component	Date of Sample			
	December 12	January 28	March 15	April 30
Epimatium	6,23	3,81	10,53	2,85
Female Gametophyte	1,63	21,71	5,55	5,88
Embryo Sporophyte	-	1,95	1,04	0,76
Whole Seed	7,86	26,57	17,12	9,49

per dry epimatium, Fig. 2.1). The paper chromatograms in Fig. 2.2 show that the cytokinins present were of two main types, a slow- and a fast-moving form respectively. The slow-moving form co-chromatographed with glucosylzeatin and the fast-moving form co-chromatographed with ribosylzeatin and zeatin. There was more of the fast-moving cytokinin present at this time. This is probably linked to the fact that this tissue is developing rapidly at this time as is reflected by marked increases in dry mass at the next sampling in late January (Table 1.1).

At the same time, the female gametophyte contained relatively low levels of cytokinins (Fig. 2.3) all of which were fast-moving and co-chromatographed with ribosylzeatin and zeatin. BANERJEE (1968) reports that in the developing seeds of *Ginkgo biloba* L. the levels of cytokinin-like substances were at a maximum when the gametophytic cells were dividing. The micrographs of the female gametophytic cells show that at this stage cell division has ceased and cell expansion is taking place (Plate 1.1). This could explain the relatively low levels of cytokinin at this time when estimated on a per seed component dry mass basis. An interesting feature of the data of BANERJEE (1968) was that the cytokinin levels fluctuated in the female gametophyte throughout the period of development. When considered on a per gramme dry mass basis, the levels of cytokinins were quite high in this seed component. THOMAS, KUGRENS and ROSS (1980) report that in the cotyledons of radish (*Raphanus sativus* L.) applied zeatin enhanced the breakdown of protein bodies. The levels of protein decreased in the female gametophyte between the December and

Figure 2.1. Changes in the cytokinin content of the embryo sporophyte O-O, female gametophyte X-X, and epimatium ●-● of developing *Podocarpus henkelii* seeds. The values indicating cytokinin activity were derived by expressing the callus yield from bioassays in Figs. 2.2, 2.3 and 2.4 as nanogramme zeatin equivalents (ZE) per gramme dry mass. Standards were included at the time of each assay for each separate bioassay. The values of ZE represent the means of two paper bioassays.

Fig. 2.1

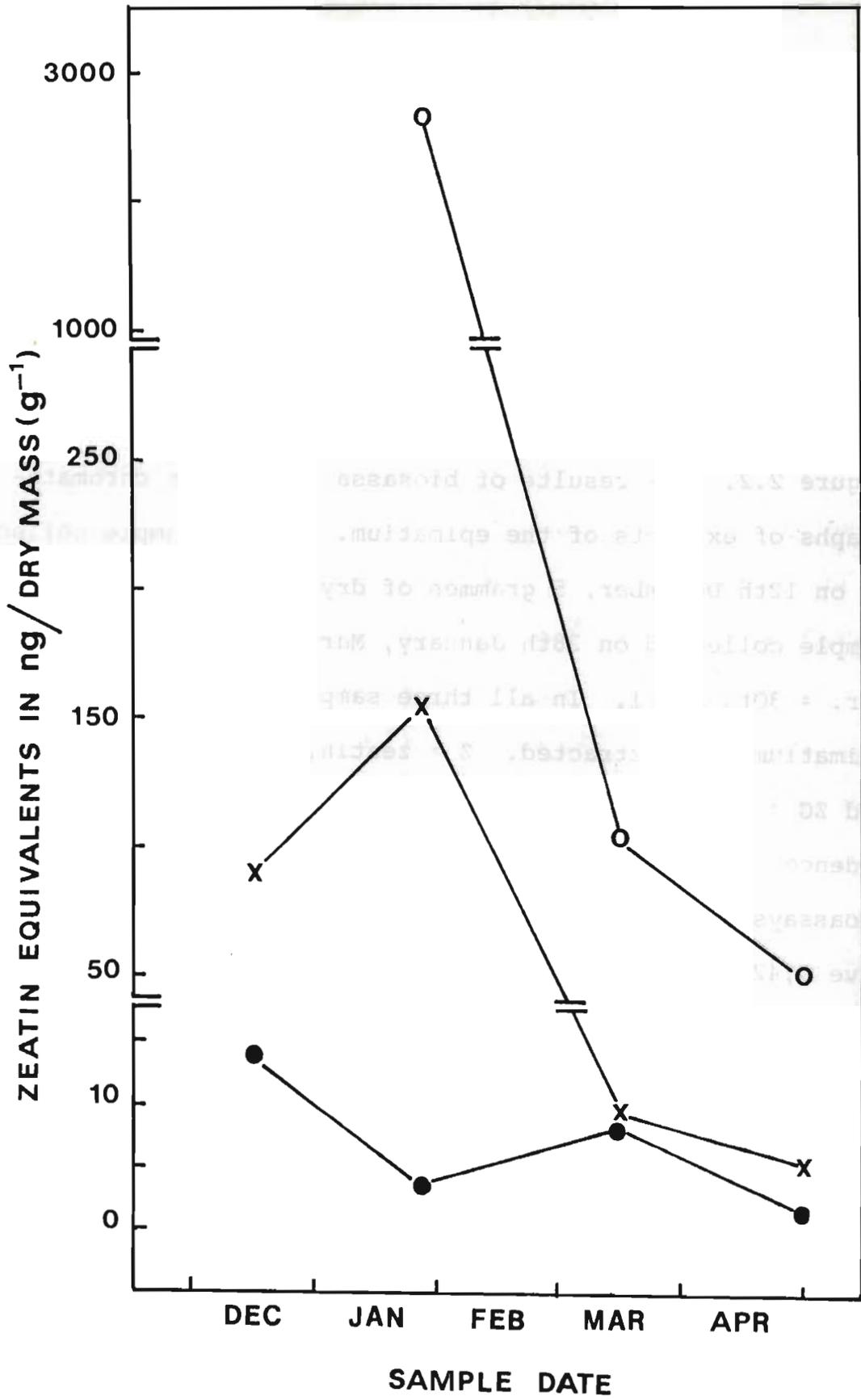
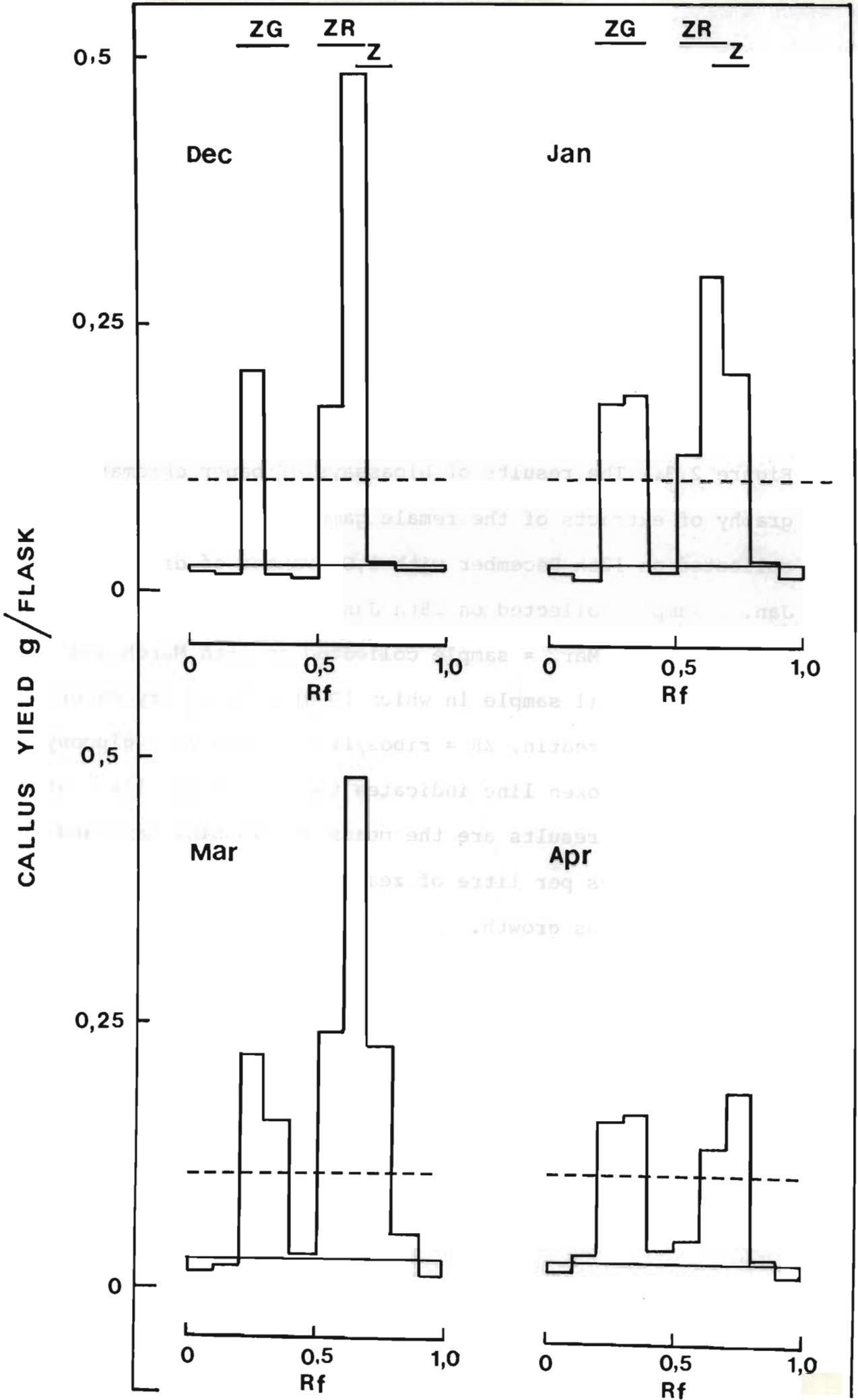


Figure 2.2. The results of biosassays of paper chromatographs of extracts of the epimatium. Dec. = sample collected on 12th December, 5 grammes of dry material. Jan. = sample collected on 28th January, Mar. = 15th March and Apr. = 30th April. In all three samples 10 grammes of dried epimatium were extracted. Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin. The broken line indicates the confidence limit at $P = 0,01$. The results are the means of two bioassays and 1,0 microgrammes per litre of zeatin standard gave 0,42 grammes of callus growth.



Fig. 2.2



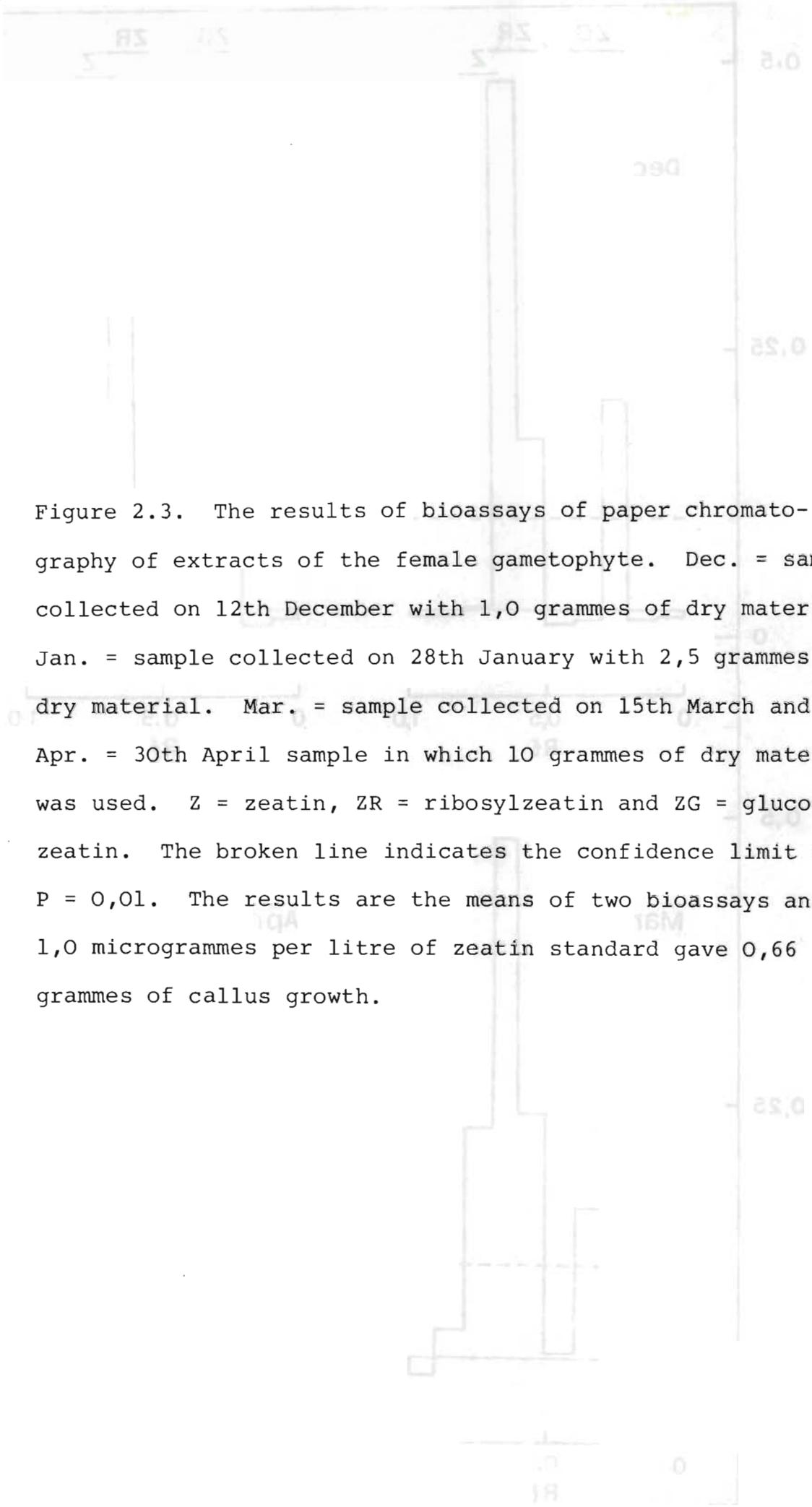
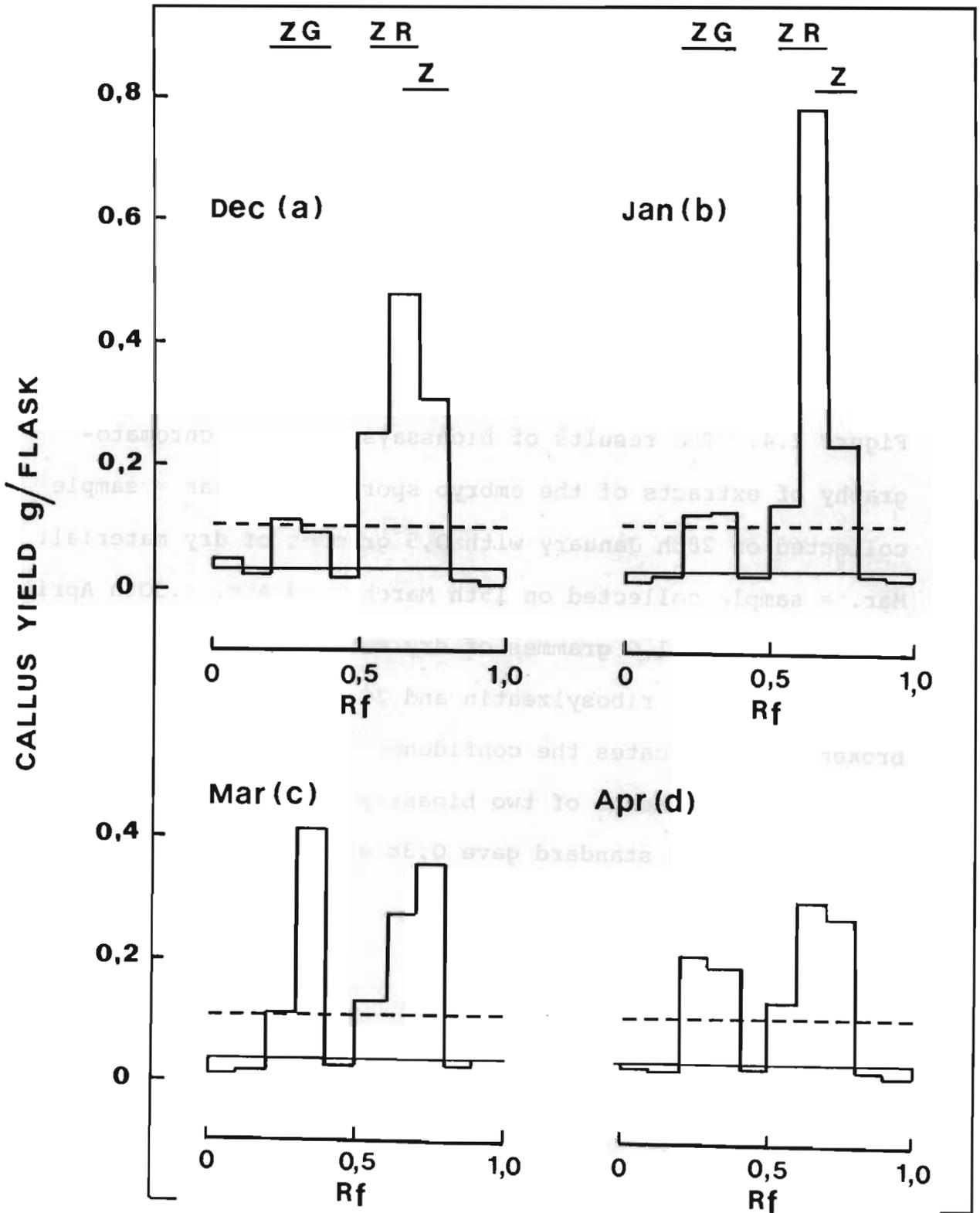


Figure 2.3. The results of bioassays of paper chromatography of extracts of the female gametophyte. Dec. = sample collected on 12th December with 1,0 grammes of dry material. Jan. = sample collected on 28th January with 2,5 grammes of dry material. Mar. = sample collected on 15th March and Apr. = 30th April sample in which 10 grammes of dry material was used. Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin. The broken line indicates the confidence limit at $P = 0,01$. The results are the means of two bioassays and 1,0 microgrammes per litre of zeatin standard gave 0,66 grammes of callus growth.

Fig.2.3



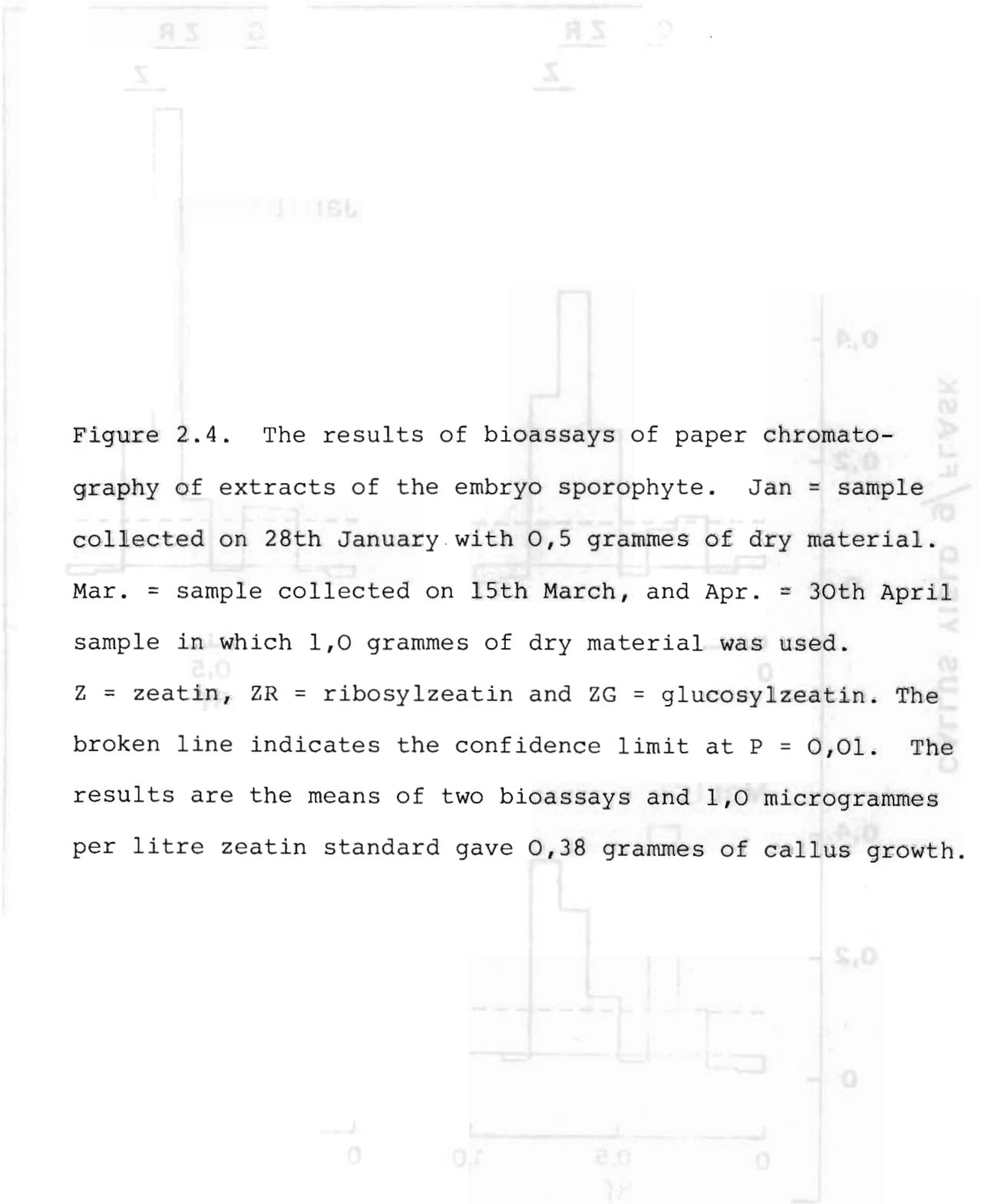
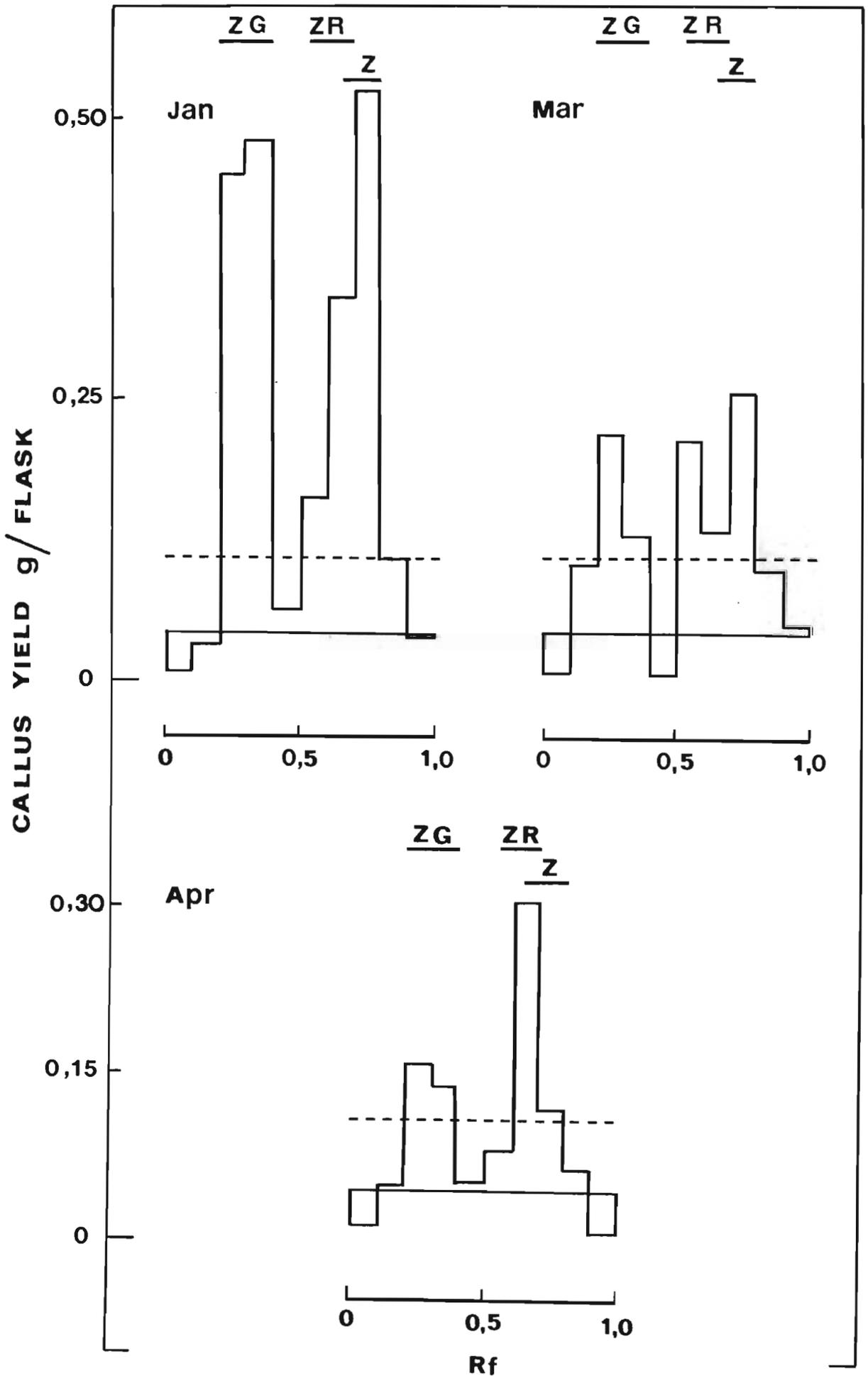


Fig.2.4

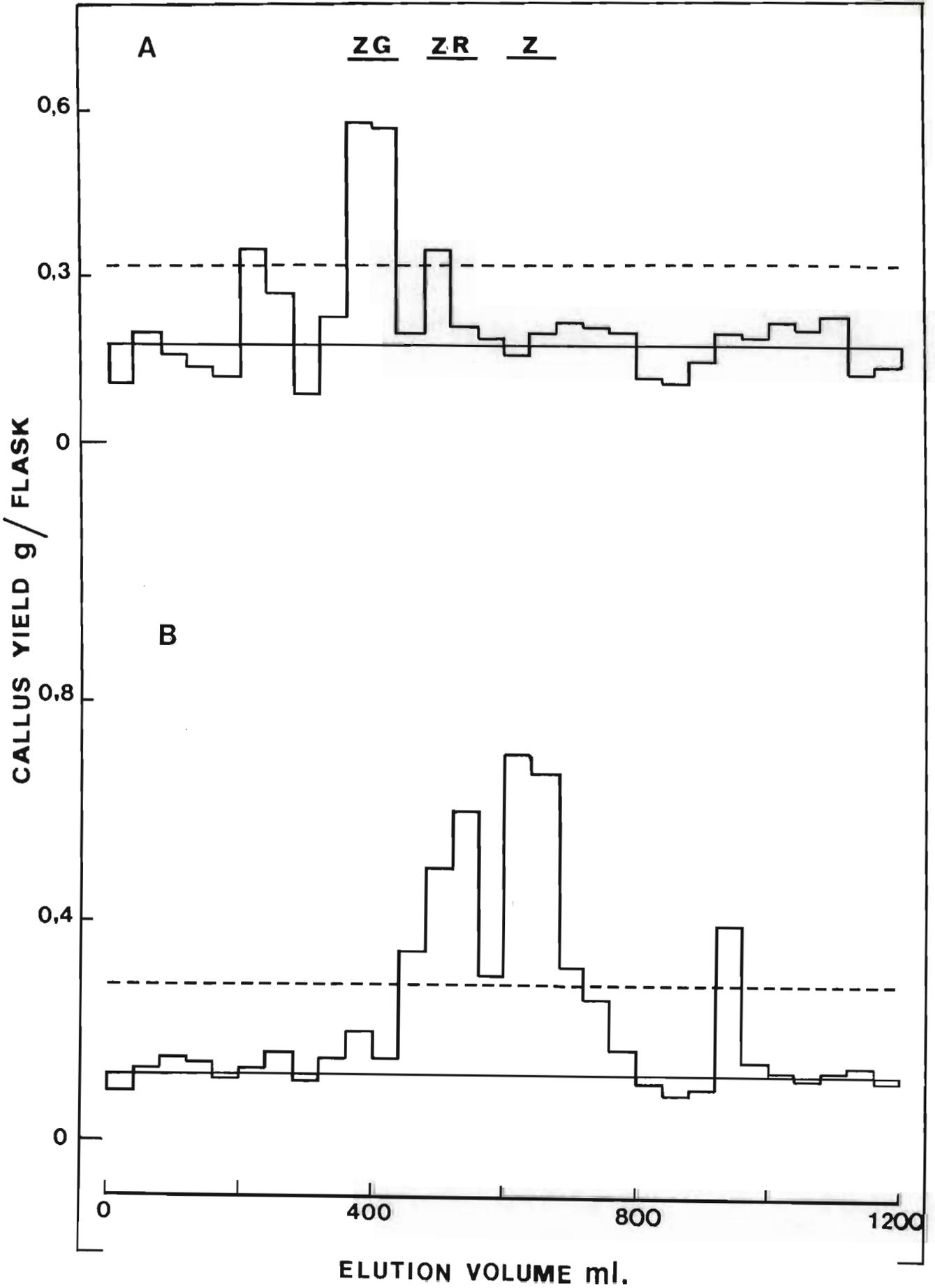


January samplings. The cytokinins present could thus be involved in this process. Another cellular event starting at this time was the deposition of starch in the plastids.

In the second sample collected in late January the levels of cytokinins in the epimatium were low (Table 2.1). There were also slight changes in the types of cytokinin present with an increase in the amount of cytokinin co-chromatographing with glucosylzeatin. The decrease in cytokinins could be associated with the slowing down in growth of this seed component (Table 1.1). At this time there were increases in cytokinins in both the female gametophyte and embryo sporophyte. The epimatium does constitute over 88 per cent of the dry mass of the seed at this stage, and could supply cytokinins to the remainder of the seed. Consequently the cytokinin level in this seed component would decrease. Experiments with ^{14}C labelled zeatin in Chapter five show that this compound is readily translocatable between the seed components in the germinating *P. henkelii* seed. At this time the female gametophyte showed an increase in cytokinin content. On paper the cytokinins present co-chromatographed with glucosylzeatin (slow-moving fraction) and ribosylzeatin and zeatin (fast-moving fraction). With column chromatographic fractionation four peaks of cytokinin activity were evident (Fig. 2.5). In the A fraction (slow-moving on paper Rf 0,1-0,5) a peak of activity co-eluting with glucosylzeatin (360-440 millilitres) was recorded. In the B fraction (fast-moving on paper Rf 0,6-0,9) three peaks of activity were noted. One peak co-eluted with ribosylzeatin (480-560 millilitres), the second co-eluted with zeatin (600-680 millilitres) and the third

Figure 2.5. Cytokinin activity in 2,5 grammes of dry female gametophyte material collected on the 28th of January. The cytokinins were eluted from Rf's 0,1-0,5 (fraction A) and Rf's 0,6-0,9 (fraction B) of paper chromatograms and then fractionated separately on a Sephadex LH-20 column eluted with 35 per cent ethanol. Authentic Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin co-eluted in the volumes indicated by the bars. The broken line indicates the confidence limit at $P = 0,01$.

Fig.2.5



peak eluted between 920 and 960 millilitres. Cellular activity at this stage consisted mainly of expansion. This was accompanied by the accumulation of starch and soluble sugars (Figs. 1.5 and 1.8). These processes were accompanied by an increase in both fresh and dry mass (Figs. 1.1 and 1.2). The higher cytokinin levels could have resulted in the increased cellular activity causing a ten-fold increment in dry mass (Table 1.1) in the female gametophyte. Studies on developing *Pisum arvense* (BURROWS and CARR, 1970) and *Lupinus albus* (DAVEY and VAN STADEN, 1979) have indicated that high levels of cytokinin activity coincided with periods when there was a large proportion of endosperm present. High levels of cytokinin activity were also recorded in the endosperm of the seeds of *Persea americana* as long as that tissue persisted (GAZIT and BLUMENFELD, 1970). ATKINS, PATE and SHARKEY (1975) have suggested that in the developing seeds of *Lupinus albus* the endosperm is responsible for the conversion and storage of nutrients entering the seed. During this period (four to six weeks after anthesis) the pools of sugars and amino acids are being formed. DAVEY and VAN STADEN (1978b) have shown that high levels of cytokinins occur at this time in white lupin seeds. In his 'competing sinks' hypothesis LUCKWILL (1977) suggests that the high concentrations of hormones found in developing seeds are necessary in order to create a strong physiological sink capable of competing with the remainder of the plant for nutrients. LUCKWILL (1977) implicated cytokinins in this process. Evidence in support of this theory comes from the work of MOTHES and ENGELBRECHT (1961) who demonstrated that sugars and amino acids can be transported preferentially to regions of high

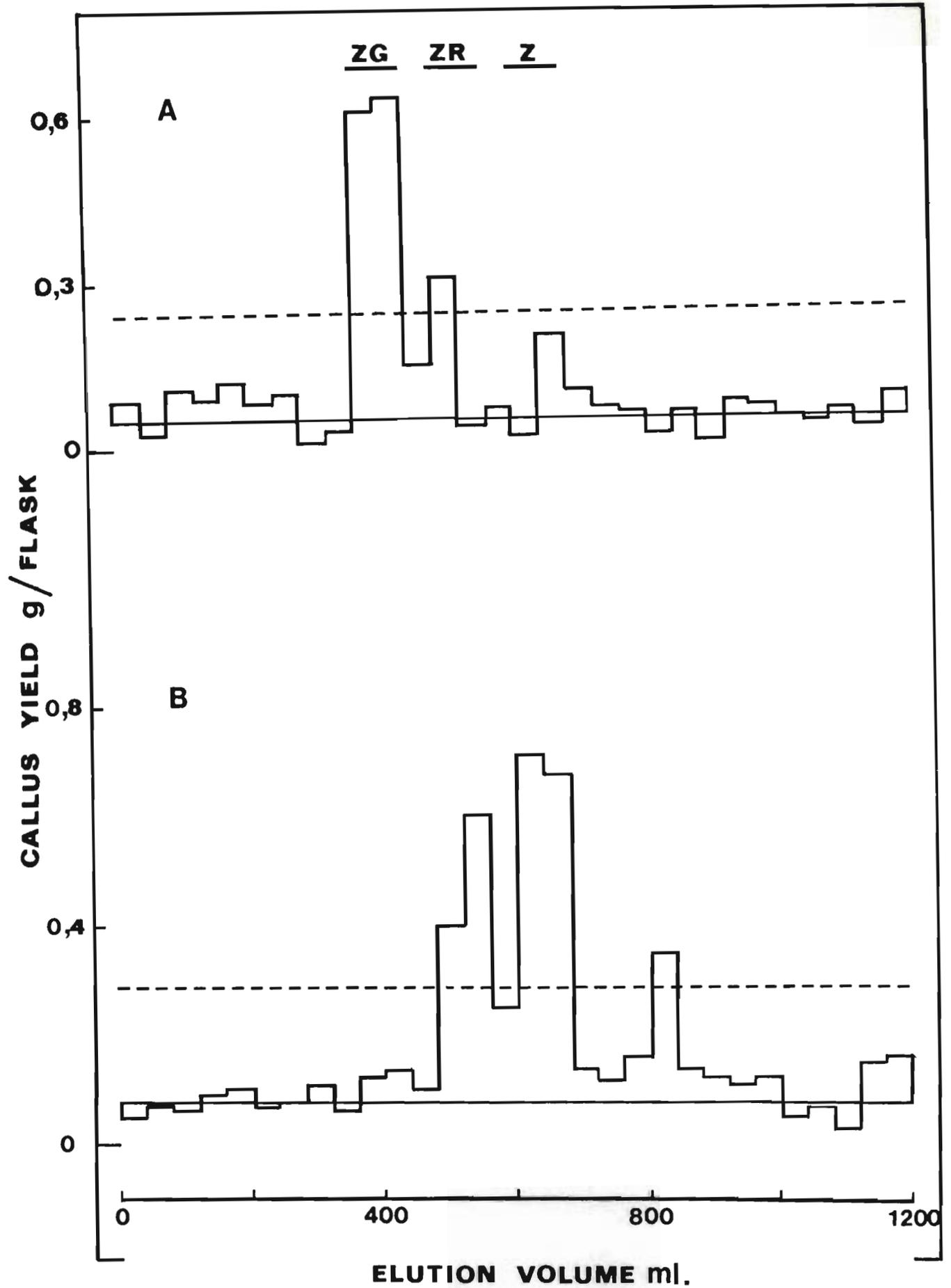
cytokinin levels. The increasing levels of nutrients occurring at the same time as high levels of cytokinins (which were detected in the female gametophyte of *P. henkelii* seeds) tend to support these assertions.

In late January the embryo sporophyte was very small with a fresh mass of only 0,002 grammes. When estimated on a per gramme dry mass basis, the quantities of cytokinin present were very high (Table 2.1). The estimate of zeatin equivalents in nanogrammes per seed component dry mass is more realistic, being 1,95 nanogrammes. This appears small but when considered in relation to a dry mass of 0,0004 grammes, the amount of cytokinin present is large compared with the other seed components. There were two peaks of cytokinin activity present with paper chromatography (Fig. 2.4). The slow-moving peak co-chromatographed with glucosylzeatin and the fast-moving peak co-chromatographed with ribosylzeatin and zeatin. The fast-moving peak is very broad and column chromatography reveals that this peak yielded cytokinin activity which co-eluted with zeatin and ribosylzeatin (Fig. 2.6 b). The slow-moving peak co-eluted with glucosylzeatin (Fig. 2.6 a).

Zeatin and ribosylzeatin are considered to be free base and nucleoside cytokinins respectively, and are probably the active forms of these hormones (ENGELBRECHT, 1972). The glucosylation of zeatin is thought to be a form of inactivation (HENSON and WAREING, 1976; VAN STADEN and PAPAPHILLIPOU, 1977) and thus rendering these cytokinins as storage forms (HEWETT and WAREING, 1973). Hence the cytokinins co-eluting with glucosylzeatin may be storage forms of cytokinin. This

Figure 2.6. Cytokinin activity detected in 0,5 grammes of dry embryo sporophyte material collected on the 28th of January. The cytokinins were eluted from Rf's 0,1-0,5 (fraction A) and Rf's 0,6-0,9 (fraction B) of paper chromatograms and then fractionated separately on a Sephadex LH-20 column eluted with 35 per cent ethanol. Authentic Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin co-eluted in the volumes indicated by the bars. The broken line indicates the confidence limit of $P = 0,01$.

Fig. 2.6



is of interest as there are such high levels of cytokinin present in the embryo sporophytes at this time. One way of limiting their action may be to store them. The very high levels of the active zeatin and ribosylzeatin could be accounted for by the fact that the embryo sporophyte is metabolically extremely active at this stage (Plate 1.2). Cell division in the root meristematic region is taking place during this stage, whilst in the other regions cells are expanding and differentiating. As cytokinins have been shown to induce both cell division (MILLER, 1965) and cell expansion (GORDON and LETHAM, 1975) it is probable that the cytokinins are utilized within this seed component to promote these activities. Studies with applied cytokinins have shown that they may be involved in the promotion of chloroplast division (BOASSON, BONNER and LAETSCH, 1972) and differentiation (HARVEY, LU and FLETCHER, 1974). Chloroplasts were differentiating within the cotyledons at this time. The highest levels of cytokinins during the development of *P. henkelii* seeds were recorded in the sample taken on the 28th of January. These data do appear to conform with that reported for other genera of seeds in which high levels of cytokinins were associated with high metabolic activity.

At the third sampling taken on the 15th of March, there was a decrease in the overall amount of cytokinin in the seed components (Table 2.1). In the epimatium there were once again two peaks of cytokinin activity. One peak co-chromatographed with glucosylzeatin while a fast-moving peak co-chromatographed with ribosylzeatin and zeatin (Fig. 2.2). The levels of cytokinin in zeatin equivalents increased

slightly. The reason for this is not clear as there is no great increase in the growth rate of this seed component at this time (Table 1.1). Concurrently there is a decrease in the cytokinin contents of both the female gametophyte and the embryo sporophyte. As subsequently mentioned ^{14}C labelled zeatin is readily translocatable between seed components in germinating *P. henkelii* seeds (Chap. 5). This increase in cytokinin in the epimatium could be a result of translocation from the embryo sporophyte and female gametophyte.

There was a marked qualitative change as well as a decrease in the cytokinins present in the female gametophyte. Paper chromatography revealed that there were both slow- and fast-moving peaks present. The former co-chromatographed with glucosylzeatin and the latter with ribosylzeatin and zeatin (Fig. 2.3). It is noticeable that as the levels of cytokinins decreased in this seed component so the amount of glucosylzeatin increased. This conforms to the aforementioned theory that this is a form of inactivating and storing of cytokinins. The growth rate of this seed component decreased at this time with the dry mass increasing from 0,14 to 0,56 grammes (Table 1.1). This was accompanied by a decrease in the pool of soluble sugars, and increases in the levels of starch and the pool of free amino acids (Figs. 1.5, 1.6, and 1.8).

In the embryo sporophyte the levels of cytokinins had decreased by mid-March. Paper chromatography (Fig. 2.4) showed that there were no qualitative changes in the cytokinins present. The two peaks showing cytokinin activity co-chromatographed with glucosylzeatin, and zeatin and ribosylzeatin.

Prior to this sample there had been a considerable amount of metabolic activity in the embryo sporophyte (Plate 1.5) with a 25-fold increase in dry mass from 0,0004 grammes to 0,01 grammes. The high level of cytokinins in this seed component thus precedes the rapid increase in growth as measured as a dry mass increase. In the female gametophyte however, the rapid (ten-fold) increase in dry mass occurred between the December and January samplings. This growth was accompanied by an increase in the levels of cytokinins (Figs. 2.3 a and b). These appear to be two conflicting observations but this is in fact not the case. The female gametophyte and embryo sporophyte differ greatly in both their structure and function (COERTZE, VAN DER SCHIJFF and SCHWEICKERDT, 1971). Consequently, the biochemical processes within each seed component would be expected to differ and this is reflected in the different levels of cytokinins at times of maximum growth. During this period there was a gradual increase in the levels of protein in both the embryo sporophyte and female gametophyte (Fig. 1.7). In the seeds of white lupin the levels of storage protein increase in the latter part of the developmental cycle. This increase occurs at the same time the cytokinin levels tend to decrease (DAVEY and VAN STADEN, 1978b). The data for the developing *P. henkelii* seeds conform to this finding.

The mature seeds of *Podocarpus henkelii* collected at the end of April contained low levels of cytokinin (Tables 2.1 and 2.2). These data conform to those obtained for other mature seeds. Mature *Pisum arvense* seeds contained low levels of cytokinin (BURROWS and CARR, 1970) as did the mature seeds of *Lupinus albus* (DAVEY and VAN STADEN, 1979). Pumpkin seeds

display similar characteristics with low levels of cytokinins in very young, and old seeds (GUPTA and MAHESHWARI, 1970).

The low levels of cytokinin activity were present in both slow- and fast-moving fractions of paper chromatograms of epimatum extracts (Fig. 2.2). The slow-moving peak co-chromatographed with glucosylzeatin and the fast-moving peak with ribosylzeatin and zeatin. Similar peaks of cytokinin-like activity were present on paper chromatograms of the embryo sporophyte and the female gametophyte extracts (Figs. 2.3 and 2.4). These chromatograms were divided into slow- (A) and fast-moving (B) fractions and then fractionated on a Sephadex LH-20 column. In both the embryo sporophyte and female gametophyte one peak of activity from fraction A was present. This co-eluted with glucosylzeatin (Fig. 2.7). In the B fractions of both embryo sporophyte and female gametophyte two peaks of cytokinin activity were present. One peak co-eluted with ribosylzeatin and the second with zeatin (Figs. 2.7 and 2.8). In the female gametophyte a third small peak of cytokinin activity was present which eluted at 880 to 960 millilitres. It is interesting to note that there was not a large increase in the amount of glucosylzeatin in any of the seed components at maturity. This could reflect a continued metabolic activity of the seeds after they fall from the trees. This is feasible as the mature seeds at fall have a very high moisture content (ca. 62 per cent). Data in Chapter three show clearly that the embryo sporophyte continues to increase in dry mass with storage. This growth could be associated with the continued presence of cytokinins.

Figure 2.7. Cytokinin activity detected in 1,0 grammes of dry embryo sporophyte material collected on the 30th of April. The cytokinins were eluted from Rf's 0,1-0,5 (fraction A) and Rf's 0,6-0,9 (fraction B) of paper chromatograms and then fractionated separately on a Sephadex LH-20 column eluted with 35 per cent ethanol. Authentic Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin co-eluted in the volumes indicated by the bars. The broken line indicates the confidence limit of $P = 0,01$.

Fig.2.7

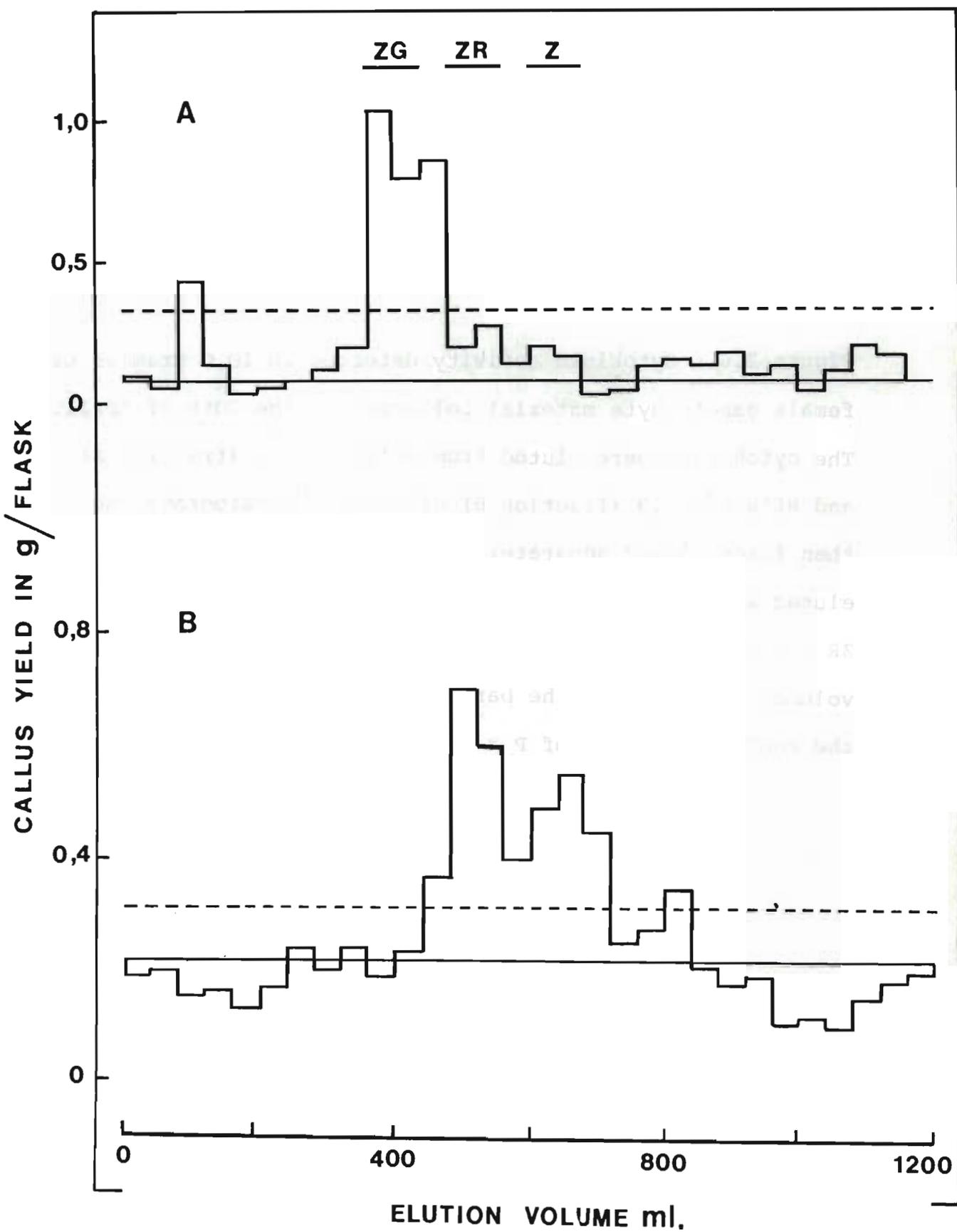
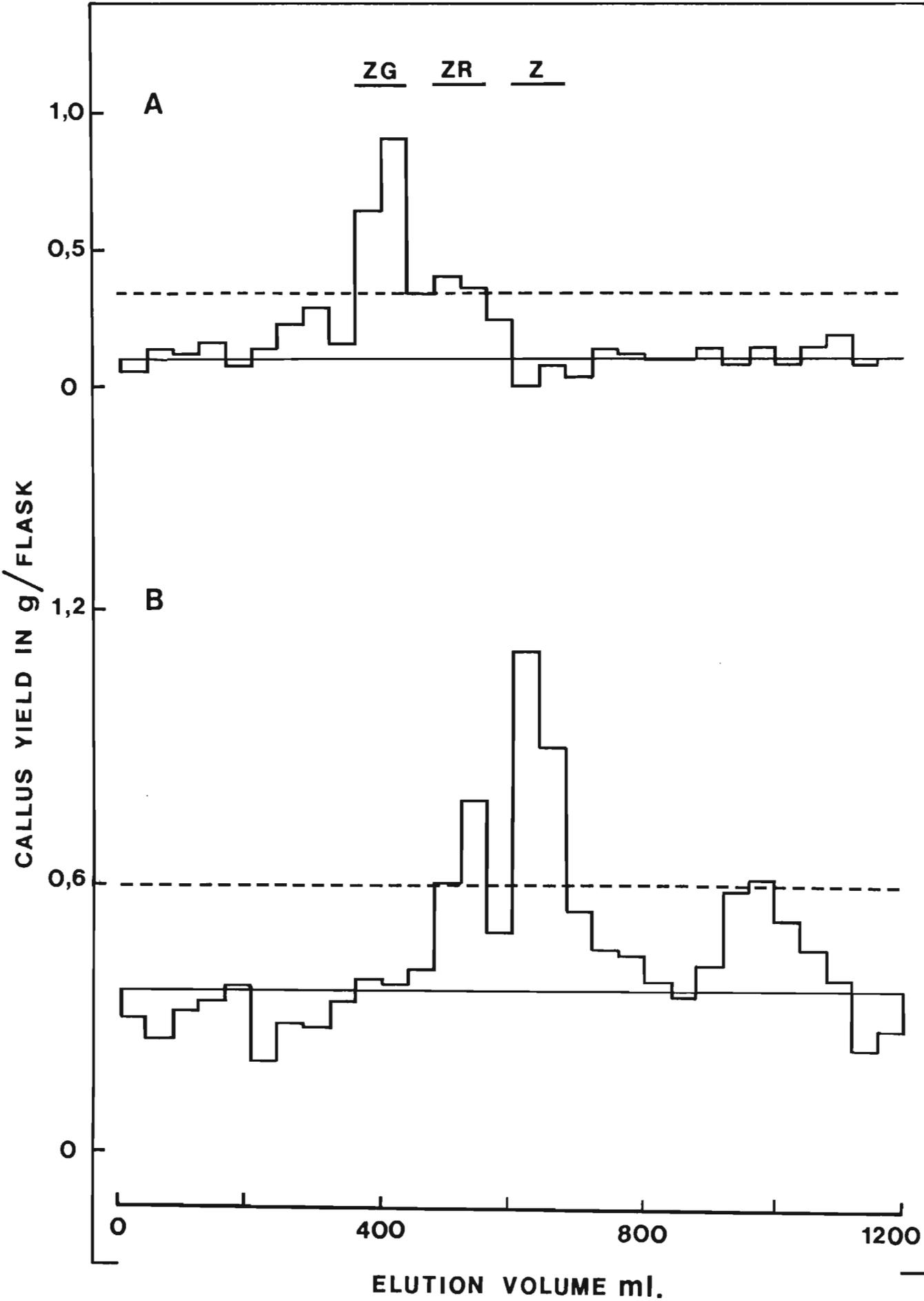


Fig. 2.8



The fluctuating qualitative and quantitative changes of cytokinin during development of *Podocarpus henkelii* seeds do not differ markedly from the data reported for similar studies on a few other seeds. The precise function of cytokinins in developing seeds is not clear. There have been suggestions that cytokinins may be involved in protein synthesis. Decreasing levels of cytokinins during protein accumulation may reflect the utilization of cytokinins in this process (DAVEY and VAN STADEN, 1978b). The data for *Podocarpus henkelii* seeds show that both high and low cytokinin levels are accompanied by metabolic and biochemical changes. Further research is necessary to reveal whether cytokinins are indeed utilized in the aforementioned processes.

Studies with plant hormones in isolation present some considerable difficulty, as it appears that the control of growth is achieved through a delicate balance of phytohormones (WARDLAW, 1965). The studies of EEUWENS and SCHWABE, (1975) on changes in phytohormones in relation to *Pisum sativum* seed development also implicate a fine balance between the levels of hormones. More recently, TREWAVAS (1979) has reviewed the role of hormones and suggests that they control the 'plastic' nature of growth and development. Cytokinins are thus thought to be closely associated with growth processes. To attempt an elucidation of the function of cytokinins in any particular process of growth or biochemical reaction requires much further precise experimentation at the molecular level. These data presented for cytokinins in *Podocarpus henkelii* seeds show that these compounds are closely associated with these developmental processes.

CHAPTER THREE

GERMINATION AND VIABILITY STUDIES IN

PODOCARPUS HENKELII SEEDS

One aspect of seed biology which is closely linked to the germination process is that of viability of seeds. Viability is the ability of seeds to remain alive for a period of time, and still be able to germinate once placed under suitable environmental conditions. The length of time for which the seeds can remain viable is variable and depends upon both the storage conditions and the type of seed (MAYER and POLJAKOFF-MAYBER, 1975). This period of viability is thus determined by both environmental and genetic factors.

A characteristic of many seeds is their drying out just prior to maturity (HARRINGTON, 1972; THOMAS, WEBB and WAREING, 1973). This period of dehydration appears to be associated with the maintenance of viability (BARTON, 1965; HARRINGTON, 1972; ROBERTS, 1972). In contrast to this, there are some seeds which lose viability rapidly once moisture is lost. The seeds of the River maple (*Acer saccharinum* L.) lose viability below moisture contents of 30 to 34 per cent (JONES, 1920).

The ability of seeds to germinate is also determined by their degree of maturity. According to EDWARDS (1980), there are two aspects to the maturity of seeds - the morphological and the physiological. Morphological maturity refers to the development of the embryo. In many tree seeds the embryo is not fully developed when the seed falls off the tree

(EDWARDS, 1980). This does not appear to be the case with the seed of *Podocarpus henkelii*. Ultrastructural studies have shown that the embryo sporophyte appears to be well developed in seed collected off the ground (Plate 1.7). Physiological maturity implies the completion of organic accumulation within the seeds as shown by no further increase in dry mass. This does not however preclude the continued development of the embryos during storage. Food reserves from the endosperm could be mobilized to provide metabolites for the embryo - this phenomenon being termed after-ripening. Other tree seeds such as those of the Noble fir (*Abies procera* Lindl.) require a period of after-ripening (EDWARDS, 1980). The sporadic germination of the seeds of *Podocarpus henkelii* (NOEL AND VAN STADEN, 1976), suggested that they may have an after-ripening requirement.

The mature seeds of *Podocarpus henkelii* have a very high moisture content. These seeds thus provided interesting material to investigate the relationship between high moisture content and the maintenance of viability. The seeds of *Podocarpus henkelii* do not undergo a period of desiccation (Table 1.1; Plates 1.6 and 1.7) with the seed tissues remaining highly hydrated at maturity. Estimated on a dry mass basis, the moisture content of the mature seeds was found to be ca. 62 per cent. The seeds fall off the trees during autumn and lie on the ground during the dry winter months. They are thus naturally subject to desiccation. As the trees produce copious quantities of seed with little natural regeneration occurring (VON BREITENBACH, 1965), there appears to be a problem with either the germination or viability of these seeds.

To determine whether or not these seeds could germinate, samples were incubated in moist vermiculite at 25°C. First germination was observed after 72 days and by 160 days a total of 68 per cent germination was recorded. This sporadic germination confirms the data of NOEL and VAN STADEN, 1976. These conditions of constant temperature and readily available moisture were not to be found under natural conditions during the winter months. When scarified and incubated under the same conditions all the seed germinated within 22 days (Fig. 3.1). Healthy seedlings were established from all the seeds which had germinated in these experiments. From these results it would appear as if the seeds did not require a period of after-ripening and that poor germination was due to the restricting influence of the epimatium. The controlling effect of this seed component in regulating seed germination was subsequently investigated in more detail.

Mature seeds which had a high moisture content of 62 per cent imbibed very little water during imbibition at 25°C (Figs. 3.2 and 3.3). This suggested that seeds with such a high moisture content did not require much additional water for germination. Support for this view was obtained by scarifying and incubating mature seeds without any water in closed polythene bags. Within 18 days 80 per cent of these seeds had germinated. This indicated that upon shedding the seeds had sufficient water for germination but that the epimatium restricted germination either mechanically or by limiting gaseous exchange. Under natural conditions however, the seeds are shed during the dry season and are immediately subjected to desiccation. Seeds desiccated to a moisture content of 53

per cent failed to germinate without the addition of water. Both in the field and during storage in the laboratory the moisture contents of *P. henkelii* seeds and their component tissues dropped rapidly (Table 3.1). In all cases the female gametophyte lost the highest percentage of moisture, followed by the embryo sporophyte and the epimatium. These results show that all seed components lose water but that the embryo sporophyte always has the highest moisture content. This is perhaps indicative of a buffer effect that the epimatium and female gametophyte provide against desiccation of the embryo sporophyte. An indication of the rapidity of the desiccation experienced under natural conditions is shown in Table 3.1. Seed collected from beneath trees had dropped in moisture content from 62 per cent to 49 per cent in 30 days.

An investigation of the water uptake and germination of desiccated seeds revealed that with desiccation, water uptake, and the rate of germination increased when compared with the mature seeds (Figs. 3.1, 3.2 and 3.3). From this it would seem that a degree of after-ripening occurred. During storage the dry mass of the embryo sporophyte increased from 0,015 (\pm 0,005) grammes at shedding to 0,026 (\pm 0,003) grammes after 16 weeks at 4°C. This slow increase in size could be necessary under natural conditions for the embryo sporophyte to rupture the epimatium for germination to ensue.

The rapid loss of water from the seeds under natural conditions posed the question as to what the lowest moisture content was at which they remained viable. This was investigated by desiccating the seeds for varying lengths of time and

Figure 3.1. Germination of *Podocarpus henkelii* seeds. Control of mature seeds upon shedding with a 62 per cent moisture content ●-●; seeds stored for six weeks at 4°C with a 53 per cent moisture content ○---○; seeds stored for sixteen weeks at 4°C with a 49 per cent moisture content ■—■. Four replicates of 25 seeds each were used in each experiment and the bar represents the maximum 95 per cent confidence limit.

Figure 3.2. Water uptake of scarified seeds of *Podocarpus henkelii* incubated at 25°C. Control of mature seeds upon shedding with a 62 per cent moisture content ●-●; seeds stored for six weeks at 4°C with a 53 per cent moisture content ○---○; seeds stored for sixteen weeks at 4°C with a 49 per cent moisture content ■—■. Four replicates of 25 seeds each were used in each experiment and the bar represents the maximum 95 per cent confidence limit.

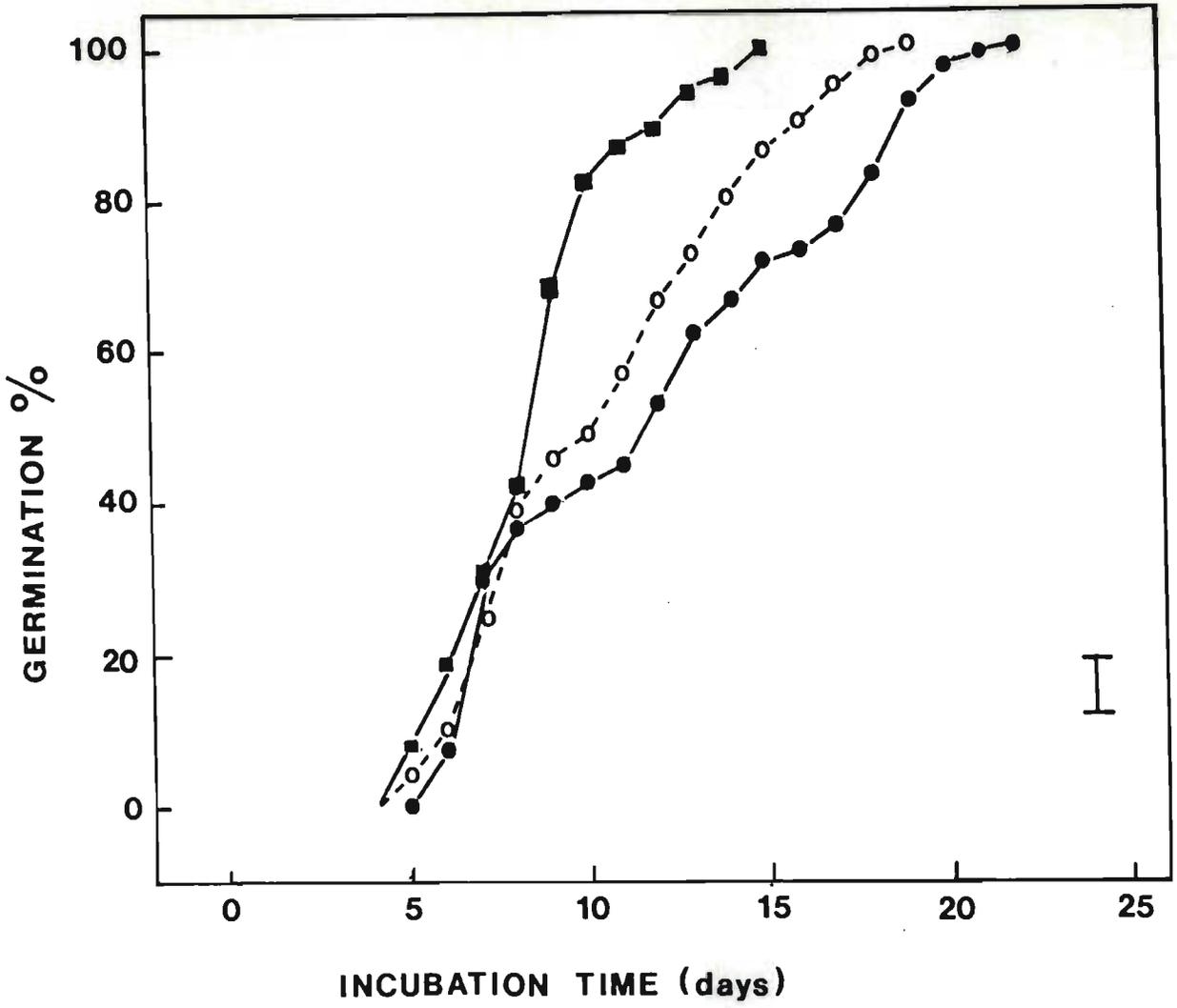


Fig.3.2

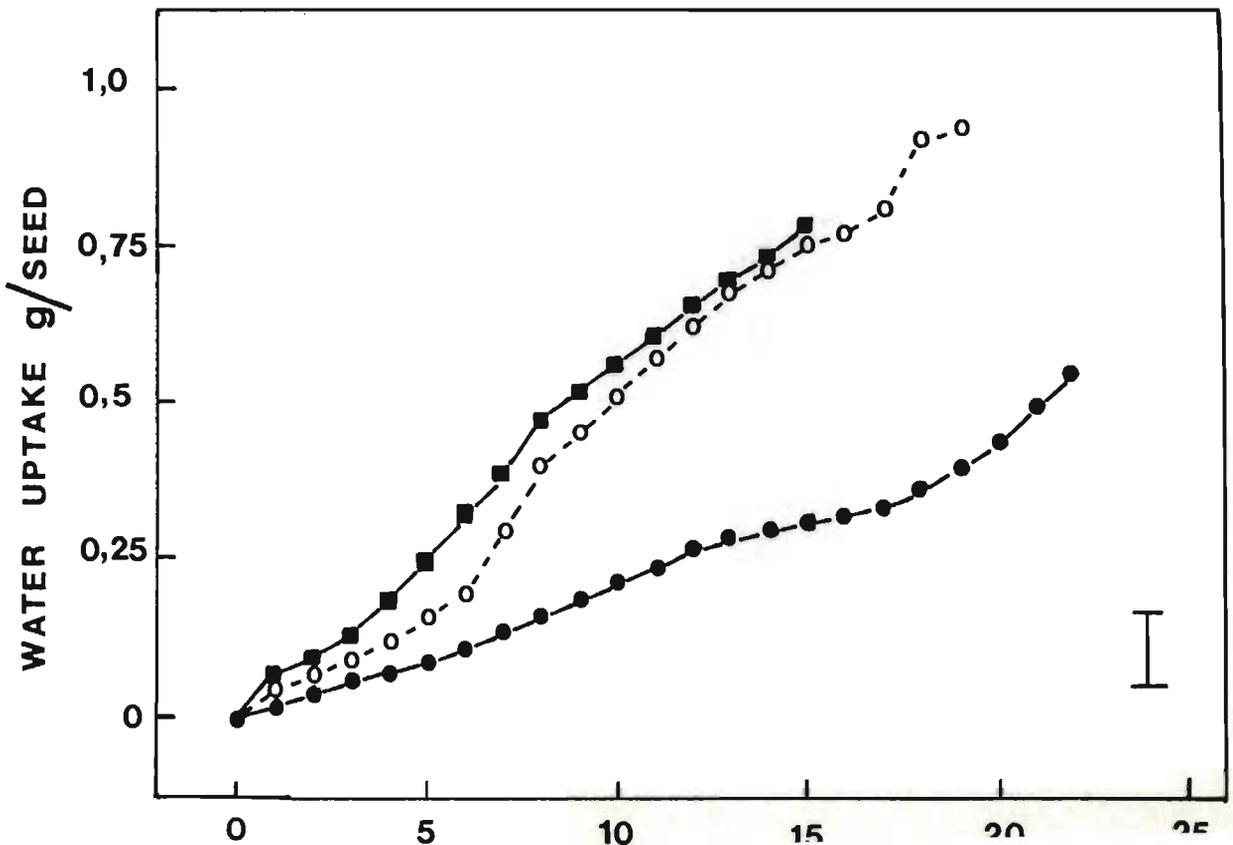


Table 3.1. Changes in moisture content (%) of *Podocarpus henkelii* seeds under different storage conditions. Figures in parentheses represent the standard error.

Plant material	Seed age (days) and storage condition				
	0	30	30	86	86
	Mature seed upon shedding	$\pm 4^{\circ}\text{C}$ polythene bag	Collected from the field	$\pm 4^{\circ}\text{C}$ polythene bag	$\pm 22^{\circ}\text{C}$ polythene bag
Epimatium	59,4	55,3	49,8	54,7	49,6
% loss	-	7,0	16,0	7,9	16,5
Female gametophyte	62,8	48,6	39,7	42,1	40,9
% loss	-	22,6	36,7	32,9	34,7
Embryo sporophyte	75,0	62,1	52,8	58,7	53,4
% loss	-	17,1	29,4	21,6	28,7
Whole seed	62,0 \pm 1,9	52,8 \pm 2,6	46,3 \pm 3,8	48,2 \pm 3,3	45,9 \pm 4,
% loss	-	14,2	24,9	21,2	25,5

then testing their germinability. The results in Table 3.2 show that at a moisture content of 34 per cent germination decreased to 58 per cent. Below this moisture content no germination was recorded. These data concur with that of *Acer saccharinum*, the seeds of which have a moisture content of 58 per cent when shed from the tree (JONES, 1920). This moisture content decreases rapidly and the seeds lose viability at water contents below 30 to 34 per cent. Other hardwood forest trees' seeds such as *Quercus*, *Fagus*, *Aesculus* and *Castanea* display similar characteristics (HOLMES and BUSZEWICZ, 1958). These data suggest that the abovementioned seeds and those of *Podocarpus henkelii* have not developed the capacity to dehydrate during their maturation. This dehydration appears to be necessary to enhance longevity in the face of environmental hazards such as extreme temperatures and desiccation (ROBERTS, 1972).

The high moisture content of *P. henkelii* seeds suggested that during storage their metabolic activity would be high. The seeds are green and have been shown to have stomata on the surface of the epimatium (NOEL and VAN STADEN, 1976). It thus seemed logical to establish whether or not they were able to photosynthesize and thus supplement their food reserves. Chlorophyll extracts from the leaves and epimatium showed almost identical absorption spectra (Fig. 3.5). When separated into its constituent parts by thin layer chromatography (TLC) three main pigments were found in both the leaves and the epimatium (Table 3.3). The absorption spectra obtained correspond with those reported for carotene, chlorophyll a and chlorophyll b (ROBERTS and WHITEHOUSE, 1976). To determine

Figure 3.3. The percentage increase in fresh mass of scarified seeds of *Podocarpus henkelii* incubated at 25°C. Control of seeds with a 62 per cent moisture content ●-●; seeds with a 53 per cent moisture content ○---○, and seeds with a 49 per cent moisture content ■--■

Figure 3.4. The moisture uptake ●-● of scarified seeds of *Podocarpus henkelii* in relation to the increase in CO₂ evolution X-X with germination. Arrow denotes the time germination was first recorded.

Fig. 3.3

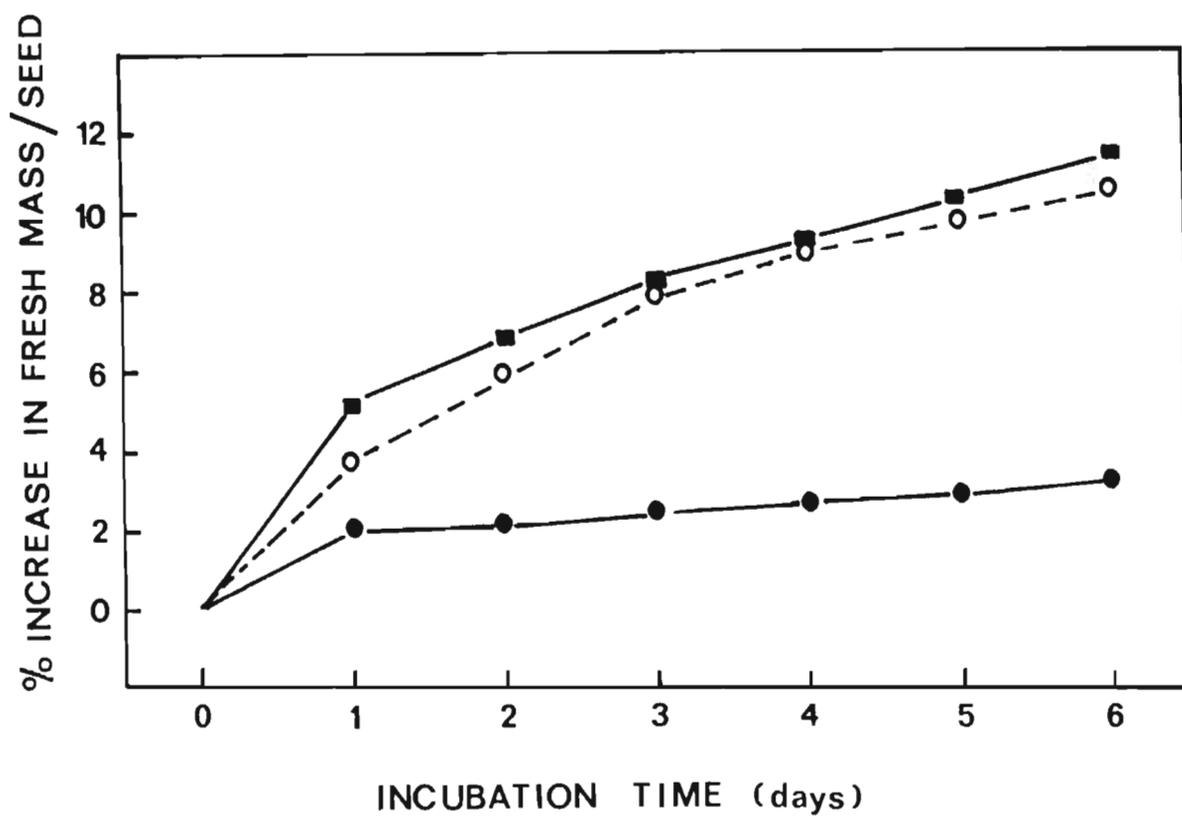


Fig. 3.4

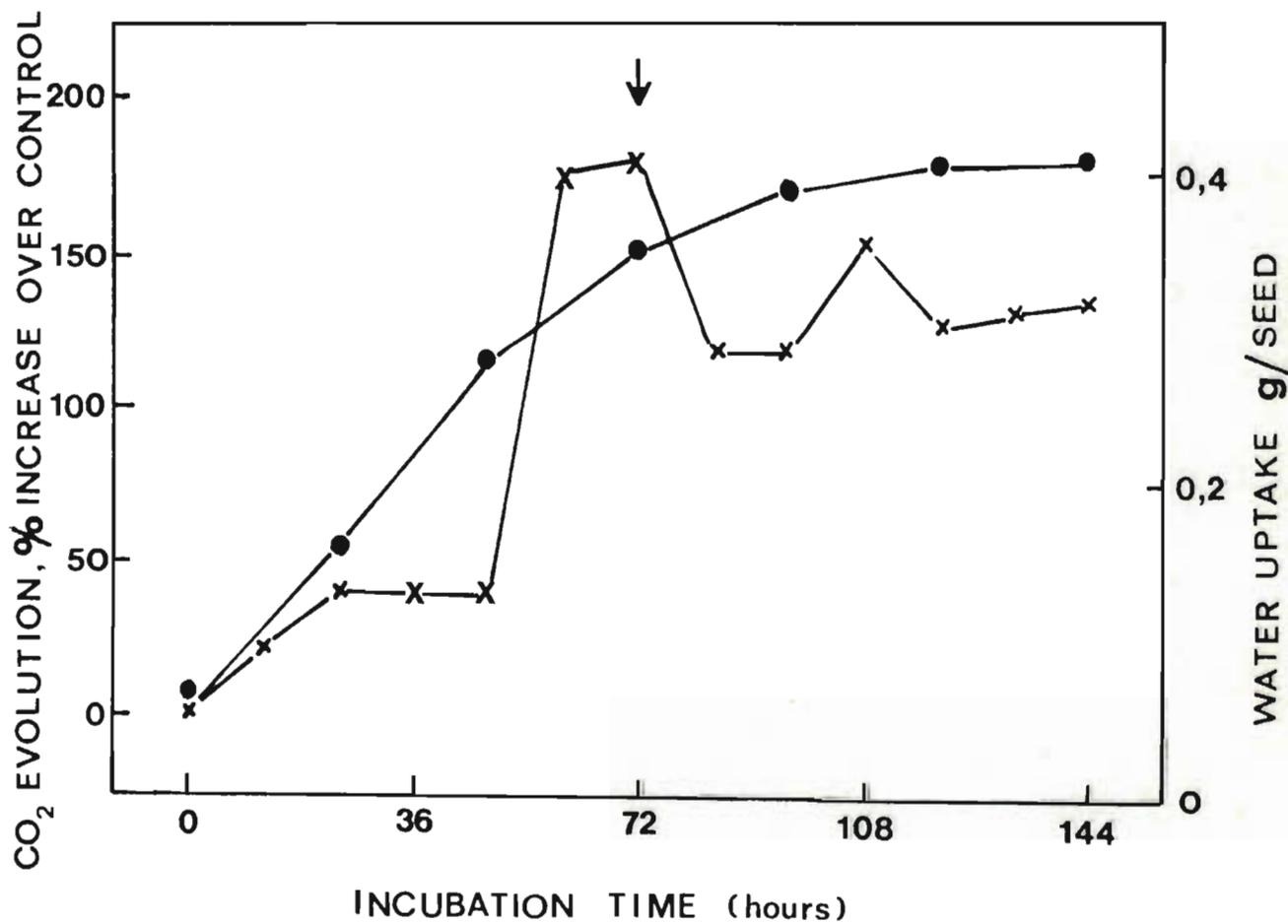


Table 3.2. The effect of desiccation on the moisture content of whole seed and seed components of *Podocarpus henkelii*. Figures in parentheses represent the standard error.

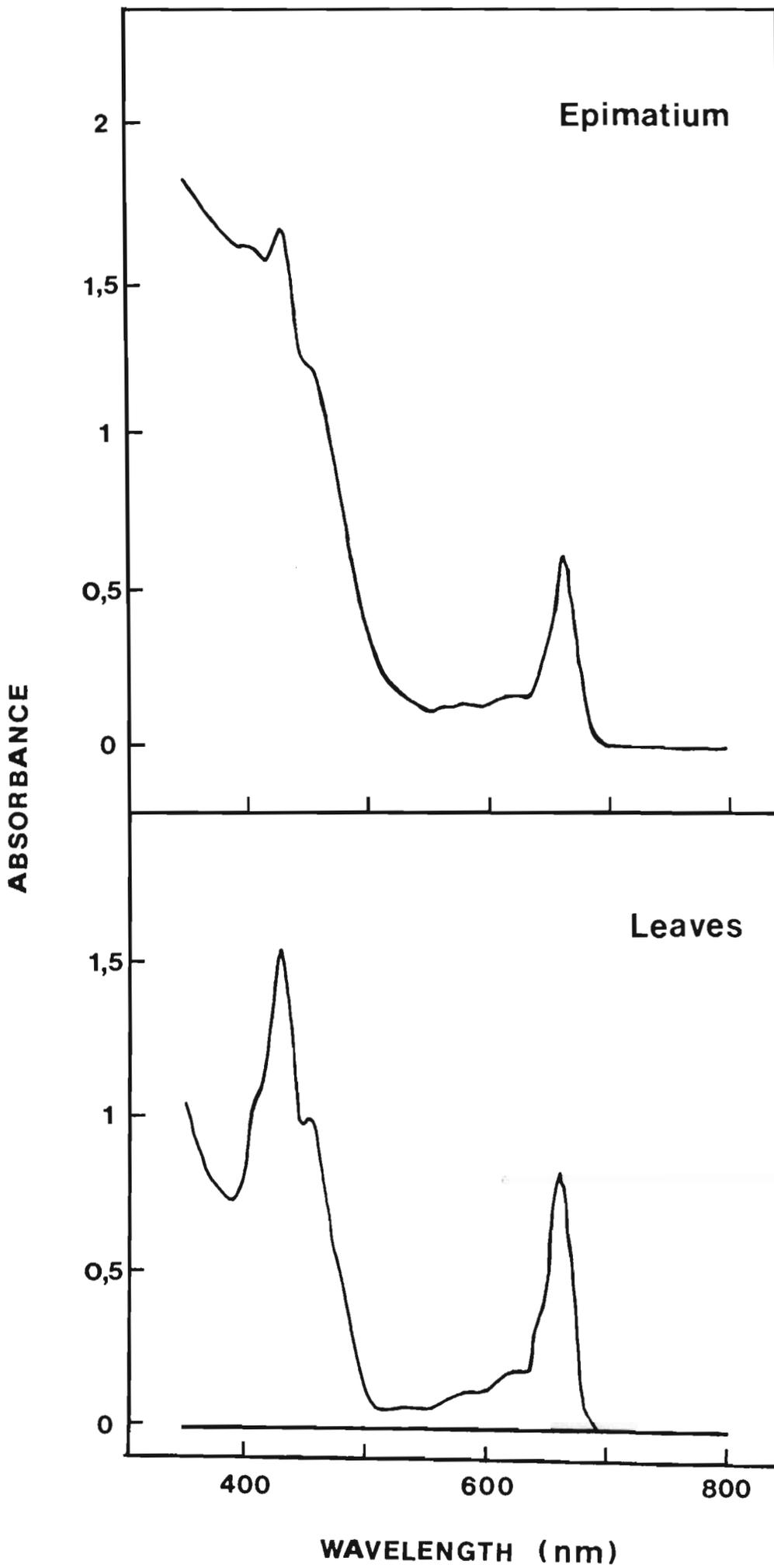
Whole seed	Moisture content (%)			Germination (%)
	Epimatium	Female gametophyte	Embryo sporophyte	
54,5 ± 3,2	57,2	50,5	61,6	100 ± 0
44,0 ± 2,7	47,9	38,6	54,7	100 ± 0
39,0 ± 1,7	43,8	32,7	42,3	72 ± 6,8
34,0 ± 1,9	36,1	31,0	41,9	58 ± 18,9
31,5 ± 2,2	32,0	30,5	39,9	0 -
25,0 ± 1,3	24,8	25,8	26,8	0 -

Table 3.3. Physical data of the three major pigments extracted from the epimatium of seeds of *Podocarpus henkelii*. Acetone extracts were separated by TLC and the pigments eluted and their absorption spectra recorded in a UV-Vis spectrophotometer.

Pigment	Rf-value	Absorption peak (nm)
Carotene	0,97	450
Chlorophyll a	0,38	410,660
Chlorophyll b	0,34	435,640

Figure 3.5. Absorption spectra of pigments in acetone extracts from the leaves and epimatium of seeds of *Podocarpus henkelii*.

Fig. 3.5



whether or not the seeds could photosynthesize, samples were incubated in the light in the presence of $^{14}\text{CO}_2$. These seeds did incorporate substantial quantities of labelled carbon (Table 3.4). There was no fixation of labelled carbon by seeds incubated in the dark (Table 3.4). With time, the labelled carbon was transported from the epimatium to the female gametophyte and embryo sporophyte. These data concur with the transport of ^{14}C labelled sucrose between the various seed components in Chapter four. Chromatographic analyses of the ethanol soluble sugars indicated that most of the radio-activity co-chromatographed with ribose. Radio-activity that co-chromatographed with sucrose was detected in the embryo sporophyte only (Table 3.5). This could be due to the fact that this disaccharide is the common transport sugar in plants and was being utilized in this seed component to synthesize further storage reserves. These data indicate firstly, that gases can readily penetrate the epimatium and secondly, that the seeds can photosynthesize and that the photosynthates are translocated to the embryo sporophyte.

As $^{14}\text{CO}_2$ was able to penetrate the epimatium readily, it would appear that this seed component did not exert its influence over germination by limiting gaseous exchange to the embryo sporophyte. To investigate the role water played in desiccated seeds the following experimentation was undertaken. Seeds were desiccated to 49 per cent, scarified on their sides, and placed scarified side surface upwards or downwards onto moist vermiculite at 25°C . The latter treatment produced 70 per cent germination within 16 days whereas in the former no germination was recorded. These results confirm the observa-

Table 3.4. Radioactivity (dpm g⁻¹ dry mass) detected in seeds of *Podocarpus henkelii* incubated in ¹⁴CO₂ for different periods of time.

Time exposed to ¹⁴ CO ₂ in light (hours)	Radioactivity (dpm g ⁻¹ dry mass) in different seed components			
	Epimatium	Female gametophyte	Embryo sporophyte	Whole seed
24	19266	0	0	19266
48	10966	5450	0	16416
72	14000	2730	2500	19230
Dark				
72	0	0	0	0

Table 3.5. Radioactivity (dpm g⁻¹ dry mass) detected on paper chromatograms of soluble sugar extracts from seeds of *Podocarpus henkelii* incubated with ¹⁴CO₂ for 72 hours.

Sugars that co-chromatograph with:	Radioactivity (dpm g ⁻¹ dry mass) in different seed components		
	Epimatium	Female gametophyte	Embryo sporophyte
Sucrose	0	0	600
Glucose	80	730	700
Fructose	110	120	900
Ribose	60	2180	1200

tions of NOEL and VAN STADEN (1976) that the epimatium is a barrier to water uptake, but not to loss.

Water uptake is thus an important factor in the germination of *Podocarpus henkelii* seeds once they have started to desiccate. The water uptake curves depicted in Fig. 3.2 are of interest as they do not follow the classical triphasic water uptake curves for germinating seeds (BEWLEY and BLACK, 1978; POLLOCK, 1972). These curves also do not follow the water uptake curve for other gymnospermous seeds such as *Pinus lambertiana* (STANLEY, 1958). The seed tissues of *Podocarpus henkelii* were always in a relatively high state of hydration. Thus, the amount of water necessary for germination at a particular time was dependent on the level to which the moisture content had decreased. Despite this, there was no evidence of any triphasic uptake of water (Fig. 3.2). It is apparent that the water uptake curve consists only of the third phase of the triphasic water uptake curve. It is during this phase that germination in dry seeds normally occurs. In unscarified seeds of 52 per cent moisture content, and which took 72 days to first germination, the amount of water imbibed was only 0,44 grammes. These seeds thus display exactly the same water uptake characteristics as the scarified *P. henkelii* seeds.

To determine when the metabolism of scarified seeds of *P. henkelii* (which had a moisture content of 49 per cent) increased during incubation, the quantities of CO_2 evolved were measured. The results obtained are shown in Fig. 3.4 and reveal that the metabolism of the seeds increased markedly after 60 hours, with first germination being recorded after

72 hours. Water uptake increased steadily up to the fifth day from incubation and then remained at a fairly constant level. By this time, some 56 per cent of the seed had germinated. The levels of carbon dioxide evolved decreased slightly after 72 hours, and remained at approximately 125 per cent above the control levels. This pattern of respiration, although only measured in terms of CO_2 evolution, does not resemble that of other germinating seeds such as *Pisum sativum*. KOLLÖFFEL (1967) reported that in pea seeds the pattern of respiration follows quite closely that of the classical triphasic water uptake curve up to the end of stage three. There follows a fourth stage characterized by a marked decline in respiration which coincides with the disintegration of the cotyledons following the depletion of the stored reserves. In *Podocarpus henkelii* the respiration rate appears to follow that of the water uptake curve, with a slight decrease after 72 hours (Fig. 3.4).

The water uptake of these 'wet' *P. henkelii* seeds is markedly different from the curves recorded for 'dry' seeds. A study of the increase in moisture content of the individual seed components of scarified seeds revealed that there was only a small increase upon incubation (Table 3.6). These results are very different from those achieved in germinating *Zea mays* seeds, where the moisture content of the seed as a whole increased to 51 per cent. The embryo alone achieved an increase in moisture content of 261 per cent (BLACKLOW, 1972). In *Pinus lambertiana* seeds the gametophytic tissue increased in moisture content by 125 per cent during imbibition (STANLEY, 1958). In contrast to this, the embryo sporophyte of *Podo-*

Table 3.6. Change in the moisture content (%) of the component parts of scarified seeds of *Podocarpus henkelii* during incubation at 25°C. Figures in parentheses represent the standard error.

Incubation time (days)	Moisture content (%) of seed components			
	Whole seed	Epimatium	Female gametophyte	Embryo sporophyte
0	52,8 ± 1,8	52,7	48,6	61,3
3	52,7 ± 1,1	55,3	49,8	62,8
6	53,5 ± 0,9	58,3	51,4	64,2
9	55,4 ± 1,2	59,0	51,5	65,9

carpus henkelii showed an increase of only 7,4 per cent moisture content during nine days of incubation. These data show that seeds of *Podocarpus henkelii* at a moisture content of 53 per cent were in a highly hydrated condition and that the only water necessary for germination was apparently that required for the swelling and emergence of the radicle.

Under natural conditions the embryo sporophyte has somehow to rupture the thick tough epimatium. NOEL and VAN STADEN (1976) suggested that the water uptake by the seeds of *P. henkelii* is dependent upon the formation of peristomatal fissures in the cuticle as the seeds shrink, presumably due to desiccation. This change could be accompanied by a softening of the epimatium which would aid radicle protrusion. MAYER (1977) suggests that seed coats which act as mechanical barriers may be dissolved by chemical means or ruptured physically. NABORS and LANG (1971) suggest that the physical thrust is due to vacuolation in the embryo. How the epimatium of *P. henkelii* is ruptured under natural conditions is as yet unsolved. It is evident in these seeds that the epimatium acts as a barrier to germination both physically and by limiting water uptake. This seed component was also very effective in restricting water uptake but did not appear to be as competent in limiting the loss of water from the seed. Consequently the viability of the seed appears to be dependent upon the balance between the water gained from the substrate and that lost to the environment through desiccation.

The high moisture content of the seeds of *Podocarpus henkelii* thus makes them very vulnerable and is apparently

the major factor limiting their longevity and viability in nature. 'Dry' seeds have evolved the ability to dehydrate and thus withstand the extremes of the climate without deteriorating. This enables them to remain viable for much longer periods of time. The deterioration of seeds may be described as an irreversible degenerative change in the quality of a seed after it has reached its maximum quality level (ABDULBAKI and ANDERSON, 1972). The length of time which a seed takes to reach a stage of deterioration obviously varies with species but it seems to be much quicker in those seeds which do not dehydrate. As described previously, the completion of seed maturation is often marked by a rapid dehydration, during which the water content of the seed falls to a very low level. This process often has marked effects on the ultrastructure of the seed (BAIN and MERCER, 1966a; ÖPIK, 1968). At the same time decreases in respiration and protein synthesis have also been recorded (KOLLÖFFEL, 1970; MARRÉ, 1967). These many biochemical and cytological changes that accompany the reduced water content are thought to be a direct result of the reduced availability of the aqueous medium in which the many enzymes and cell structures usually function.

The effects of desiccation on maturing seeds can be observed in many ways. The O_2 consumption falls gradually during the latter part of *Ricinus communis* maturation (MARRÉ, 1967). Enzymes of the glycolytic pathway, pentose phosphate pathway, Krebs cycle and cytochrome system are all reported to decline during desiccation (MARRÉ, 1967). There are also reports of decreased mRNA activity in *Phaseolus vulgaris* seeds (ÖPIK, 1968). PAYNE and BOULTER (1969) reported that in the

seeds of *Vicia faba* there are changes in the endoplasmic reticulum and a loss of membrane bound polysomes during maturation, and these may account for the change in protein synthesis in late maturation. Other ultrastructural studies have established that in desiccated seeds major cellular organelles such as nuclei and mitochondria are present (BAIN and MERCER, 1966a; KLEIN and POLLOCK, 1968; ÖPIK, 1968). These organelles are however often in a diffuse, ill-defined state. These ultrastructural studies leave the impression of biochemical inactivity, but there are few ways of verifying this. When comparing the mature 'wet' seeds of *P. henkelii* (Plates 1.6 and 1.7) with the data for mature *Pisum sativum* seeds (BAIN and MERCER, 1966a), they do not give the impression of biochemical inactivity. The high hydration state of *P. henkelii* seeds possibly results in higher metabolic rates than in 'dry' seeds, resulting in the utilization of valuable food reserves. These can to some extent be supplemented by the ability to fix carbon. This would however, only benefit the carbohydrate metabolism. Nitrogen metabolism would have no source of replenishment. As these seeds are not programmed to remain viable under low moisture contents, they are very susceptible to desiccation and this appears to be the limiting factor in their distribution and viability. Seeds of *Podocarpus henkelii* were successfully stored at 4°C in sealed polythene bags for 18 months. During this time their moisture contents dropped to only 58 per cent and 95 per cent germination was recorded after scarification and incubation at 25°C. This concurs with the storage conditions for acorns of *Quercus* which must be stored at a relatively high moisture level if

viability is to be maintained (EDWARDS, 1980).

Autecological studies are necessary to confirm whether or not these laboratory generated data explain the poor natural regeneration of *Podocarpus henkelii* and the very localized distribution range of this species.

CHAPTER FOUR

THE ULTRASTRUCTURE AND BIOCHEMISTRY OF GERMINATING

PODOCARPUS HENKELII SEEDS

A large volume of information on the fine structure and metabolism of germinating seeds has been amassed in recent years. Most authors however, have concentrated on the metabolic and ultrastructural changes in storage organs and embryos of seeds belonging to the angiosperms. Seeds of Leguminosae and Gramineae have been particularly well investigated mainly because of their economic importance (ROST, 1972). The gymnospermous seeds (which provide a large variety of seed forms) have, with a few notable exceptions been largely neglected. CHING (1965) has studied the germinating seeds of Douglas fir. DURZAN, MIA and RAMAIAH (1971) have reported on the metabolic and ultrastructural changes in germinating Jack pine (*Pinus banksiana* Lindl. and Gord.). The fine structure of *Pinus sylvestris* and *Picea abies* has been well documented by SIMOLA (1974; 1976). The only other gymnospermous seed to receive a detailed ultrastructural investigation is *Welwitschia mirabilis* (BUTLER, 1975; BUTLER, BORNMAN and JENSEN, 1979a and b). Fine structural observations of the mature seed of *Podocarpus henkelii* have been reported by VAN STADEN, NOEL and GILLILAND (1976). These seeds have been shown to differ from other gymnosperms with regard to their morphology and high moisture content. For these reasons, as well as their interesting germination characteristics (Chap. 3) an ultrastructural and biochemical study was instigated.

Seeds of *Podocarpus henkelii* were germinated in the same manner as reported in the previous chapter. Seed for this study had been stored for six weeks at 4°C by which time their moisture content had decreased to 54 per cent. The seed was scarified and incubated at 25°C in moist vermiculite and sampled on days 0, 3, 6 and 9 of incubation. A sample was collected on day 12 of incubation for additional ultrastructural investigation. The seed samples were divided up for ultrastructural studies, biochemical analyses and cytokinin determinations. All samples were drawn from the same batches of seed, and only seed which had germinated on days six and nine of incubation were sampled. Consequently meaningful interpretations of the germination process in this seed can be obtained. The changes in fresh and dry mass of the three seed components and change in size of the embryo sporophyte are listed in Table 4.1. The change in moisture content of the three seed components is listed in Table 3.6. There is very little change in the fresh mass of the epimatium and female gametophyte over the nine days of study. First germination was recorded on day five of incubation and by day nine, 45 per cent of the seed had germinated (Fig. 4.1). There was a slight decrease in the dry mass of both these seed components during the nine days of study. The embryo sporophyte increased in both size, and fresh and dry mass between the third and ninth day of incubation (Table 4.1). The fact that only 45 per cent of the seed had germinated by day nine is reflected in the larger standard errors for both length ($36,23 \pm 8,1$ millimetres) and fresh mass ($0,164 \pm 0,049$ grammes) of the embryo sporophyte. The moisture content of these three seed components did not increase much during

Fig.4.1

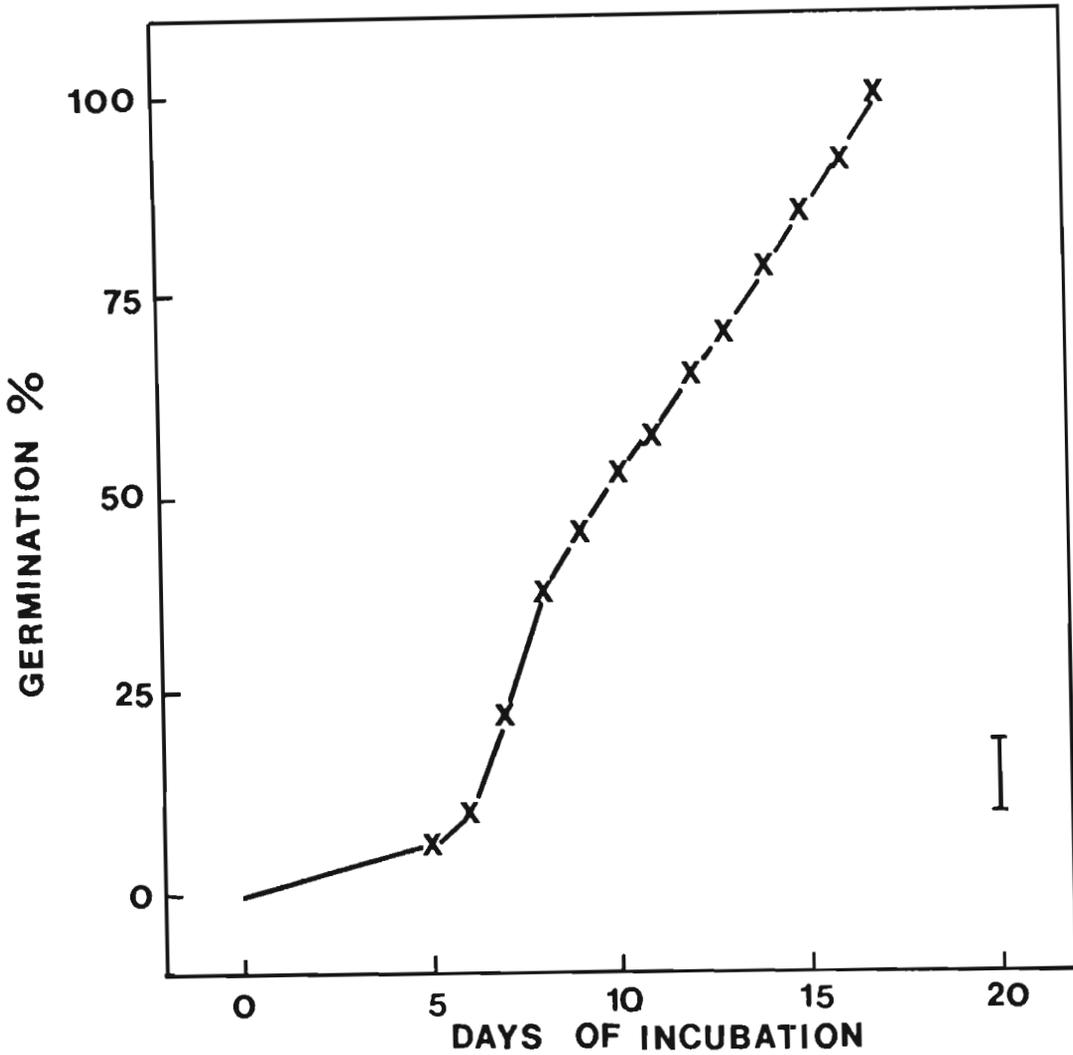


Figure 4.1. Germination of scarified seeds of *Podocarpus henkelii* on a moist substrate at 25°C. The seeds were subsequently sampled for biochemical and cytokinin extractions. Bar represents the maximum 95 per cent confidence limit.

Table 4.1. Changes in the fresh and dry mass of seed components of scarified *Podocarpus henkelii* seeds upon incubation on a moist substrate at 25°C. Each sample is the mean of fifty seeds and the figures in parentheses represent the standard error

Seed Component		Days of Incubation			
		0	3	6	9
Epimatium	Fresh mass grammes	3,28 ± 0,27	3,09 ± 0,29	3,03 ± 0,32	3,12 ± 0,31
	Dry mass grammes	1,49	1,39	1,35	1,14
Female gametophyte	Fresh mass grammes	2,58 ± 0,26	2,54 ± 0,30	2,83 ± 0,45	2,76 ± 0,32
	Dry mass grammes	1,22	1,12	1,03	1,02
Embryo sporophyte	Length millimetres	16,82 ± 2,46	17,66 ± 2,58	22,93 ± 6,3	36,23 ± 8,1
	Breadth millimetres	2,89 ± 0,40	3,06 ± 0,31	3,18 ± 0,67	3,24 ± 0,37
	Fresh mass grammes	0,063 ± 0,019	0,065 ± 0,024	0,099 ± 0,045	0,164 ± 0,04
	Dry mass grammes	0,023	0,024	0,033	0,045

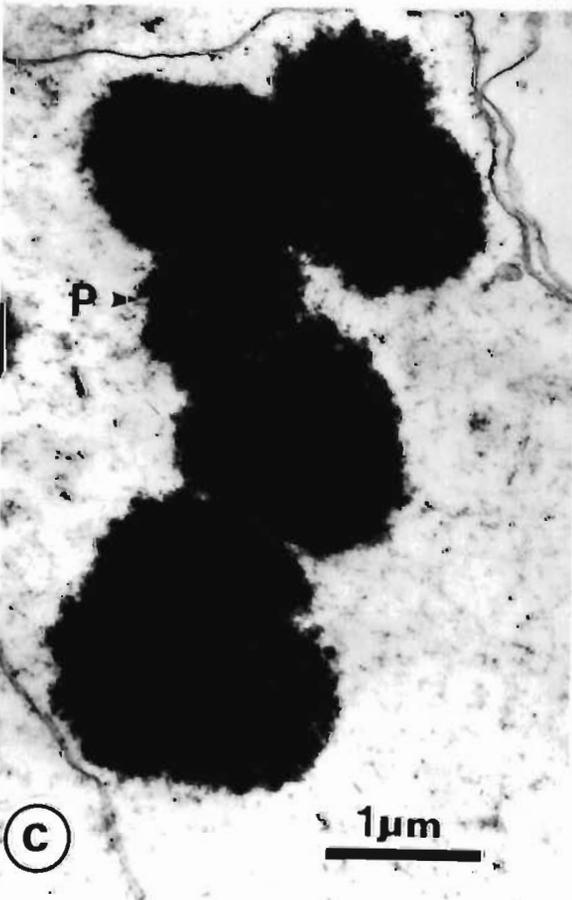
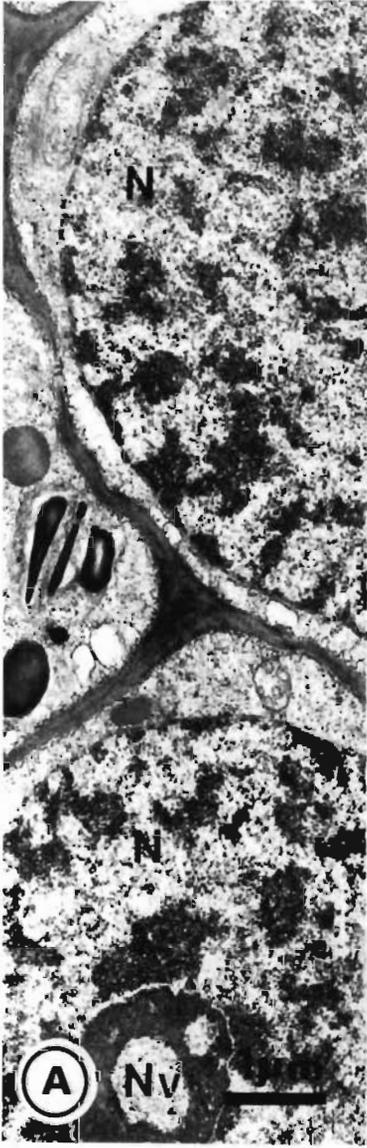
the nine days of incubation (Table 3.6 and Fig. 3.2). This is a reflection on the initial high state of hydration in the *Podocarpus henkelii* seeds. The cellular detail of 'dry' seeds such as *Pinus sylvestris* changes markedly with imbibition (SIMOLA, 1974). After one day's imbibition, rootlet cells of *Pinus sylvestris* exhibit changes. Protein bodies formed vacuoles and compound amyloplasts became abundant. Mitochondria, dictyosomes and some endoplasmic reticulum had developed. Numerous ribosomes were also present in the cytoplasm. By the second day the amount of cytoplasm had increased substantially and in the cotyledonary cells, protein material was being catabolized. Food reserve mobilization in the endosperm cells commenced only after five days of imbibition.

The fine structure of mature seeds of *Podocarpus henkelii* has been described previously and is illustrated in Plates 1.6 and 1.7. The ultrastructure of seeds sampled after six weeks in cold storage is shown in Plate 4.1. The moisture content of these seeds had decreased during storage to 54 per cent. An obvious feature of the female gametophyte was that there had been little change in the cellular detail during the six weeks of storage (Plate 4.1 d). The thick cell walls were still in evidence as were the reserves of starch, lipid and protein (Plate 4.1 c and d). The regions of cytoplasm close to the cell periphery contained most of the reserve food material. The background cytoplasm adjacent to the plasmalemma was filled with numerous ribosomes in some areas, but very little endoplasmic reticulum was present. The protein was stored as granules within small vacuoles which lay in close proximity to the cell periphery. The contents of the large cell vacuole were

PLATE 4.1

ULTRASTRUCTURAL DETAILS OF MATURE *PODOCARPUS HENKELII*
SEEDS AFTER SIX WEEKS STORAGE AT 4°C

- A: Cotyledonary cells with large nuclei (N), nucleolar vacuoles (Nv) and an active cytoplasm.
- B: A radicle cell showing an active cytoplasm with dictyosomes (D), starch (S), lipid (L) and nucleus (N).
- C: Protein (P) contained in vacuoles in the female gametophyte cells.
- D: A typical female gametophyte cell with thickened cell wall (CW), lipid bodies (L) and starch grains. The cytoplasm appeared to be relatively inactive with little endoplasmic reticulum or dictyosomes.



very electron-transparent. An interesting observation of this seed component was that the cellular fine structure was all very similar, no matter from which region of this vast tissue the sample was obtained.

The fine structure of the embryo sporophyte had not changed much during the six weeks of storage (Plate 4.1 a and b). There was evidence from both the ultrastructural studies and the physical measurements (Table 4.1) that this seed component had been metabolically active during storage. This was not evident from any increase in size of the embryo sporophyte, but from the fact that this seed component had increased from 0,015 grammes to 0,045 grammes in dry mass during this time. Although no standard errors are available for these data, they conform to the experimentation in Chapter three. A detailed study showed an increase in dry mass of the embryo sporophyte from 0,015 (\pm 0,005) grammes at shedding to 0,026 (\pm 0,003) grammes after 16 uninterrupted weeks at 4°C. The seed stock used for experimentation in this chapter was often removed from the cold store for short periods of time to obtain seeds. This change in temperature could account for the greater increase in dry mass during the six weeks of storage compared with the 16 week sample.

The fine structure of the tissue observed suggests that metabolic activity occurred. The tissue shown in Plate 4.1 a was situated deep within the cotyledons and showed signs of recent cell division. Both the nuclei are large and there is a prominent nucleolus with nucleolar vacuole in one cell. The latter occurrence is consistent with the observations of

JORDAN (1971) in which nucleolar vacuoles are associated with tissue which has been active and is in the process of slowing down. The cytoplasm contained active mitochondria with well defined cristae. Numerous ribosomes were present together with a few polyribosomes but little endoplasmic reticulum was observed (Plate 1.4 a). The meristematic region of the root tip also displayed signs of metabolic activity. The micrograph in Plate 4.1 b shows a cell that was situated in the cortex in the region just above the meristem. The nucleus had a smooth outline and the cytoplasm was filled with ribosomes and polyribosomes. Dictyosomes were present but they appeared to be relatively inactive as there were few dictyosomal vesicles. In common with the cotyledonary cells there was very little endoplasmic reticulum present. These observations are similar to those of COLBORNE, MORRIS and LAIDMAN (1976) for germinating *Triticum vulgare* and LORD (1978) for germinating *Ricinus communis*. These authors showed that small amounts of endoplasmic reticulum are present in mature seeds. In the castor bean endosperm and the aleurone of wheat the endoplasmic reticulum proliferates 24-72 hours after seed imbibition. Subsequent seedling growth is accompanied by an increase in the endoplasmic reticulum of the storage cells. Other work however, has shown that at the start of germination of Mungbean (*Vigna radiata*) (L.) Wilczek.) cotyledons have extensive endoplasmic reticulum (HARRIS and CHRISPEELS, 1980). The amount of endoplasmic reticulum present decreases with seedling growth. This illustrates that there are clear patterns but no general rules for ultrastructural and metabolic events in germinating seeds.

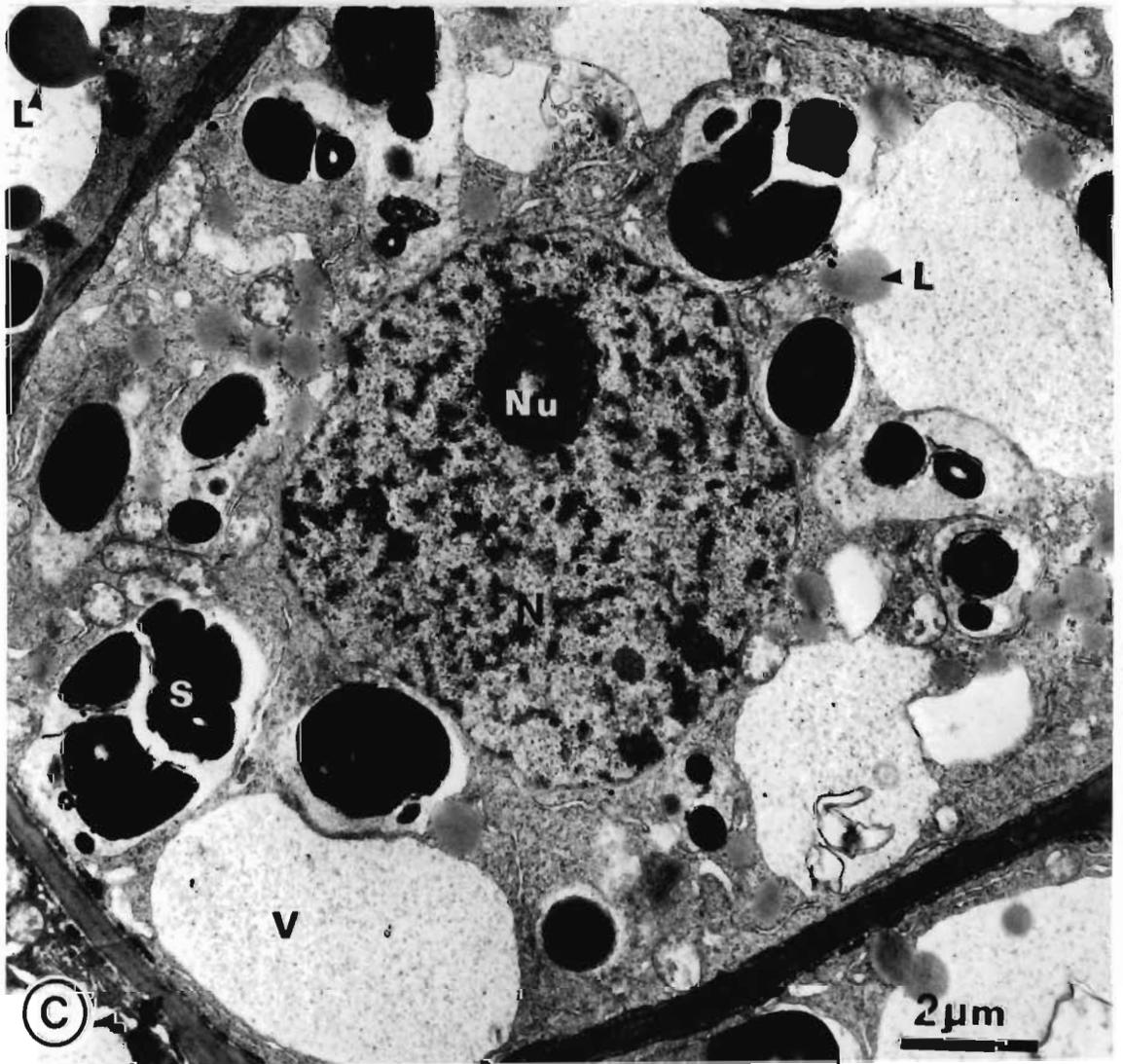
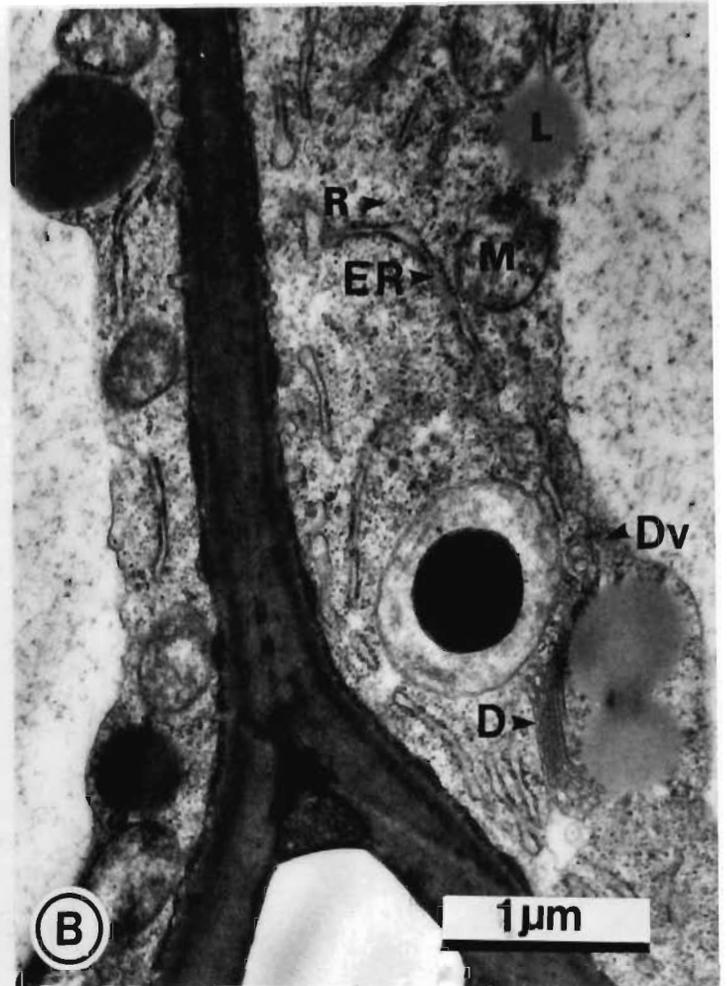
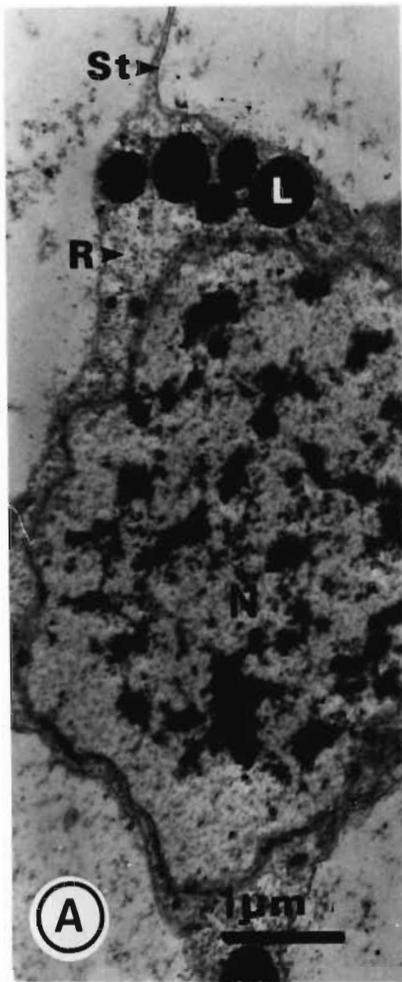
The root tip cells contained numerous starch grains and lipid bodies. The different staining properties of the lipid bodies in the female gametophyte compared to the embryo sporophyte are very clear (Plate 1.1 b and d). All these ultrastructural observations show that the embryo sporophyte is metabolically active during storage, and substantiate the suggestion that there is an after-ripening effect with storage. This continued metabolic activity would explain the increase in size of the embryo sporophyte with storage and the subsequent increased germination rate compared to fresh seed (Fig. 3.1).

After three days of incubation the three seed components had increased slightly in moisture content (Table 3.6). There was a slight decrease in both the fresh and dry mass of the epimatium and female gametophyte during this time. Ultrastructural observations showed little change in the fine structure of the female gametophyte. In some cells, portions of the nucleus appeared in the sheets of the cytoplasm which permeate the large central vacuole (Plate 4.2 a). No endoplasmic reticulum or dictyosomes were observed in these regions but some ribosomes were present. These cytoplasmic strands often contained starch and lipid. The protein which remained in vacuoles associated with the cell periphery appeared to be less dense. The function of the vacuolar compartments which arose from these cytoplasmic strands is not clear. There is obviously a need for some nuclear control, hence the appearance of fingers of nuclear material within the strands. When compared with the equivalent tissue in 'dry' seeds such as *Pinus sylvestris* or *Picea abies* (SIMOLA, 1974; 1976) which

PLATE 4.2

DETAILS OF THE FINE STRUCTURE OF SEED COMPONENTS THREE DAYS AFTER THE START OF INCUBATION.

- A: In the female gametophyte, sheets of cytoplasm (St) which permeate the central vacuole contain fingers of nuclear material (N), ribosomes (R) and lipid bodies (L).
- B: In the embryo sporophyte the radicle cells in the region of the meristem were very active containing flat dictyosomes (D) with associated dictyosomal vesicles (Dv), endoplasmic reticulum (ER), ribosomes (R) and mitochondria (M).
- C: In some radicle cells the lipid bodies (L arrowed) were being incorporated into the vacuoles (V). It is thought that since no glyoxysomes could be found the lipid was catabolized in these vacuoles.



had been imbibed for one day, the female gametophyte appears relatively inactive. In *Welwitschia mirabilis* seeds, which take only seven hours to imbibe fully, there are distinct changes observable in the female gametophyte within 24 hours of imbibition. The nucleus and cytoplasmic organelles show increased clarity of membranes. In the mitochondria the cristae appear to become more organized and the matrix loses its translucent appearance (BUTLER, 1975). The cytoplasm is less active and no endoplasmic reticulum, polyribosomes or dictyosomes could be observed at this stage (BUTLER, 1975). The female gametophyte of *Podocarpus henkelii* appears relatively inactive even after three days of incubation. These data agree with the biochemical results which showed that reserve mobilization does not begin until three and six days after incubation (Fig. 4.2) with the exception of protein which commences between zero and three days (Fig. 4.5).

Three days after the start of incubation, the embryo sporophyte appeared to be very active. Its length had increased to 17,66 (\pm 2,5) millimetres and the dry mass to 0,024 grammes. The root tip was the most active region of this seed component. Numerous flat dictyosomes were present along with their associated vesicles. Strands of endoplasmic reticulum, some of which were rough, spread through the cytoplasm which contained numerous ribosomes and polyribosomes (Plate 4.2 b and c). The nuclei had fairly irregular outlines and some contained nucleoli which were very electron-dense, indicative of nuclear activity (HYDE, 1967). The numbers of vacuoles increased in these cells perhaps contributing to the overall increase in length of this organ. An unusual feature of the root tip

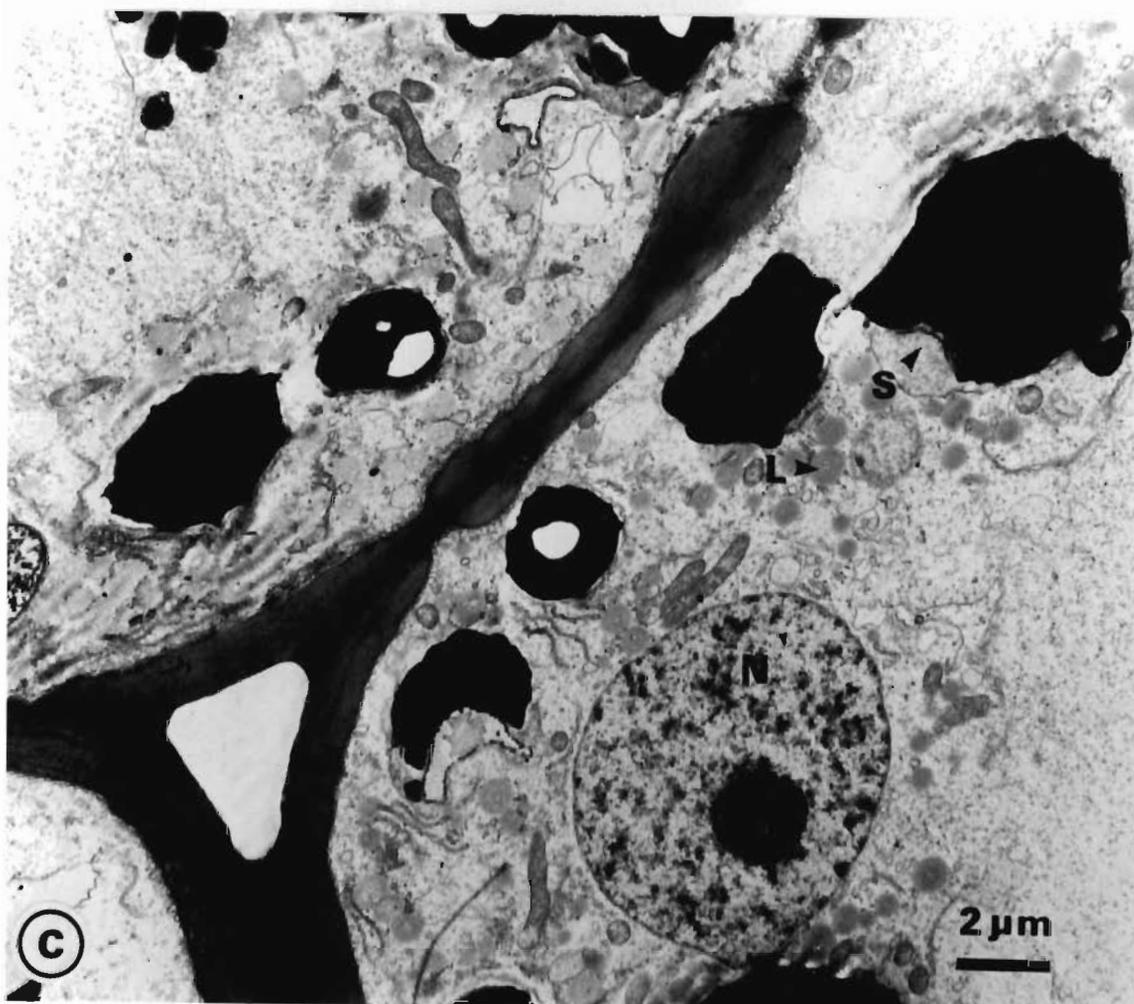
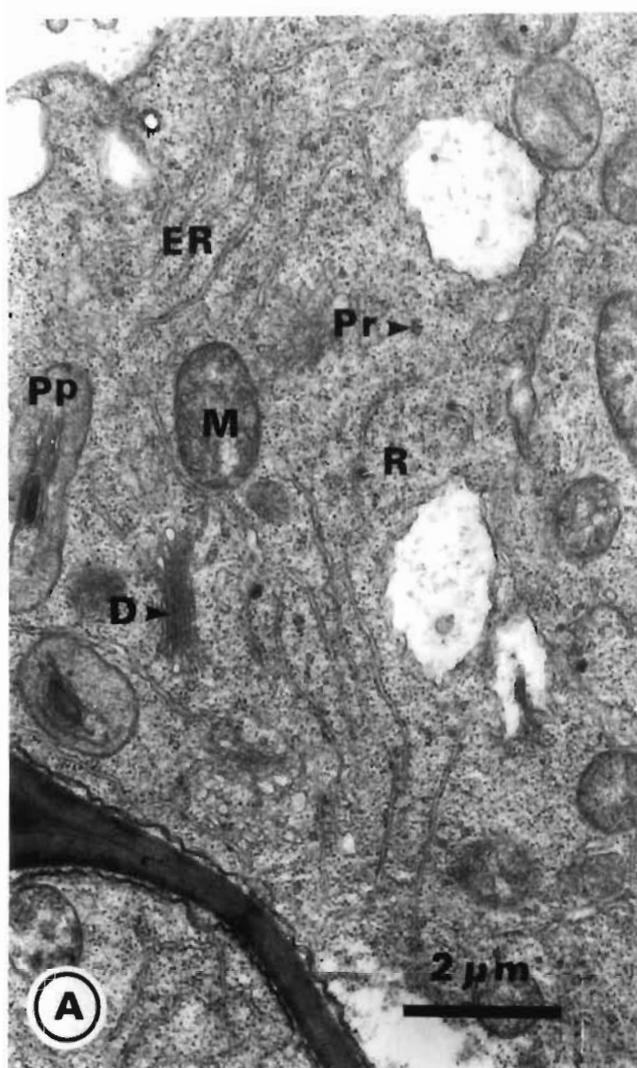
tissue was the appearance of lipid bodies in the vacuoles (Plate 4.2 c). As no glyoxysomes could be found in any of the embryo sporophyte tissues, it is suggested that the digestion of the lipid reserves occurs in these vacuoles. This phenomenon of lipid digestion occurring in autophagic vacuoles has been reported in the germinating seeds of pecan nut (*Carya illinoensis* Koch) which contains large amounts of lipid as a food reserve (GILLILAND and VAN STADEN, 1977). In the embryos of germinating *Picea abies* and *Pinus sylvestris* seeds glyoxysomes are very rare or absent. Glyoxysomes were to be found in the gametophytic tissues of the abovementioned seeds. This led SIMOLA (1976) to suggest that in gymnosperms the embryo sporophyte and female gametophyte may differ in lipid metabolism. The aforementioned results conform to the biochemical data showing that the lipid mobilization in the embryo sporophyte occurs after the third day of incubation. No protein vacuoles such as those observed in Plate 1.7 could be found in the embryo sporophyte tissue at this time. This pattern of reserve mobilization, with protein reserves being utilized before the lipid is in accordance with the findings in *Pinus sylvestris* (SIMOLA, 1974) and *Protea compacta* R. Br. (VAN STADEN and GILLILAND, 1974).

Six days after the start of incubation, ten per cent of the seed had germinated (Fig. 4.1). During this period there had been a small increase in the moisture content of the female gametophyte and a decrease in the dry mass (Table 3.6 and Fig. 4.1). There had been some change in the fine structure of the female gametophyte during the preceding three days. The nuclei were small and had smooth outlines. In some cases

PLATE 4.3

SIX DAYS AFTER THE START OF INCUBATION TEN PER CENT OF THE SEED HAD GERMINATED.

- A: In the cotyledonary cells of the embryo sporophyte signs of increased metabolic activity were evident. Dictyosomes (D), ribosomes (R), polyribosomes (Pr), mitochondria (M) and endoplasmic reticulum (ER) some of which was rough, was present. The first proplastids (Pp) were in evidence.
- B: In the hypocotyledonary cells similar cellular changes were evident with numerous polyribosomes (Pr).
- C: In the female gametophyte some of the starch grains (S arrowed) appeared to have a rough outline as if they were being digested. Some of the lipid bodies (L arrowed) had the appearance of containing small vacuoles.



nucleoli were present which were very electron-dense. The cytoplasm contained mitochondria, dictyosomes, ribosomes and very small amounts of rough and smooth endoplasmic reticulum (Plate 4.3 c). Some lipid bodies (Plate 4.3 c arrowed) appeared to contain a small vacuole. This could be associated with the metabolism of this reserve material, particularly as no glyoxysomes could be observed in this tissue. Some of the starch grains (Plate 4.3 c arrowed) had rough outlines suggesting that they were being digested slowly.

After six days of incubation the embryo sporophyte had increased in length from 17,66 (\pm 2,5) to 22,93 (\pm 6,3) millimetres, and the dry mass had increased to 0,033 grammes. In both the cotyledonary and radicle cells there was evidence of intense metabolic activity (Plate 4.3 a and b). In the cotyledons the cytoplasm of some cells was filled with numerous ribosomes, polyribosomes and rough and smooth endoplasmic reticulum. Active dictyosomes with numerous associated dictyosomal vesicles were also evident. The small vacuoles in the cotyledonary cells which had previously contained aggregations of protein were now empty except for vacuolar sap. Proplastids were developing in the peripheral regions of the cotyledonary cells. Similar changes in cellular fine structure were to be found in the root tip cells close to the radicle meristem (Plate 4.3 b). All these changes were indicative of intense metabolic activity which must accompany the growth of the embryo sporophyte during germination.

The female gametophyte showed signs of increased metabolic activity after nine days of incubation. During this time,

the dry mass of this tissue had decreased and 45 per cent of the seed had germinated. In the female gametophyte cells close to the corrosion cavity, some regions of the cytoplasm contained long sheets of endoplasmic reticulum. Some of these sheets were studded with ribosomes (Plate 4.4 a). Polyribosomes and ribosomes together with apparently active dictyosomes were also present in the cytoplasm. This increase in metabolism of the female gametophyte tissue after a similar increase in the embryo sporophyte, agrees with observations by SIMOLA (1974) with germinating *Pinus sylvestris* seed.

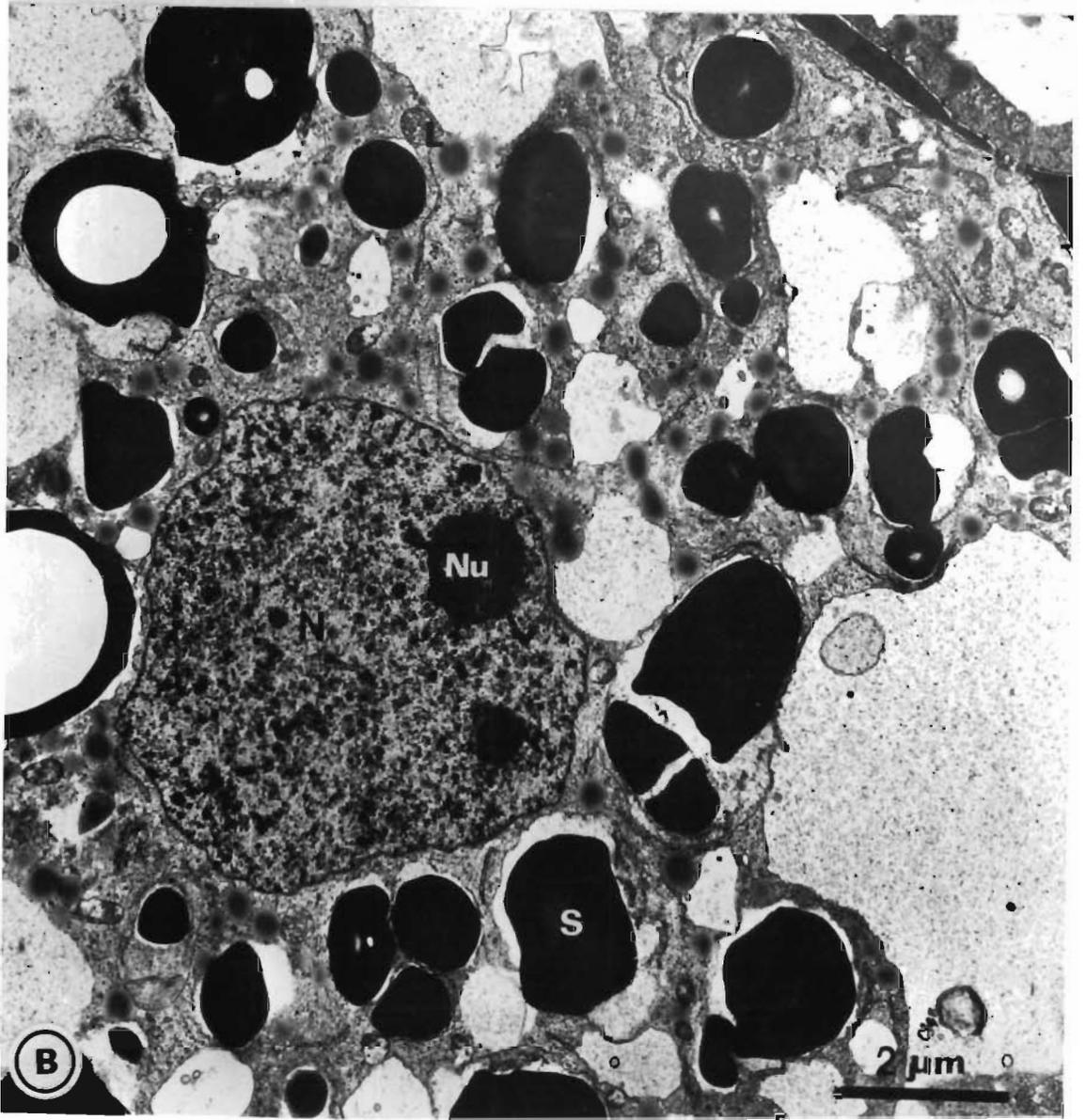
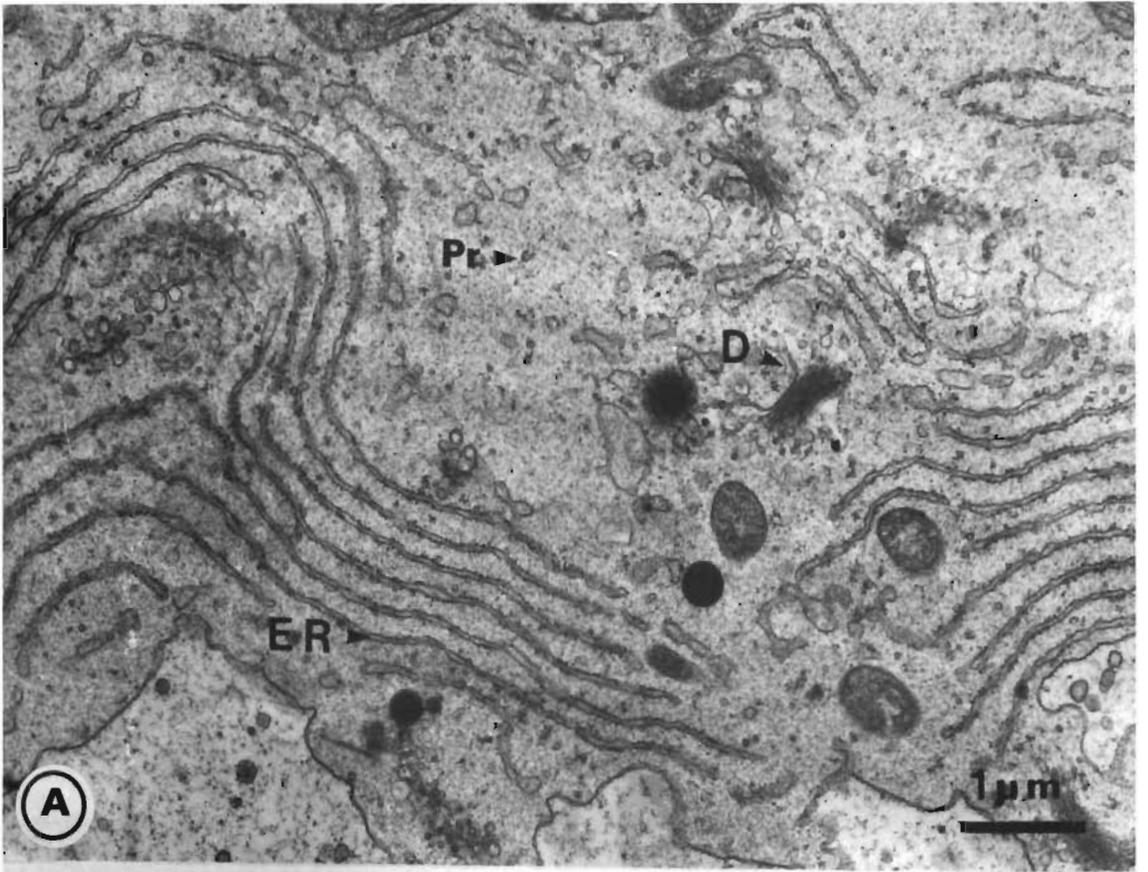
The embryo sporophyte had continued to increase in both length and dry mass during the sixth and ninth day of incubation (Table 4.1). The most noticeable feature of the fine structure was the increase in vacuolation, particularly in the root tip cells above the meristem (Plate 4.4 b). Cell division continued in the root meristem also contributing to the increase in length of this organ. The cells above the meristem were still very active with the cytoplasm containing numerous ribosomes, polyribosomes and endoplasmic reticulum. There were many lipid bodies lying closely pressed against the vacuoles and some of the starch grains had rough outlines, this being indicative of digestion. The nuclei had irregular outlines and in some cases two electron-dense nucleoli were present (Plate 4.4 b).

Samples of germinating *P. henkelii* seeds were collected after 12 days of incubation for ultrastructural studies only. The seeds had achieved 65 per cent germination by this stage. The most noticeable feature of the female gametophyte was the mobilization of the food reserves from the corrosion

PLATE 4.4

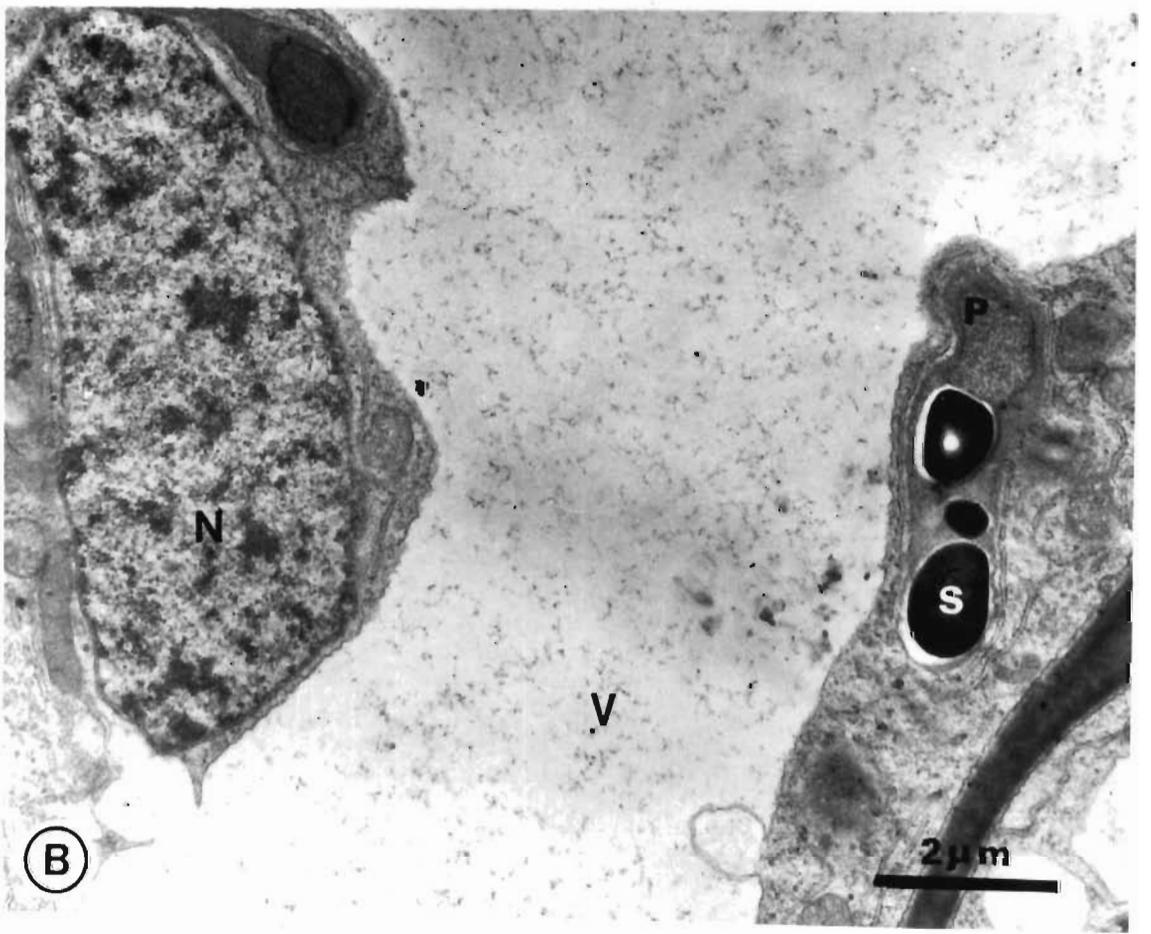
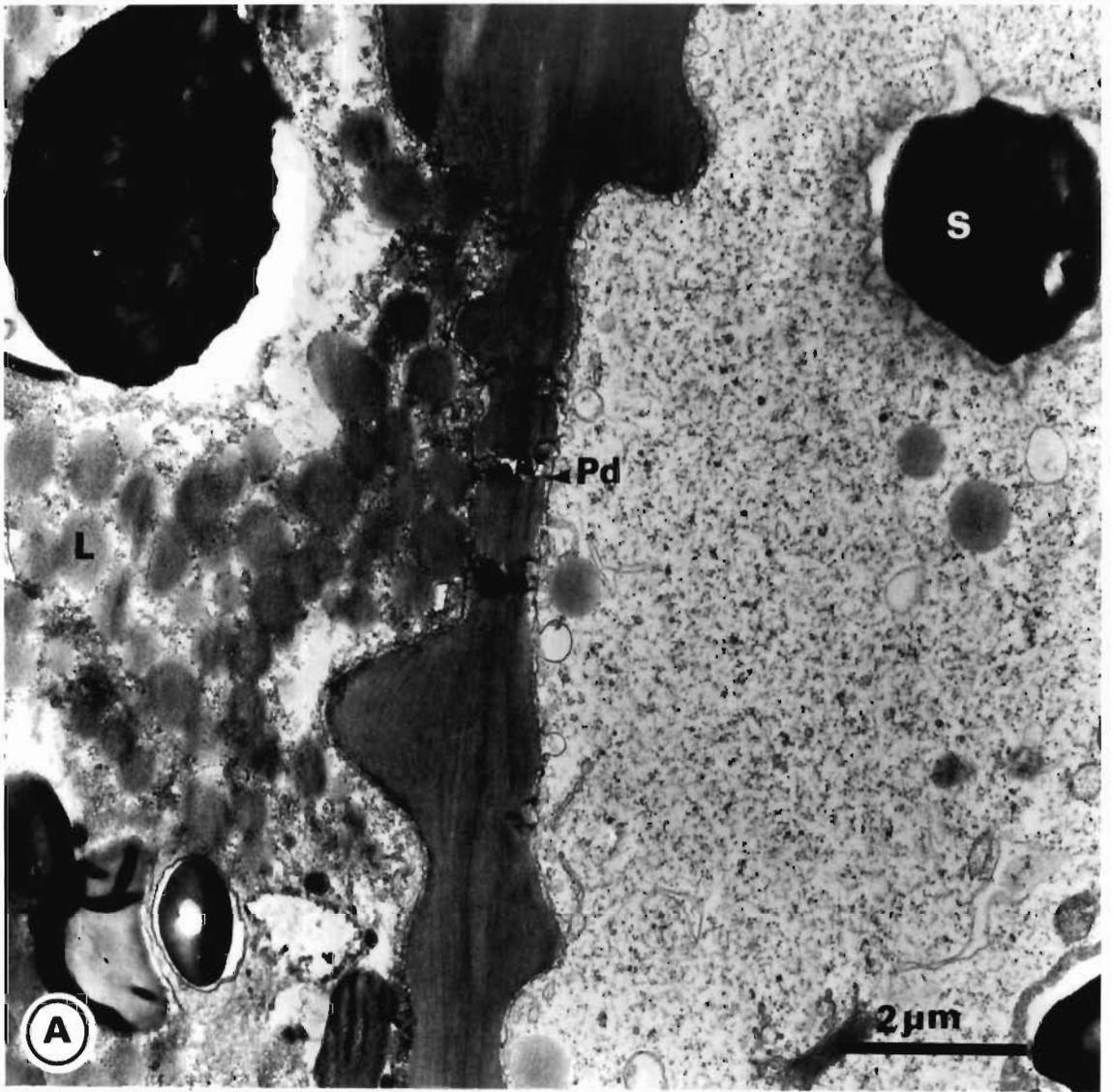
NINE DAYS AFTER THE START OF INCUBATION FORTY FIVE PER CENT OF THE SEED HAD GERMINATED

- A: In the female gametophyte cells close to the corrosion cavity, there was evidence of increased metabolic activity with dictyosomes (D), endoplasmic reticulum (ER), polyribosomes (Pr) and dictyosomes (D).
- B: In the radicle cells of the embryo sporophyte some of the starch grains had irregular outlines and the lipid bodies (L) had a halo-like outline.



cavity outwards. In Plate 4.5 a the cells shown are two and three cells in from the corrosion cavity (arrowed). The cells appear to lose their lipid reserves before the starch is metabolized. Some of the starch was however being mobilized as shown by the irregular outlines of certain grains. The cell walls were still very irregularly shaped and the hemicelluloses stored therein must only be metabolized at a later stage. No evidence for any transfer tissue could be found along any of the surfaces of the female gametophyte in contact with the embryo sporophyte. Transfer tissue has been reported at the interface of the female gametophyte and the feeder of the embryo sporophyte of germinating *Welwitschia mirabilis* seeds (BUTLER, 1975). The pathway of reserve translocation from the female gametophyte to the embryo sporophyte is not clear. The interface between these two seed components becomes even more restricted with cells crushed by the growing embryo sporophyte, presenting a large barrier to translocation.

The embryo sporophyte continued to grow and the most noticeable feature was the continued vacuolation of the radicle (Plate 4.5 b) and hypocotyledonary cells. An unusual feature of the embryo sporophyte and female gametophyte was the relatively large amounts of food reserve material still present at this stage. It is postulated that these reserves are utilized for subsequent seedling establishment. This is feasible as the cotyledons stay in contact with the female gametophyte for up to two months after germination by which time the hypocotyl alone may exceed five centimetres in length.



The biochemical studies of germinating *Podocarpus henkelii* seeds are complementary to the ultrastructural studies, and concur with the fine structural observations. The lipid content of the germinating seeds is shown in Fig. 4.2. An interesting observation is that the levels of the lipids in both the embryo sporophyte and female gametophyte had decreased during the six weeks of storage. The percentage lipid on a dry mass basis decreased from 11 per cent to 9,2 per cent in the embryo sporophyte and from 4 per cent to 3,5 per cent in the female gametophyte. This food reserve had obviously been metabolized during storage. From the data in Tables 1.5 and 4.2 it appears that there was little qualitative change in the fatty acid components during the period of storage. In the embryo sporophyte the main fatty acids were oleic (18,3%), linoleic (43,2%) and linolenic (12,3%) acids. The major changes in concentrations of the fatty acids with germination occurred with decreases in the amounts of linoleic and linolenic acids. The amounts of icosadienoic acid and icosatetraenoic acids increased over the nine days of germination. In the female gametophyte the major fatty acids were oleic, linoleic and icosadienoic acids. The major changes during the period of incubation were decreases of oleic acid and linoleic acid, and an increase in the amount of icosadienoic acid. These changes in fatty acid levels differed from those observed in the embryo sporophyte. They tend to support the suggestion of SIMOLA (1976) that the metabolism of lipid may be different in the embryo sporophyte compared to the female gametophyte.

These results agree with those for lipid utilization in germinating Douglas fir seeds (CHING, 1966), where the

Fig.4.2

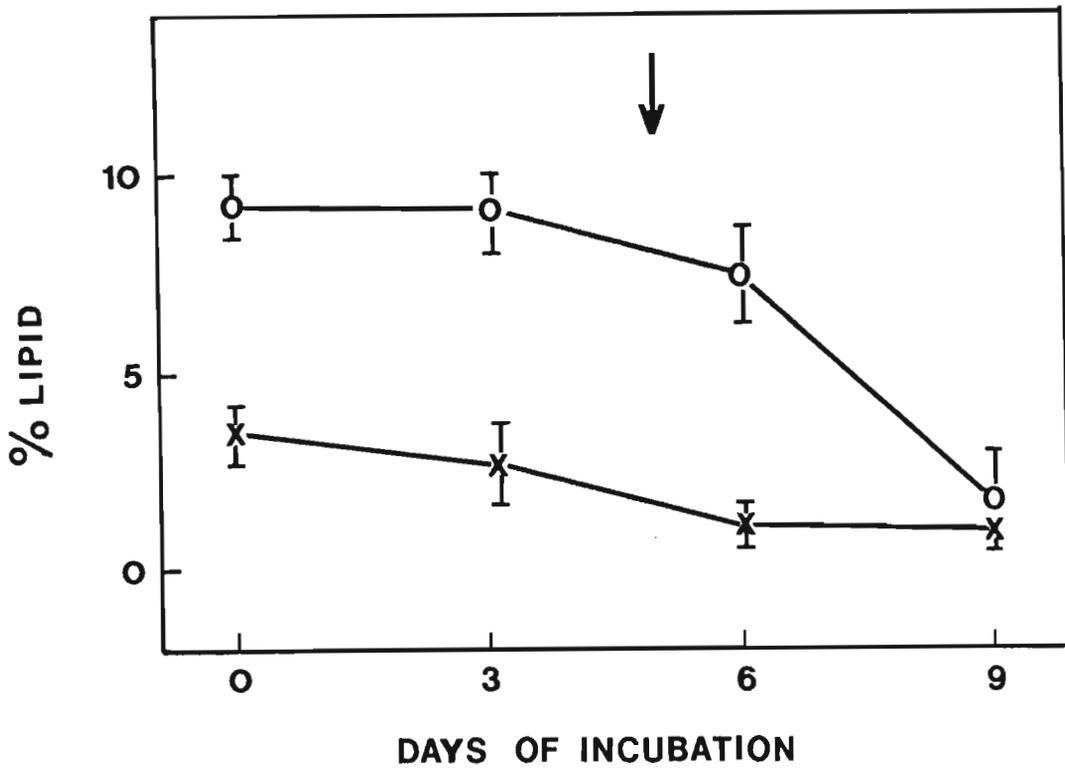


Figure 4.2. The lipid content expressed as a percent of the embryo sporophyte O-O and female gametophyte X-X of germinating *Podocarpus henkelii* seeds. Lipids were extracted with light petroleum ether in a Soxhlet apparatus. Bars represent the standard error and the arrow the first day germination was recorded.

Table 4.2. Fatty acids of the constituent lipids of germinating *Podocarpus henkelii* seeds. Δ represents the position of the double bond in the fatty acid molecule. The results are expressed as percentages of the total fatty acid content.

Fatty Acid	Symbol	Embryo Sporophyte		Female Gametophyte	
		Days of Incubation			
		0	9	0	9
Palmitic	16:0	8,2	7,2	5,5	5,1
Palmitoleic	16:1	0,4	0,2	0,2	0,2
Stearic	18:0	1,8	0,9	0,9	0,8
Oleic	18:1	18,3	17,0	21,1	15,4
Linoleic	18:2	43,2	29,4	30,8	28,4
Linolenic	18:3	12,3	4,1	4,2	5,0
Nonadecanoic	19:0	0,6	0,2	0,3	0,3
Nonadecadienoic	19:2	0,4	0,7	0,6	0,9
Arachidic	20:0	0,8	0,5	0,5	0,5
Gadoleic	20:1	0,4	1,4	1,6	1,4
Icosadienoic	Δ 8, 11 20:2	2,1	9,9	10,2	10,2
Icosadienoic	Δ 11, 14 20:2	5,8	16,2	13,4	16,1
Icosatrienoic	Δ 11, 14, 17 20:3	0,1	0,7	0,6	0,9
Icosatetraenoic	20:4	5,3	11,6	9,7	12,5
Behenic	22:0	0,1	0,2	0,2	0,1
Erucic	22:1	0,3	0,1	0,2	0,2

levels of lipid decreased rapidly during the early stages of germination. At the same time, the quantities of starch and free sugars increased. CHING (1963b) identified the fatty acids present in these seeds. Palmitic, oleic and linoleic acids were the most important fatty acids present and the levels of these changed during germination. The metabolism of lipids in germinating seeds has been fairly well researched. The lipids which are made up of triglycerides are broken down by lipases, firstly to a diglyceride and a fatty acid. These compounds are then further catabolized to a monoglyceride and two fatty acids and then ultimately to glycerol and three fatty acids. All these reactions are thought to occur in the cytoplasm. The free fatty acids may then be degraded by β -oxidation to compounds containing fewer carbon atoms. Ultimately, acetyl-CoA is produced which is incorporated into the glyoxylate pathway. The latter two reactions are thought to occur in the glyoxysome. The succinate which is produced is translocated to the mitochondrion where oxalacetate is produced. This is transported to the cytoplasm and ultimately converted to sucrose. The pathway for this conversion has been reported by CHING (1972), ORY (1969) and YAMADA and STUMPF (1965). As no glyoxysomes were observed in the germinating seeds of *Podocarpus henkelii* the pathway for gluconeogenesis in this seed may differ. The observations in Plate 4.2 c which show lipid bodies moving into vacuoles tend to support this suggestion.

The ethanol soluble fraction which contained the soluble sugars and free amino acids showed little change in either the female gametophyte or embryo sporophyte during the first nine days of incubation (Fig. 4.3). The sugars and

Figure 4.3. The ethanol soluble fraction, expressed as a percent of the embryo sporophyte O-O and female gametophyte X-X of germinating *Podocarpus henkelii* seeds. Bars represent the standard errors and the arrow the first day germination was recorded.

Figure 4.4. The total free sugars extracted with ethanol from the embryo sporophyte O-O and female gametophyte X-X of germinating *Podocarpus henkelii* seeds. The results are expressed as milligrammes of sugar per gramme dry mass. The sugars were separated by paper chromatography and quantified with the Anthrone technique. Bars represent the standard error and the arrow the first day germination was recorded.

Fig. 4.3

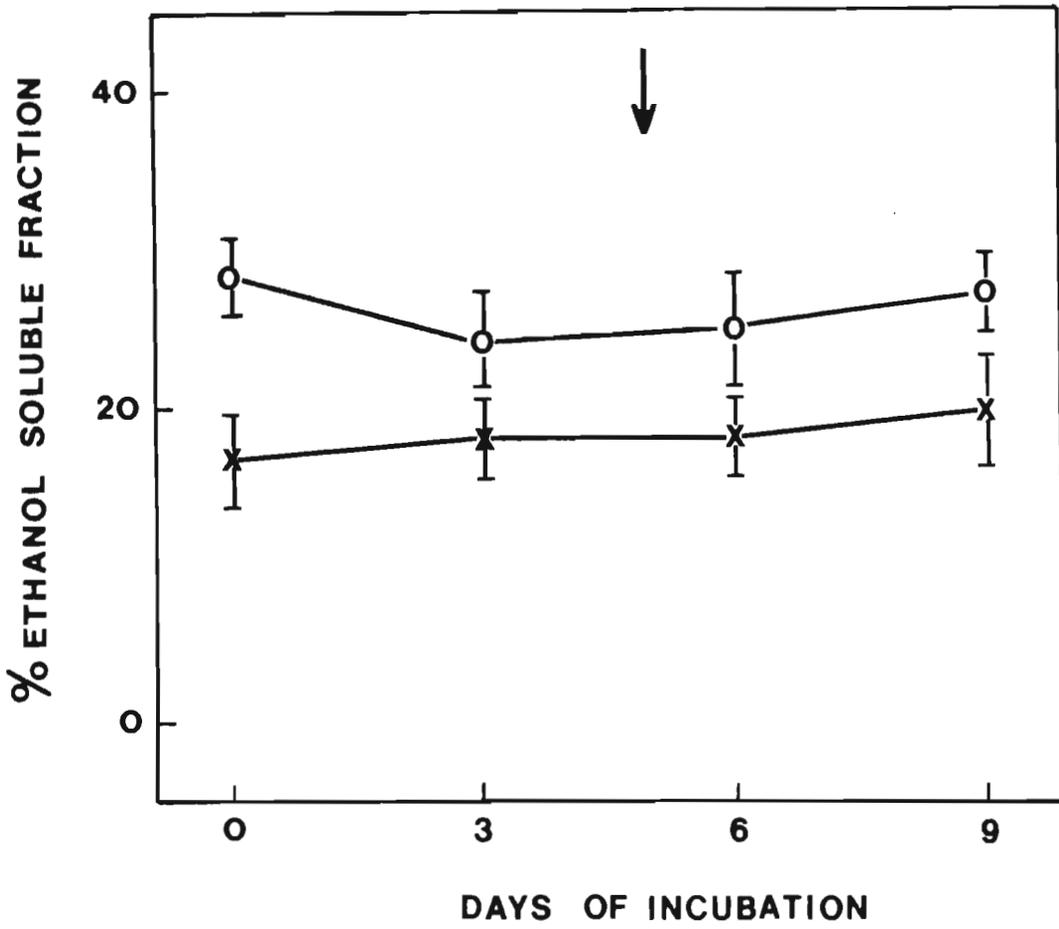
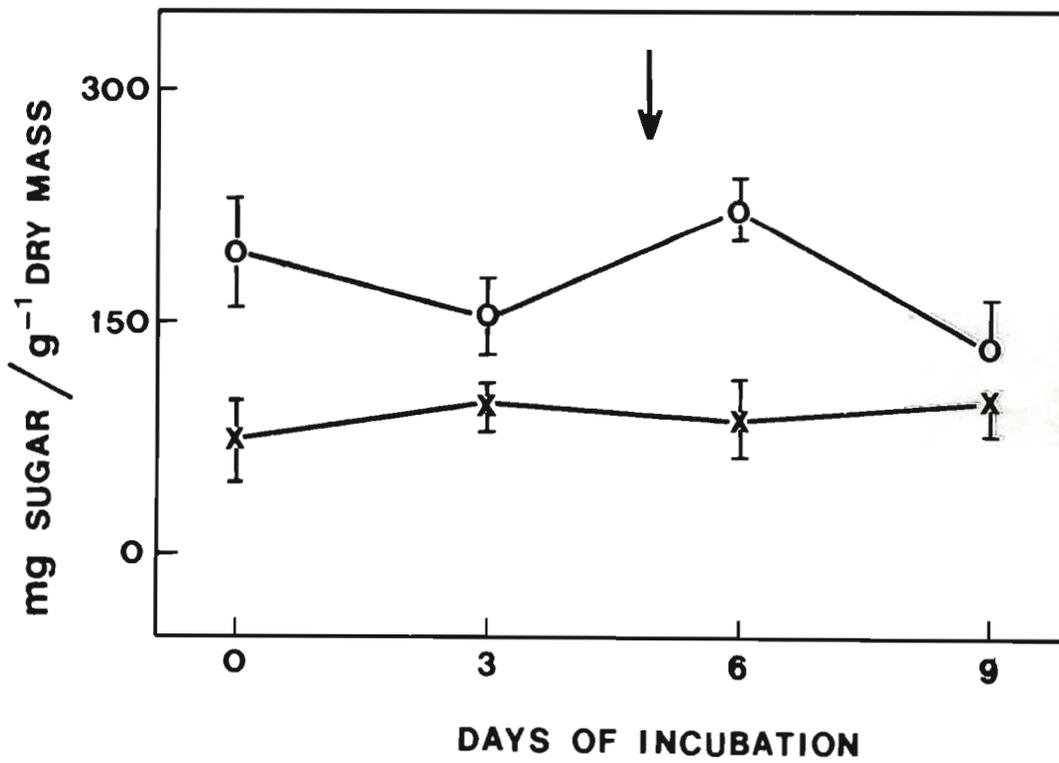


Fig. 4.4



amino acids were separated from each other using a cation exchange resin. Paper chromatography revealed that the important sugars detected in the embryo sporophyte were sucrose, maltose and ribose. The levels of sucrose increased in this seed component during the nine days of incubation (Table 4.3). Sucrose has been shown to be an important disaccharide in promoting growth of excised *Zea mays* embryos in culture (BURGHARDOVÁ and TUPÝ, 1980). Sucrose could also play an important role in the germinating *Podocarpus henkelii* embryo sporophytes. The levels of ribose fluctuated during the period of study. This was perhaps indicative of the central role that this sugar may play in the metabolism of this seed. The relatively low levels of glucose in the embryo sporophyte during germination tend to suggest that assimilation of this sugar could be important during this process. These data are similar to the reported hexose utilization in *Hordeum distichum* embryos (CAMERON-MILLS and DUFFUS, 1979).

The changes in levels of soluble sugars in *Podocarpus henkelii* seeds with germination (Fig. 4.4) are similar to those reported in *Pinus taiwanensis*, *Cunninghamia lanceolata* (KAO, 1973), *Pseudotsuga menziesii* (CHING, 1966) and *Phaseolus vulgaris* (METIVIER and PAULILO, 1980). In the aforementioned seeds, the levels of the sugars increase in the embryos during germination. At the same time the levels of the soluble sugars decrease in the endospermic tissue. There is however, little change in the level of soluble sugars in the female gametophyte of *P. henkelii* during germination. This is probably a reflection on the fact that at maturity, this seed component is ca. 73 times the size of the embryo sporophyte

Table 4.3. Soluble sugars extracted from germinating *Podocarpus henkelii* seed components. The individual sugars were separated by descending paper chromatography with butanol : ethanol : water (45:5:50) and quantified using the anthrone reagent. The free sugars are expressed as milligrammes per gramme seed component dry mass.

Free Sugar Detected	Embryo Sporophyte				Female Gametophyte			
	Days of Incubation							
	0	3	6	9	0	3	6	9
Sucrose	8,37	44,30	27,65	43,45	9,10	15,05	7,20	30,45
Glucose	0,82	15,30	19,75	9,30	40,65	33,95	23,85	30,70
Fructose	0	3,80	25,65	27,60	1,80	15,10	7,20	13,40
Maltose	38,35	23,65	22,05	44,60	5,75	7,65	8,85	6,80
Ribose	148,79	65,40	127,50	9,40	17,60	23,30	39,75	16,75
TOTAL	196,30	152,45	222,60	134,35	74,90	95,05	86,85	98,10

on a dry mass basis (Table 1.1). Consequently only a small change in the sugars in this tissue would be large in relation to the embryo sporophyte. It is interesting to note that there is an increase in the levels of starch in the female gametophyte on the sixth day of incubation. This is not accompanied by a marked decrease in the levels of soluble sugars. This increase can be accounted for by the catabolism of lipid at the same time. The products of this catabolism can be used to supplement the pool of free sugars, or for the synthesis of starch.

The levels of the free amino acids changed very slightly in the seeds of *Podocarpus henkelii* during storage (Tables 1.7 and 4.4). Little change was detected in the constituent free amino acids in either the female gametophyte or embryo sporophyte. After nine days of incubation there were increases in the levels of amino acids in both seed components. In the female gametophyte the levels of cysteine and leucine showed the largest increase with incubation. In the embryo sporophyte the levels of aspartic acid, cysteine and glycine increased during incubation. Serine was the most important amino acid in both the female gametophyte and embryo sporophyte during the period of germination studied. These increases in quantity of amino acids with germination are similar to the data of CHITTENDEN, LAIDMAN, AHMAD and WYN JONES (1978), for germinating *Triticum aestivum* kernels. As with *Podocarpus henkelii*, the largest increase in free amino acids in the germinating wheat grains occurred in the embryo.

These increases of free amino acids during germination of *P. henkelii* seeds are linked to the decline in protein

Table 4.4. Free amino acids detected in the female gametophyte and embryo sporophyte of germinating *Podocarpus henkelii* seeds. The results are expressed in μ moles per gramme dry mass.

Amino Acid	Female Gametophyte		Embryo Sporophyte	
	Days of Incubation			
	0	9	0	9
Alanine	0,474	0,596	1,150	1,230
Arginine	0,949	1,141	1,189	1,250
Aspartic acid	0,325	0,373	0,536	1,223
Cysteine	0,095	0,298	0,077	0,344
Glutamic acid	0,972	0,887	1,120	1,213
Glycine	0,055	0,046	0,042	0,162
Histidine	1,181	1,210	0,780	0,810
Iso-leucine	0,152	0,287	0,127	0,210
Leucine	0,130	0,317	0,118	0,181
Lysine	0,800	0,791	0,143	0,180
Methionine	0,051	0,036	tr.	0,050
Phenylalanine	0,048	0,228	0,028	0,084
Proline	0,105	0,230	1,079	0,640
Serine	1,250	1,250	1,230	1,248
Threonine	0,055	0,098	0,169	0,169
Tyrocine	0,025	0,132	0,169	0,143
Valine	0,037	0,036	0,210	0,840
Unknown 1	0,067	0,039	0,074	0,077
2	0,012	0,029	0,056	0,021
3	0,030	0,055	0,056	0,014
4	0,041	0,048	0,042	0,067
5	0,047	0,062	0,056	0,069
TOTAL	6,901	8,189	8,451	10,225

levels (Fig. 4.5) in both the female gametophyte and embryo sporophyte. Protein had been utilized by the seed during storage as the levels decreased during this time (Figs. 1.7 and 4.5). This is substantiated by the ultrastructural studies in which no protein was to be found in the embryo sporophyte after six weeks of storage (Plates 4.1 and 4.2). The levels of protein in the embryo sporophyte remained fairly high during the period of incubation. This contrasts with the data for germinating *Pinus taiwanensis*, *Cunninghamia lanceolata* (KAO, 1973) and *Pseudotsuga menziesii* (CHING, 1966). In these three species the levels of nitrogen increase markedly in the embryo sporophyte during germination. This reflects the increased metabolic activity of this seed component associated with rehydration and the rapid growth which accompanies germination. The relatively small changes of protein level in the embryo sporophyte of *Podocarpus henkelii* seeds during germination were probably a consequence of the high moisture content. Compared to the dry seeds, this seed is always in a fairly high state of metabolic activity. Thus when germination ensues little change in the requirement for enzymes and structural protein occurs. The levels of protein in the female gametophyte decreased during incubation and these were probably catabolized to amino acids which could supplement the amino acids and proteins of the embryo sporophyte. In germinating *Zea mays* kernels the breakdown of protein in the endosperm occurs rapidly during germination (MOUREAUX, 1979). This decrease in proteins was accompanied by an increase in non-protein nitrogen and an increase of protein in the embryo.

Figure 4.5. The protein content of the embryo sporophyte O-O and female gametophyte X-X of germinating seeds of *Podocarpus henkelii*. The results are expressed as milligrammes protein per gramme dry mass. The protein was extracted with alcoholic NaOH and quantified with the Biuret technique using B S A as a standard. Bars represent the standard error and the arrow the first day germination was recorded.

Figure 4.6. The starch content of the embryo sporophyte O-O and female gametophyte X-X of germinating *Podocarpus henkelii* seeds. The results are expressed as milligrammes starch in glucose equivalents per gramme dry mass. The starch was converted to glucose with enzymes and quantified using the Anthrone technique. Bars represent the standard error and the arrow the first day germination was recorded.

Fig. 4.5

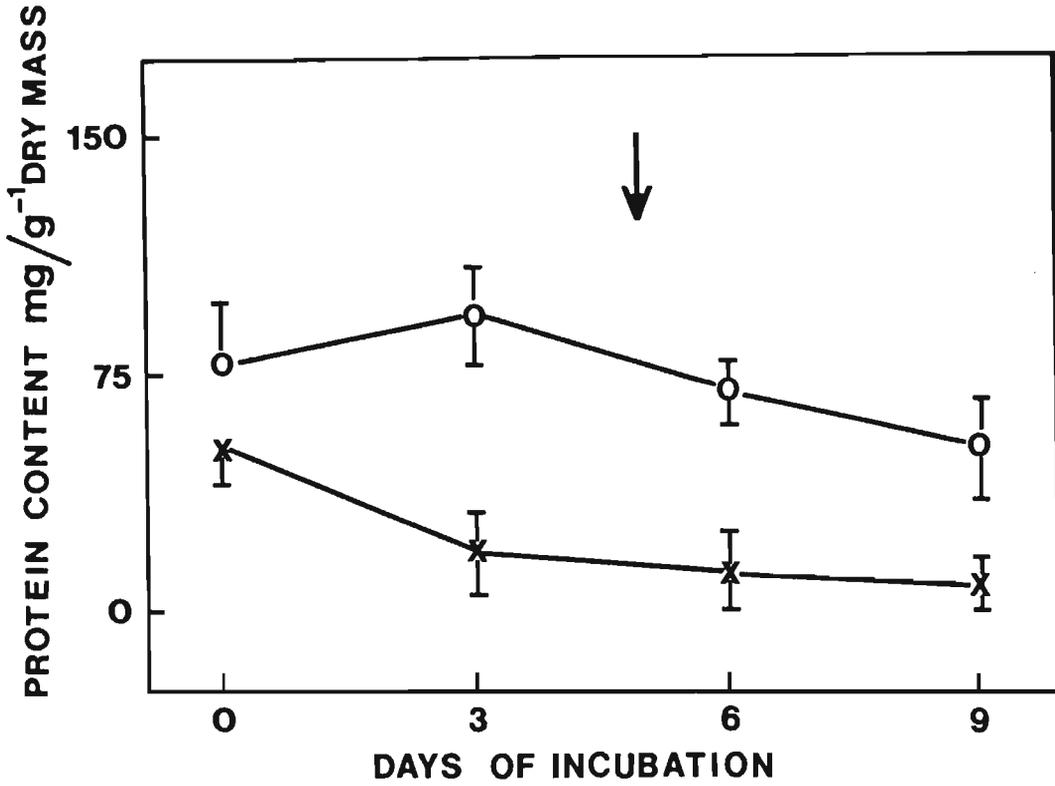
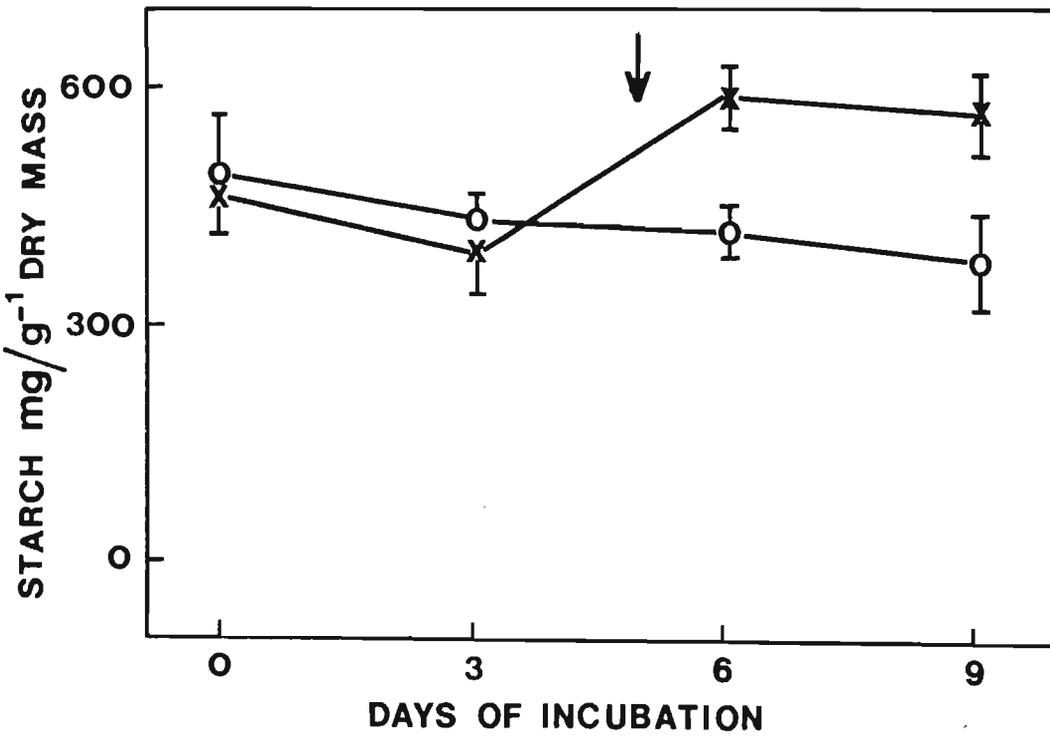


Fig. 4.6



The starch content of the germinating *Podocarpus henkelii* seeds is shown in Fig. 4.6. There was comparatively little change in the quantities over the first nine days of germination. The increase in level of starch in the female gametophyte could be accounted for by the products of lipolysis being synthesized into starch for later use. A similar pattern of lipid conversion to starch occurs in the scutellum of germinating *Hordeum vulgare* (BEWLEY and BLACK, 1978). The utilization of starch as a transitory reserve has been reported in the developing seeds of *Glycine max* by ADAMS, RINNE and FJERSTAD (1980). In other gymnospermous seeds such as *Pseudotsuga menziesii* the levels of starch in the female gametophyte decrease during germination whilst the starch content of the embryo increases (CHING, 1966).

The overall impression gained from the study of germination in *Podocarpus henkelii* seeds is of the limited utilization of the stored food reserves. In this respect, these seeds may resemble those of *Pisum sativum*. The isolated axes of these seeds are not dependent upon the reserve materials of the cotyledons for the initial stages of radicle development. The cotyledonary reserves are only drawn upon for further development of the root and shoot systems (BAIN and MERCER, 1966b). It would appear that the reserve materials remaining in both embryo sporophyte and female gametophyte after germination are utilized for subsequent seedling establishment. This is feasible, as the cotyledons remain in contact with the female gametophyte for up to two months after germination. During this time the development of the root

system is extensive and the hypocotyl alone can achieve a length of over 100 millimetres. The first leaves appear from the apical meristem around this time. The food reserves remaining in the female gametophyte and embryo sporophyte after germination are in all probability utilized for this subsequent development.

In order to establish whether or not food reserves could be translocated between both female gametophyte and embryo sporophyte in 'dormant' and germinating *Podocarpus henkelii* seeds, use was made of ^{14}C labelled compounds. This was done to assess whether reserve transfer could take place without the apparent presence of transfer tissue. Labelled sucrose was applied in a five microlitre drop (giving 345,180 dpm) to a cut on the epimatium of some seeds and in others, it was injected into the female gametophyte and embryo sporophyte respectively. The seeds were then held at a constant 25°C in the light, and the air in the storage vessel was replenished continuously. The exhaust air was passed through a carbon dioxide trap and later analysed for the presence of $^{14}\text{CO}_2$. The results in Table 4.5 show that the ^{14}C sucrose applied to all seed components was respired. The greatest amount of radioactivity recovered from $^{14}\text{CO}_2$ came from the epimatium. Lesser amounts of $^{14}\text{CO}_2$ were evolved and recorded from the female gametophyte and embryo sporophyte. This difference could be due to one of two factors; (a) the time interval for the diffusion of the $^{14}\text{CO}_2$ from deep within the seed to the surface of the epimatium, or (b) a reflection of the possible different metabolic rates of the three seed components, or both.

TABLE 4.5. Radioactivity detected as $^{14}\text{CO}_2$ evolved from the three seed components of seed of *Podocarpus henkelii* each treated with five microlitres of ^{14}C labelled sucrose and incubated in the light at 25°C. The $^{14}\text{CO}_2$ was caught in a CO_2 trap and subsequently counted for radioactivity.

Seed Component to which ^{14}C Sucrose was applied	Total dpm in 24h	Total dpm in 48h
Epimatium	51565	22522
Female Gametophyte	6653	346
Embryo Sporophyte	686	315

The percentage radioactivity recovered from the seed system is presented in Table 4.6. The ^{14}C sucrose applied to any one seed component was translocated to both the other seed components within 24 hours. The transport of sucrose out of the female gametophyte was the most rapid with only 33,63 per cent of the recovered radioactivity remaining in this seed component 24 hours after application. The large amount of radioactivity (65,54 per cent) recovered in the epimatium 24 hours after the label was applied to the female gametophyte could reflect a contamination factor. This could have been caused by the initial application of the label which was injected through a previously made hole in the epimatium, or by $^{14}\text{CO}_2$ which was released through glucolysis. After 48 hours, the radioactivity recovered showed that there had been further translocation of the ^{14}C labelled sucrose. The most noticeable feature was that only 26,21 per cent of the radioactivity applied to the embryo sporophyte remained in that organ after 48 hours. These data show that the ^{14}C sucrose applied to 'dormant' *Podocarpus henkelii* seed components was both translocated between the various seed tissues and respired. None of the seed components appeared to receive preferential loading or act as sinks. The radioactivity could have moved out of the embryo sporophyte in response to the severe wounding caused by the application of the sucrose.

Similar experiments with ^{14}C labelled sucrose and linoleic acid were conducted with scarified *P. henkelii* seeds which were then incubated on a moist substrate. The results are presented in Tables 4.7 and 4.8. The embryo sporophyte was not labelled in either case because of the risk of reducing

Table 4.6. The radioactivity detected in the seed components of *Podocarpus henkelii* seeds. The epimatium, female gametophyte and embryo sporophyte were each treated in separate batches of seeds with 5 microlitres/seed of ^{14}C sucrose. The seeds were incubated at 25°C in the light for 24 and 48 hours. Thereafter the seeds were dissected into their three component parts which were assayed for radioactivity. The results are presented as percentages of radioactivity recovered and the figures in parentheses represent the standard error.

Percentage Radioactivity Recovered in Each
Seed Component After 24h

Seed Component Sampled	Seed Component Labelled		
	Epimatium	Female Gametophyte	Embryo Sporophyte
Epimatium	82,16 \pm 33,87	65,54 \pm 9,16	18,08 \pm 6,23
Female Gametophyte	12,08 \pm 2,20	33,63 \pm 5,15	18,08 \pm 9,48
Embryo Sporophyte	5,05 \pm 1,18	10,82 \pm 2,11	64,39 \pm 22,21

Percentage Radioactivity Recovered in Each
Seed Component After 48h

Epimatium	74,09 \pm 17,49	65,26 \pm 8,54	53,55 \pm 7,86
Female Gametophyte	17,11 \pm 5,30	19,60 \pm 1,54	20,22 \pm 4,11
Embryo Sporophyte	8,75 \pm 1,95	15,12 \pm 2,94	26,21 \pm 2,62

Table 4.7. The radioactivity detected in the three seed components of *Podocarpus henkelii* seeds. The epimatium and female gametophyte were each treated in separate batches of seeds with 5 microlitres/seed of ^{14}C sucrose. The seeds were then scarified and incubated under moist conditions at 25°C for 48, 96 and 144 hours. Thereafter the seeds were dissected into their component parts which were assayed for radioactivity. The results are expressed as percentages of radioactivity recovered and the figures in parentheses represent the standard error.

Percentage Radioactivity Recovered			
Seed Component	Period of Incubation of the Seed		
	48h	96h	144h
Sampled	Epimatium Labelled		
Epimatium	86,94 \pm 10,76	65,20 \pm 8,79	46,12 \pm 3,40
Female Gametophyte	5,05 \pm 1,09	4,76 \pm 0,73	5,31 \pm 0,53
Embryo Sporophyte	8,0 \pm 2,11	30,03 \pm 9,52	48,56 \pm 1,38
% Germination	0	10	50
	48h	96h	144h
	Female Gametophyte Labelled		
Epimatium	12,23 \pm 1,72	6,70 \pm 0,05	5,28 \pm 0,37
Female Gametophyte	57,44 \pm 10,77	23,23 \pm 3,60	8,04 \pm 2,32
Embryo Sporophyte	30,31 \pm 3,54	70,05 \pm 2,74	86,67 \pm 13,86
% Germination	0	10	40

Table 4.8. The radioactivity detected in the seed components of *Podocarpus henkelii* seeds. The epimatium and female gametophyte were each treated in separate batches of seeds with 5 microlitres of ^{14}C linoleic acid. The seeds were scarified and incubated under moist conditions at 25°C for 48, 96 and 144 hours. Thereafter the seeds were dissected into their component parts which were assayed for radioactivity. The results are presented as percentages of radioactivity recovered. The figures in parentheses represent the standard error.

Seed Component Sampled	Percentage Radioactivity Recovered		
	Period of Incubation of the Seed		
	48h	96h	144h
	Epimatium Labelled		
Epimatium	100 \pm 12,8	100 \pm 15,21	100 \pm 10,21
Female Gametophyte	0	0	0
Embryo Sporophyte	0	0	0
% Germination	0	6,5	20,0
	Female Gametophyte Labelled		
	48h	96h	144h
Epimatium	49,51 \pm 25,0	14,63 \pm 6,20	16,61 \pm 8,90
Female Gametophyte	50,49 \pm 15,01	85,37 \pm 12,18	83,39 \pm 21,12
Embryo Sporophyte	0	0	0
% Germination	0	4,5	23,0

viability. The radioactivity was translocated to the two other unlabelled seed components within 48 hours of the start of incubation. It is noticeable that after 96 and 144 hours of incubation (by which time some germination was recorded), the embryo sporophyte contained the highest percentage of radioactivity. This was indicative of preferential transport to the region of active metabolism or a sink effect.

The labelled linoleic acid was not translocated between the various seed components. The radioactivity recorded in the epimatium after the ^{14}C linoleic acid had been applied to the female gametophyte was probably due to contamination during initial treatment. No radioactivity was recorded in the embryo sporophyte in any of the treatments with ^{14}C linoleic acid. These data conform to the reports of lipolysis occurring *in situ* in the region of storage. The end products of lipolysis are then translocated to other regions. The data from the radiochemical studies show that ^{14}C sucrose (or its constituent monosaccharides, glucose or fructose) is readily transported between the various seed components in both 'dormant' and germinating *Podocarpus henkelii* seeds. This translocation is achieved despite the apparent lack of any transfer tissue between the female gametophyte and embryo sporophyte, and the barrier of cell debris found in this region (Plate 1.6 d). This cell debris accumulates as the embryo sporophyte increases in size during germination.

The germination process in the seeds of *Podocarpus henkelii* does vary from the ultrastructural and biochemical data recorded in other gymnospermous seeds. The main difference appears to be related to the high moisture content of

these seeds. At the start of germination there is no rehydration of the seed tissues, thus there would be only a small demand for amino acids and proteins for the synthesis of enzymes associated with germination. There is thus little change in the amounts of protein in the embryo sporophyte during germination. The lipid reserves in both embryo sporophyte and female gametophyte are reduced during germination, but the levels of the other reserves are hardly reduced at all during this time. This suggests that these reserves are utilized for subsequent seedling establishment. This would explain the large size of the seed which contains all the reserves necessary to establish a healthy competitive seedling on the forest floor.

CHAPTER FIVE

CYTOKININS IN THE GERMINATING SEEDS OF *PODOCARPUS HENKELII*
AND THE METABOLISM OF APPLIED {8-¹⁴C} ZEATIN

It is widely assumed that seed germination is controlled by a fine balance of phytohormones (KHAN, 1977). One group of hormones which has been implicated in this process is the cytokinins. The levels of cytokinins have been shown to change in response to many germination-promoting stimuli, and this suggests that cytokinins may play an important role in the germination process. VAN STADEN, WEBB and WAREING (1972) reported that the levels of cytokinins in the seed of Sugar maple (*Acer saccharum* Marsh.) increased with stratification, this being a necessary pre-requisite for germination. The treatment of *Leucadendron daphnoides* Meisn. and *Protea compacta* seeds with high oxygen tensions resulted in a significant increase of germination. This treatment was accompanied by a rise of endogenous cytokinins in the seeds prior to germination (BROWN and VAN STADEN, 1973). Similarly a rapid increase in the amount of extractable cytokinin was reported in the seeds of *Rumex obtusifolius* Fries. which had been exposed to red light (VAN STADEN and WAREING, 1972).

In contrast to the aforementioned data, the positive germination stimulus in some seeds is accompanied by a decrease in the level of cytokinins. When lettuce seeds (*Lactuca sativa* L.) were irradiated with red light there was a decrease in the endogenous cytokinins. This decrease con-

sisted of a large decline in the level of water soluble cytokinins and a small increase in the level of the butanol soluble cytokinins (VAN STADEN, 1973). In the seeds of *Acer pseudoplatanus* L. the amounts of cytokinin also decreased with stratification, which is necessary to promote germination (WEBB, VAN STADEN and WAREING, 1973). These data show that there are changes in cytokinin levels in seeds prior to germination. No clear pattern is evident however, as both increases and decreases in the levels were recorded in response to germination-promoting stimuli.

It has been suggested that cytokinin production in plant tissues may be a natural accompaniment of cell division (GOLDACRE, 1959). Recent work with rootless *Nicotiana tabacum* L. plants by CHEN and PETSCHOW (1978) suggested that actively dividing cells may be sources of cytokinins. As seed germination requires both cell division and cell expansion, it was of interest to investigate the cytokinin changes in *Podocarpus henkelii* seeds during germination. In addition to this, no reference could be found for the investigation of cytokinins in germinating gymnospermous seeds. For these reasons both a quantitative and a qualitative study of the cytokinins in the germinating seeds of *Podocarpus henkelii* were undertaken.

Seeds of *Podocarpus henkelii* which had been stored for six weeks at 4°C were scarified and incubated on a moist substrate at 25°C. Samples were taken on days 0, 3, 6 and 9 of incubation and dissected into the epimatium, female gametophyte and embryo sporophyte respectively. The rate of

germination of the seed is shown in Fig. 4.1. On days six and nine of incubation, only seed which had germinated was sampled for cytokinin extraction. The quantitative results are expressed in two ways. The convention is to express results in nanogramme equivalents of zeatin per gramme dry mass of material. The results have been expressed as such in Table 5.1 and Fig. 5.1 respectively. As the embryo sporophyte is relatively small in relation to the other two seed components, it appears as if the cytokinin levels are very high in this seed component. The data are therefore also presented as nanogramme zeatin equivalents per seed component dry mass (Table 5.2). This does not change the trend of the results but places them in perspective in relation to the different sizes of the three seed components. The qualitative changes in the cytokinins were determined in two ways. The Rf zones displaying cytokinin activity in the soybean bioassay were compared with the Rf zones for authentic cytokinins on paper. In an attempt to elucidate some of the cytokinins in more detail, a column chromatographic system was employed. The cytokinins were first separated by paper chromatography. These were then separated into slow- and fast-moving zones (Rf's 0,1-0,5 and 0,6-0,9) respectively, and subsequently eluted through the Sephadex column. The elution volumes displaying cell division properties were then compared with the elution volumes of authentic cytokinins.

After six weeks of storage, analyses of the three seed components showed that they all contained low levels of cytokinins (Tables 5.1 and 5.2). The cytokinin levels had decreased in all three seed components during the six weeks

Table 5.1. The cytokinin activity detected in the three seed components of germinating *Podocarpus henkelii* seeds. The callus yield, calculated from the bioassays of paper chromatograms is expressed as nanogramme zeatin equivalents per gramme dry mass of seed material.

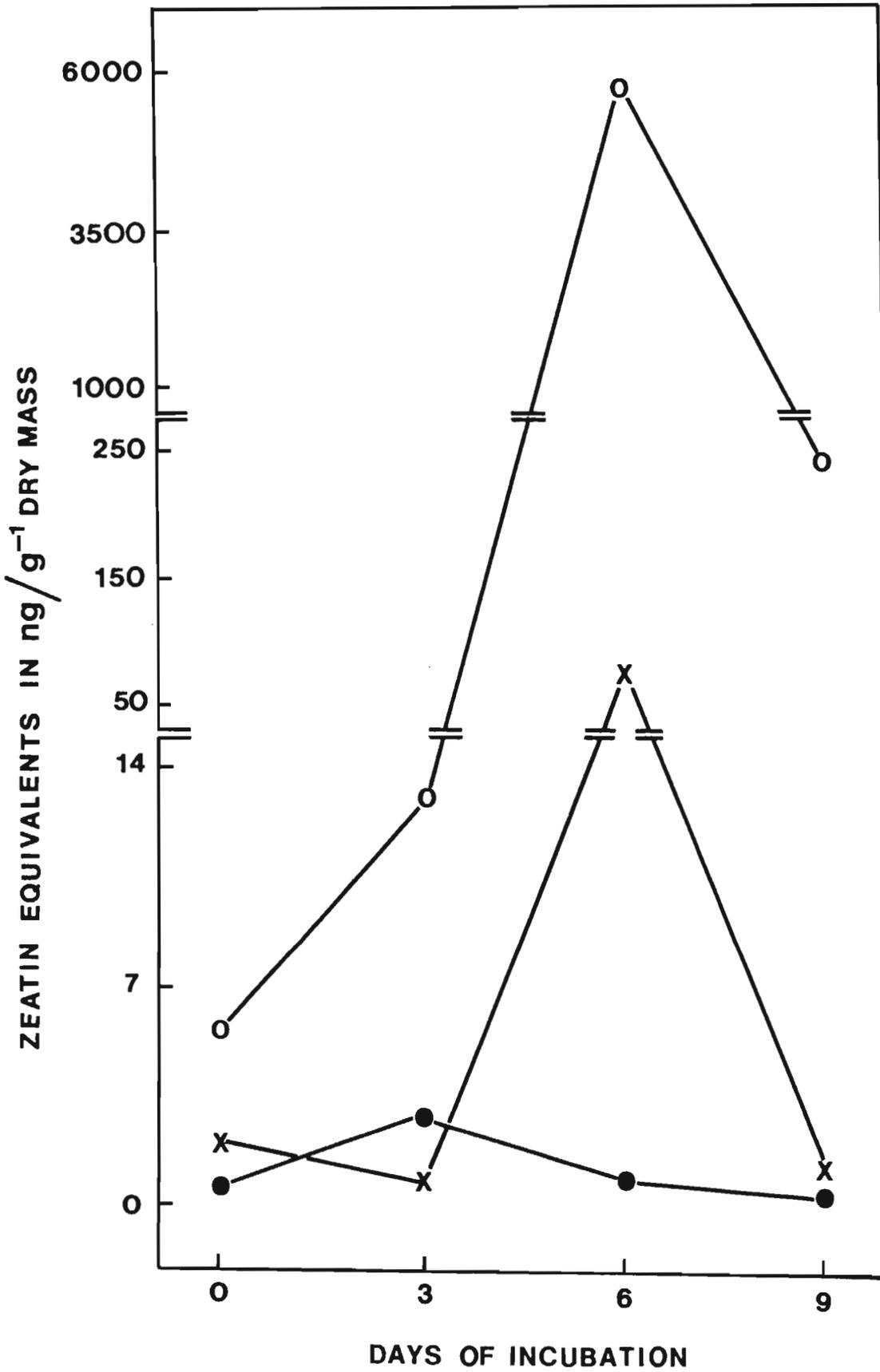
Seed Component	Days of Incubation			
	0	3	6	9
Epimatium	0,6	2,8	0,8	0,3
Female Gametophyte	2,0	0,9	75,0	1,0
Embryo Sporophyte	5,5	13,0	5750,0	240,0
Whole Seed	8,1	16,7	5825,8	241,3

Table 5.2. The cytokinin activity detected in the three seed components of germinating *Podocarpus henkelii* seeds. The callus yield, calculated from the bioassays of paper chromatograms is expressed as nanogramme zeatin equivalents per individual dry seed organelle. (See text for explanation).

Seed Component	Days of Incubation			
	0	3	6	9
Epimatium	0,9	3,9	1,0	0,3
Female Gametophyte	2,4	1,0	77,3	1,1
Embryo Sporophyte	0,1	0,3	189,8	10,8
Whole Seed	3,4	5,2	268,1	12,2

Figure 5.1. Changes in the cytokinin content of the embryo sporophyte O-O, female gametophyte X-X and epimatium ●-● of germinating *Podocarpus henkelii* seeds. The values indicating cytokinin activity were derived by expressing the callus yield from bioassays in Figs. 5.2, 5.3 and 5.4 as nanogramme zeatin equivalents (ZE) per gramme dry mass. Standards were included at time of each assay for each separate bioassay. The values of ZE represent the means of two paper bioassays.

Fig.5.1

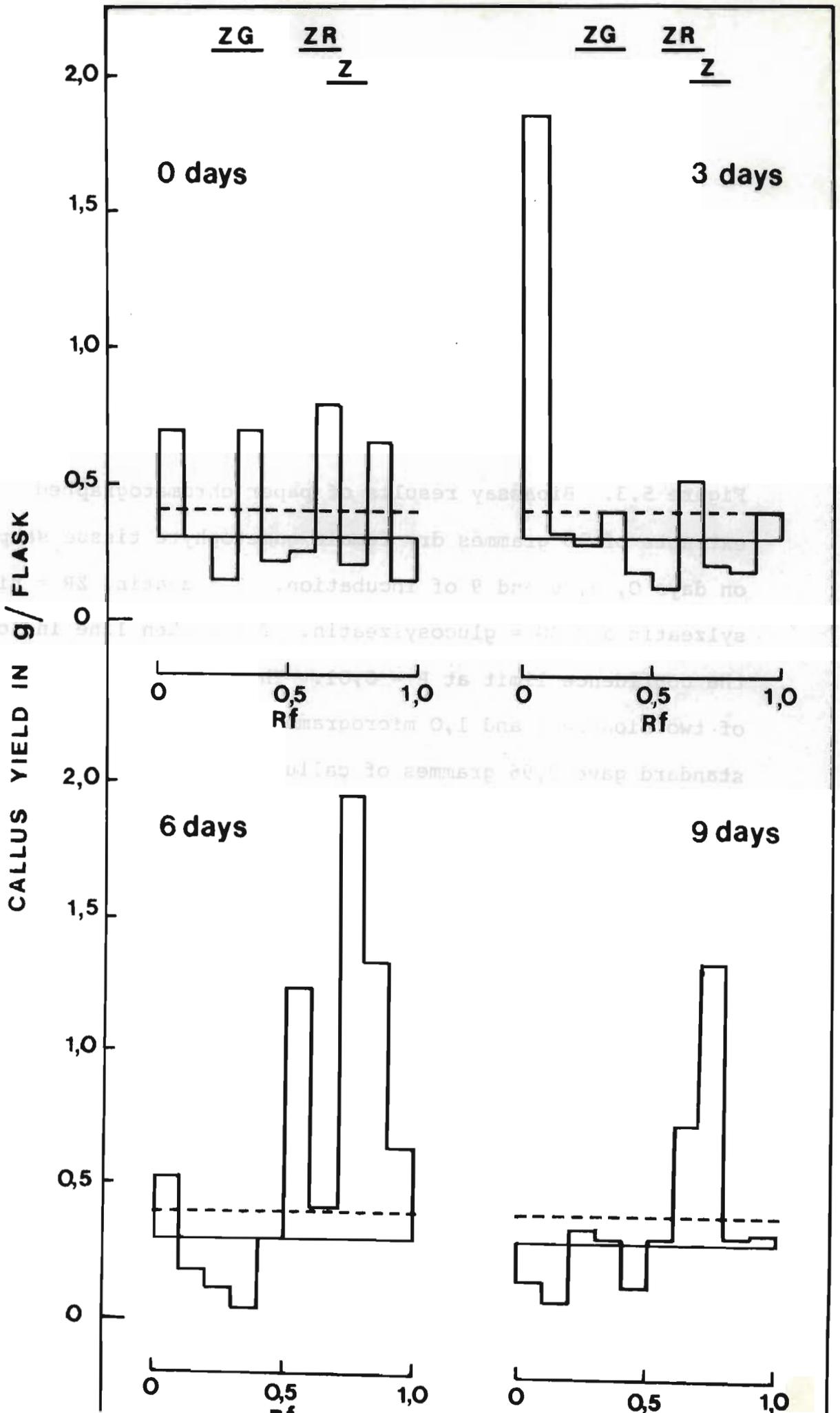


of cold storage (Tables 2.1 and 5.1). The growth or after-ripening of the embryo sporophyte reported in Chapter three was obviously not dependent upon, or accompanied by higher levels of cytokinins. Paper chromatography of the cytokinin extracts revealed that compounds which co-chromatographed with glucosylzeatin, ribosylzeatin and zeatin were present in all three seed components at this time (Figs. 5.2, 5.3 and 5.4). The levels of glucosylzeatin in all cases were lower than those recorded for the free base zeatin, and its nucleoside, ribosylzeatin. Glucosylzeatin is thought to act as a storage or bound form of cytokinin (HENSON and WAREING, 1976). The low levels of this compound in relation to the free base form are perhaps indicative of the relatively high rate of metabolic activity of this seed during cold storage. These results are in agreement with the low levels of cytokinin reported in dormant *Protea compacta* and *Leucadendron daphnoides* seeds (BROWN and VAN STADEN, 1973).

Three days after the start of incubation there were increases in the amounts of cytokinin detected in the embryo sporophyte and epimatium. The cytokinin levels in the female gametophyte decreased slightly at this time (Fig. 5.1). There was a slight change in the types of cytokinin present in the epimatium. The peak of cytokinin activity which co-chromatographed with glucosylzeatin at day zero had disappeared. The cytokinin activity detected in this seed component was all associated with ribosylzeatin and zeatin (Fig. 5.4). The increase in levels of endogenous cytokinins in the embryo sporophyte was also accompanied by a qualitative change. The peak of cytokinin activity chromatographed on paper as a very slow-moving form in Rf 0.1. In order to try and

Figure 5.2. Bioassay results of paper chromatographed extracts of 0,25 grammes dry embryo sporophyte tissue sampled on days 0,3, 6 and 9 of incubation. Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin. The broken line indicates the confidence limit at $P = 0,01$. The results are the means of two bioassays and 1,0 microgrammes per litre of zeatin standard gave 1,43 grammes of callus growth.

Fig.5.2



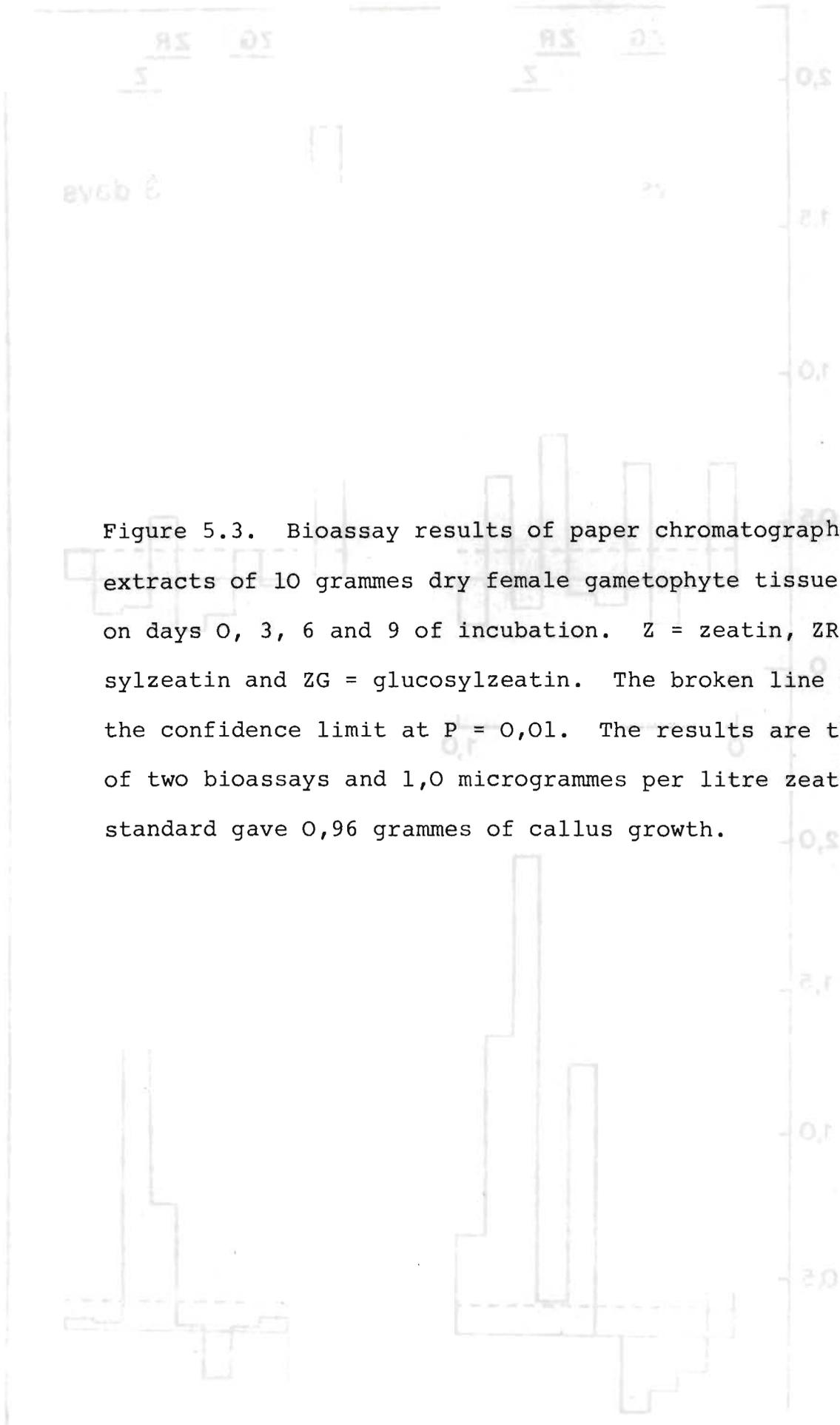
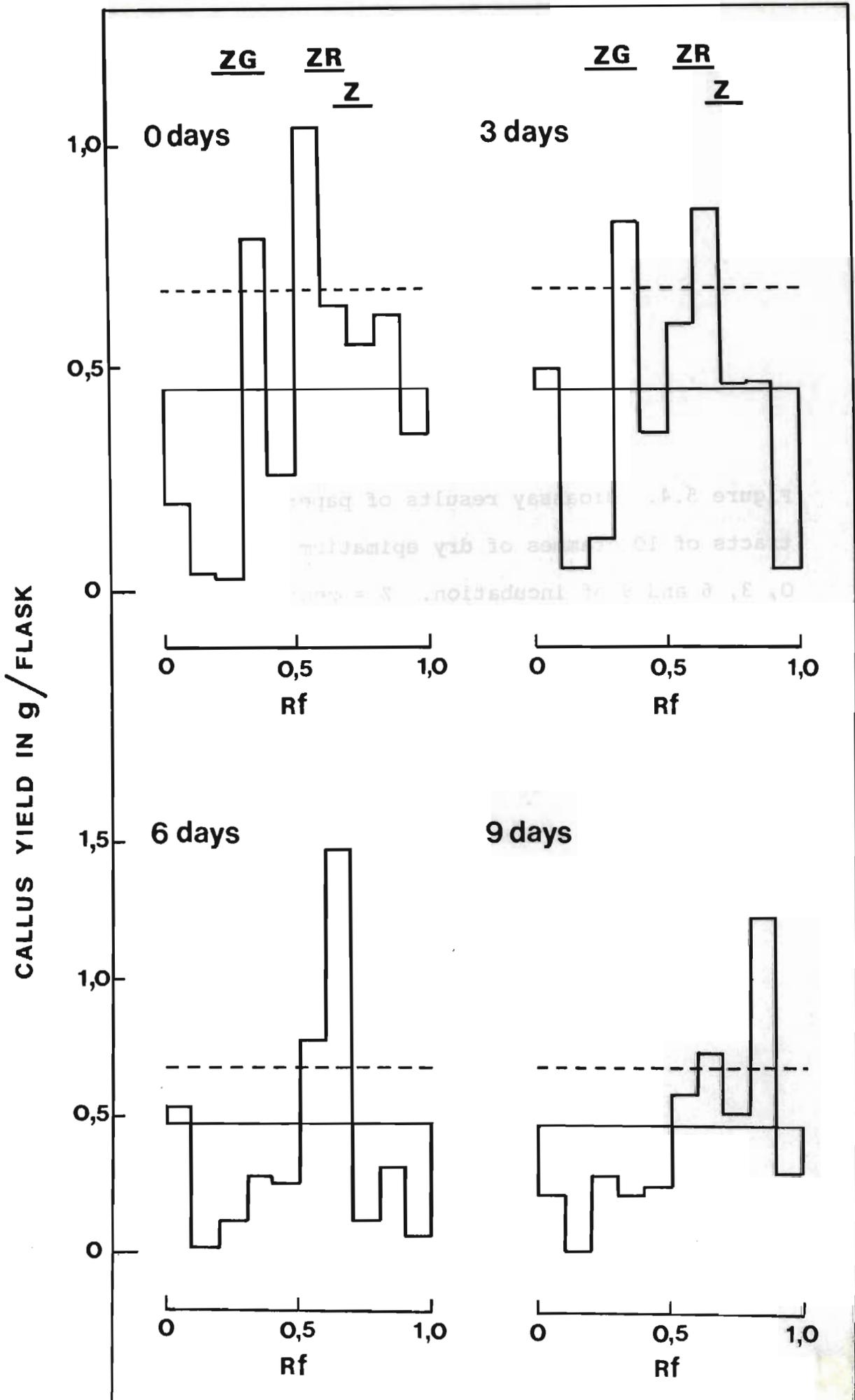


Figure 5.3. Bioassay results of paper chromatographed extracts of 10 grammes dry female gametophyte tissue sampled on days 0, 3, 6 and 9 of incubation. Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin. The broken line indicates the confidence limit at $P = 0,01$. The results are the means of two bioassays and 1,0 microgrammes per litre zeatin standard gave 0,96 grammes of callus growth.

Fig. 5.3



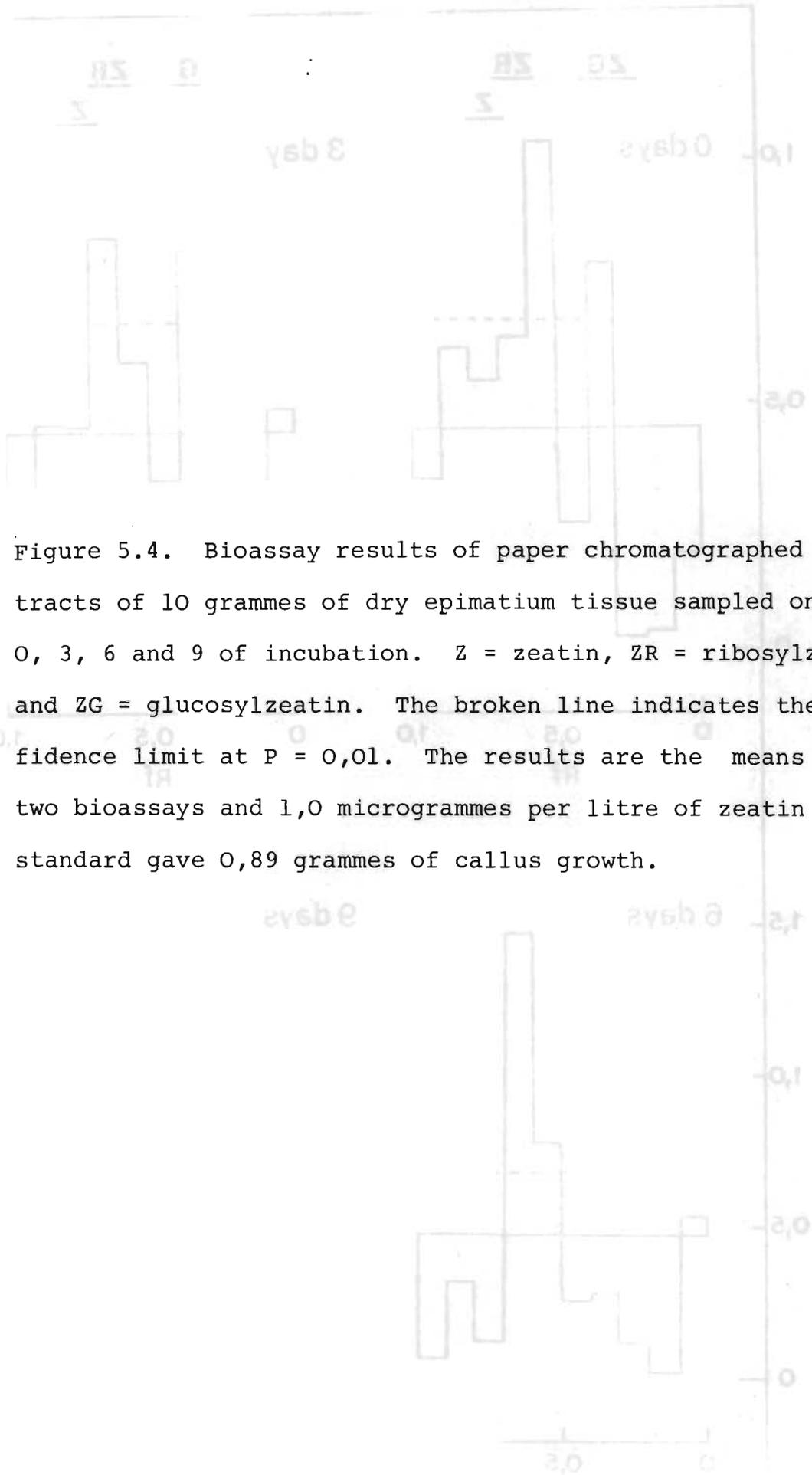
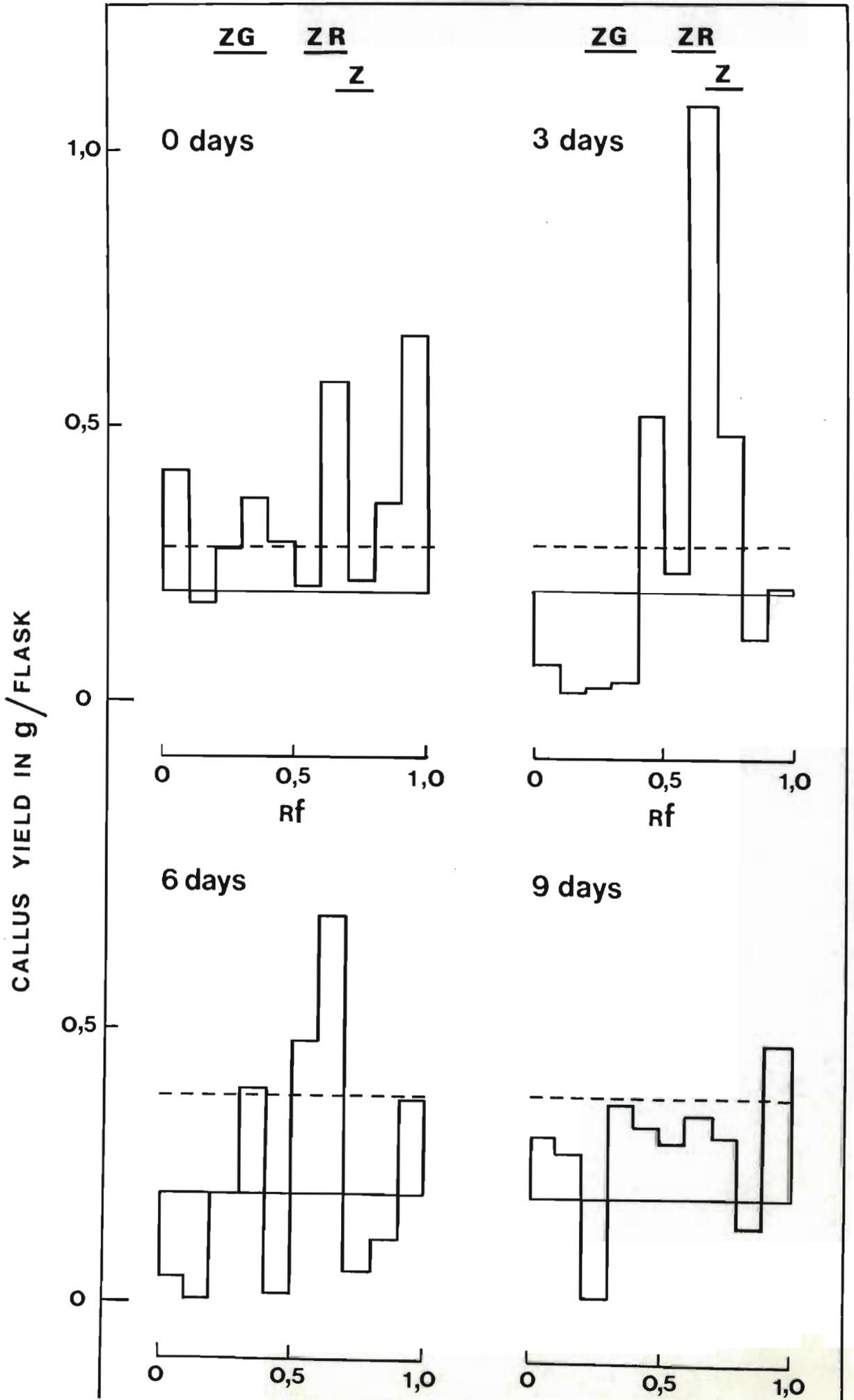


Figure 5.4. Bioassay results of paper chromatographed extracts of 10 grammes of dry epimatum tissue sampled on days 0, 3, 6 and 9 of incubation. Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin. The broken line indicates the confidence limit at $P = 0,01$. The results are the means of two bioassays and 1,0 microgrammes per litre of zeatin standard gave 0,89 grammes of callus growth.

Fig. 5.4

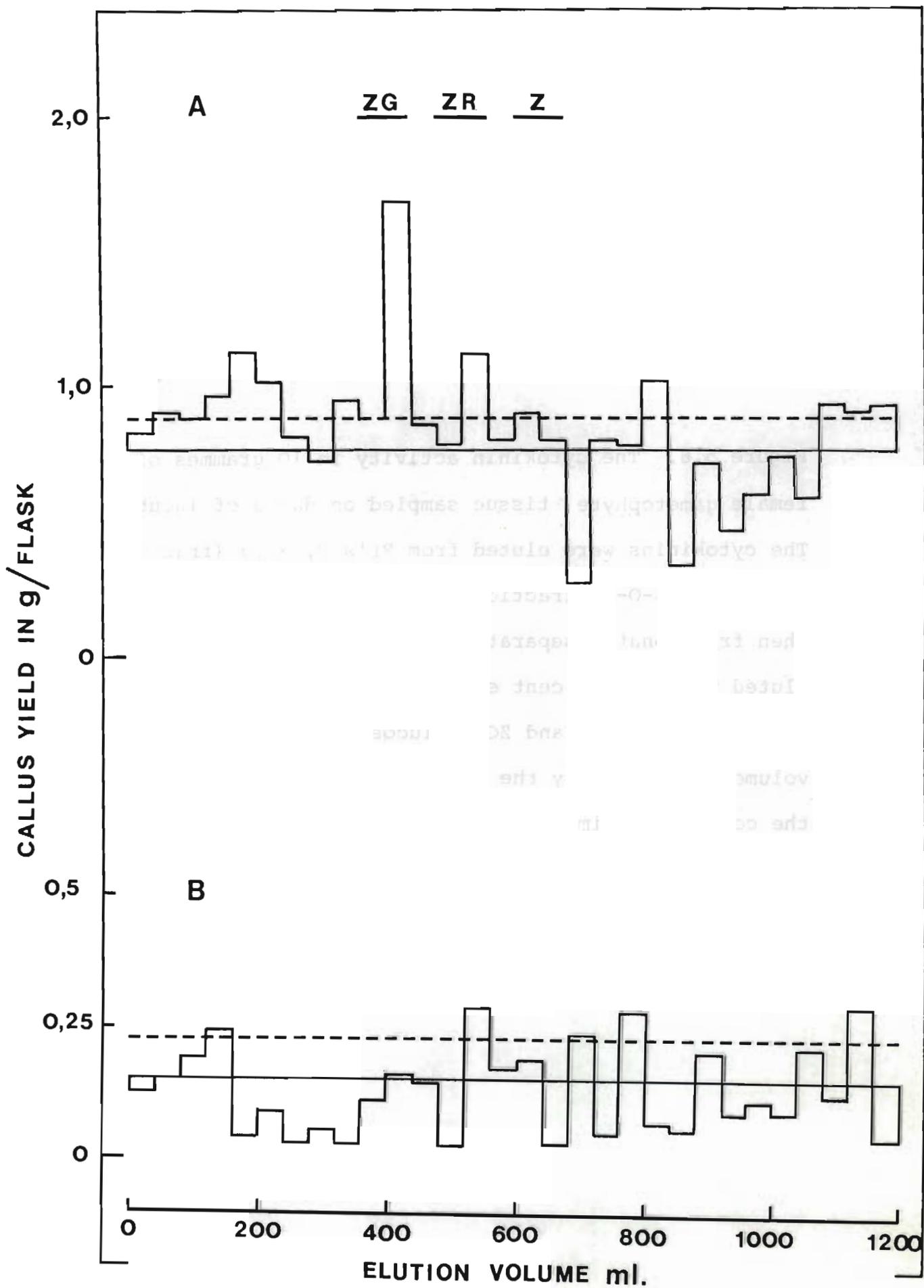


identify this peak further, the paper chromatogram was separated into a slow- (A) and fast- (B) moving fraction. The cytokinins were eluted off the paper with 35 per cent ethanol, concentrated and then eluted through a Sephadex LH-20 column with 35 per cent ethanol. The results of this exercise are shown in Fig. 5.5. Significant cytokinin activity was detected in the elution volume 160-240 millilitres. This activity arises from the peak of activity found on Rf 0,1 on the paper chromatogram. It is not known what type of cytokinin co-elutes in this volume. The other major peak of activity eluted at 400-440 millilitres and thus co-eluted with glucosylzeatin. Cytokinin activity which co-chromatographed with glucosylzeatin was not present on the paper chromatogram (Fig. 5.2). The presence of a compound which co-eluted with glucosylzeatin after Sephadex LH-20 fractionation was thus unexpected. It is suggested that the polar compound which eluted in Rf 0,1 on paper prevented clear separation of the compounds present. Thus glucosylzeatin was not able to separate as normal. The fast-moving (B) fraction displayed no really significant cytokinin activity.

The paper chromatograms of the cytokinin extract from the female gametophyte displayed cytokinin activity which co-chromatographed with glucosylzeatin, zeatin and ribosylzeatin (Fig. 5.3). This extract was also divided into both a slow- and a fast-moving fraction and subjected to column chromatography. The results obtained are shown in Fig. 5.6. In the slow-moving (A) fraction, cytokinin activity which co-eluted with glucosylzeatin (400-480 millilitres), was present. In the fast-moving (B) fraction, cytokinin activity which co-

Figure 5.5. The cytokinin activity in 0,25 grammes of embryo sporophyte tissue sampled on day 3 of incubation. The cytokinins were eluted from Rf's 0,1-0,5 (fraction A) and Rf's 0,6-0,9 (fraction B) of paper chromatograms and then fractionated separately on a Sephadex LH-20 column eluted with 35 per cent ethanol. Authentic Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin co-eluted in the volumes indicated by the bars. The broken line indicates the confidence limit at $P = 0,01$.

Fig.5.5



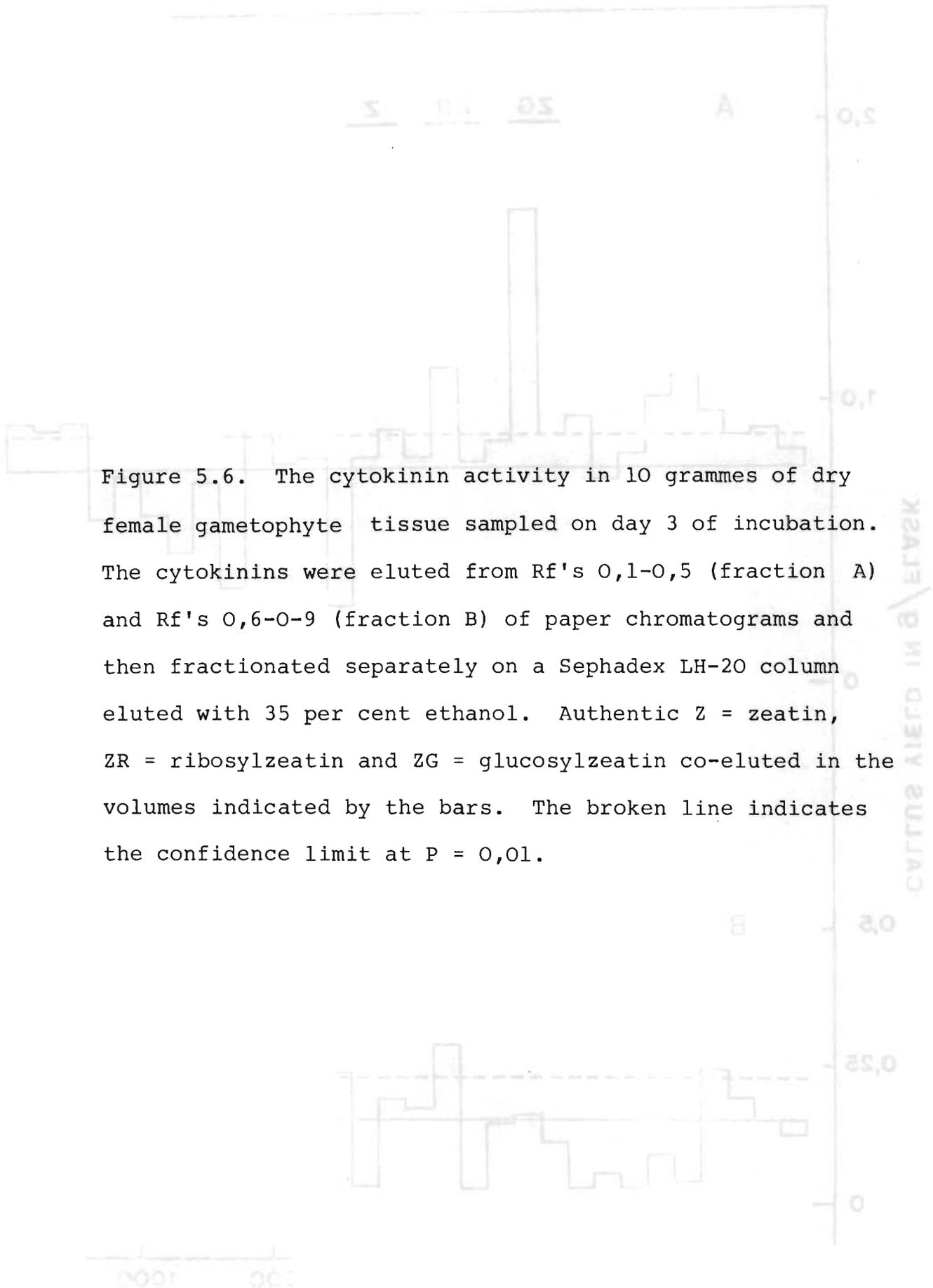
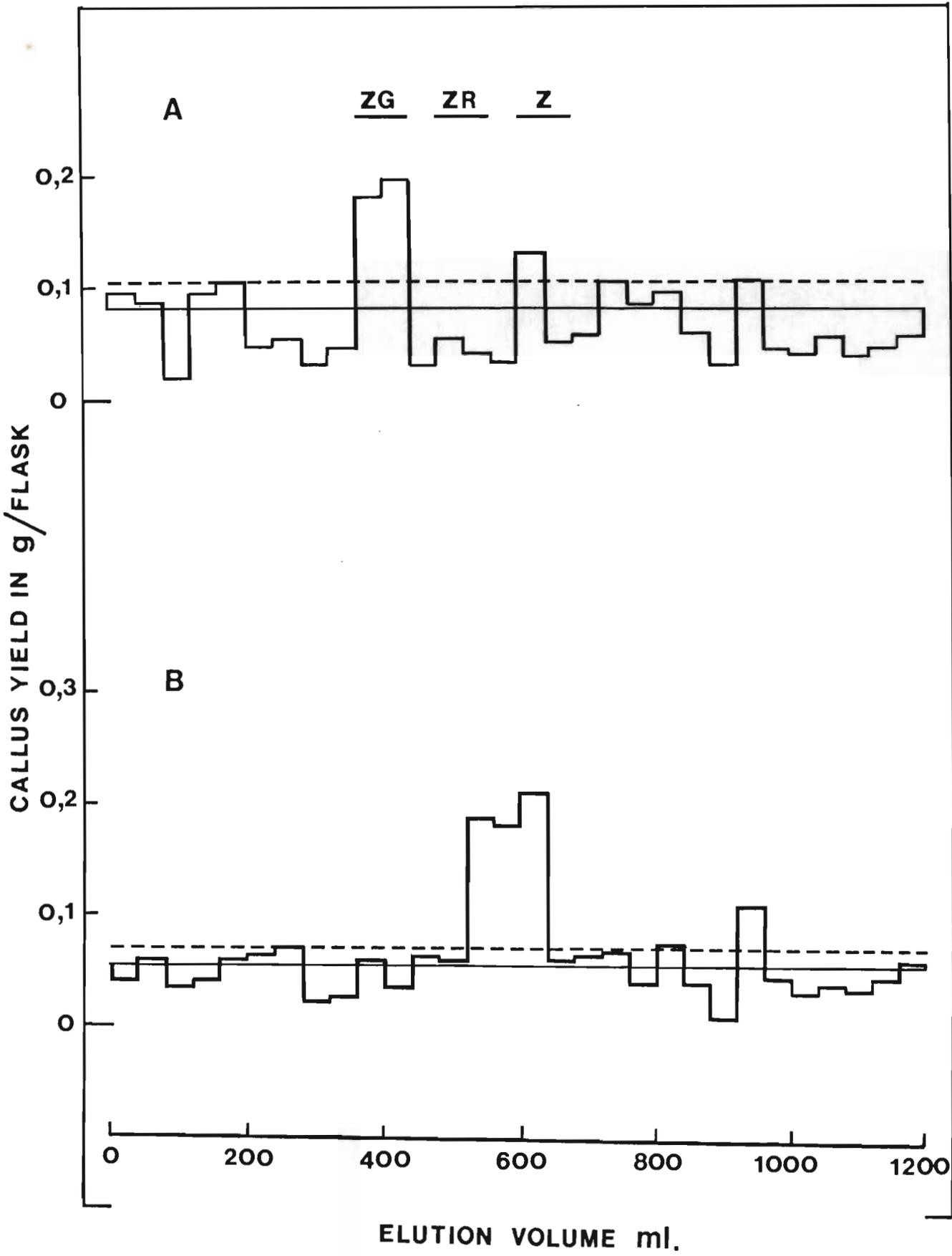


Fig. 5.6



eluted with ribosylzeatin and zeatin was present. The ultrastructural observations of the germinating *Podocarpus henkelii* seed reported in Chapter four did not show many signs of increased metabolic activity at this time.

On the sixth day of incubation ten per cent of the seed had germinated. At this time there was a substantial increase in the levels of endogenous cytokinin detected in both the female gametophyte and embryo sporophyte. At the same time the levels of the cytokinins decreased in the epimatium (Fig. 5.1). In the embryo sporophyte the increase in quantity of cytokinin was accompanied by a qualitative change as well. Compounds which on paper co-chromatographed with ribosylzeatin and zeatin were present (Fig. 5.2.). The paper chromatograms of the embryo sporophyte and female gametophyte were again separated into slow- (A) and fast-moving (B) fractions and the eluates of these were run through a Sephadex LH-20 column. The results of this study are shown in Fig. 5.7. Compounds which co-eluted with zeatin were present in the B fraction (Rf 0,6-0,9). The very high levels of this active free base cytokinin were accompanied by noticeable increases in the amounts of polyribosomes and ribosomes in the cotyledonary and hypocotyledonary cells (Plate 4.3 a and b). Similar observations of this nature have been made by WOŹNY and GWÓŹDŹ (1980) with *Cucumis sativus* L. cotyledons. These workers incubated detached cucumber cotyledons with kinetin and found that polyribosome formation was stimulated in comparison to non-kinetin treated cotyledons. CHOVEAUX, VAN STADEN

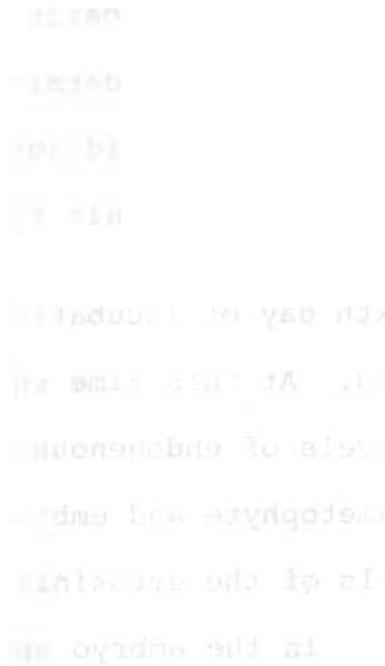
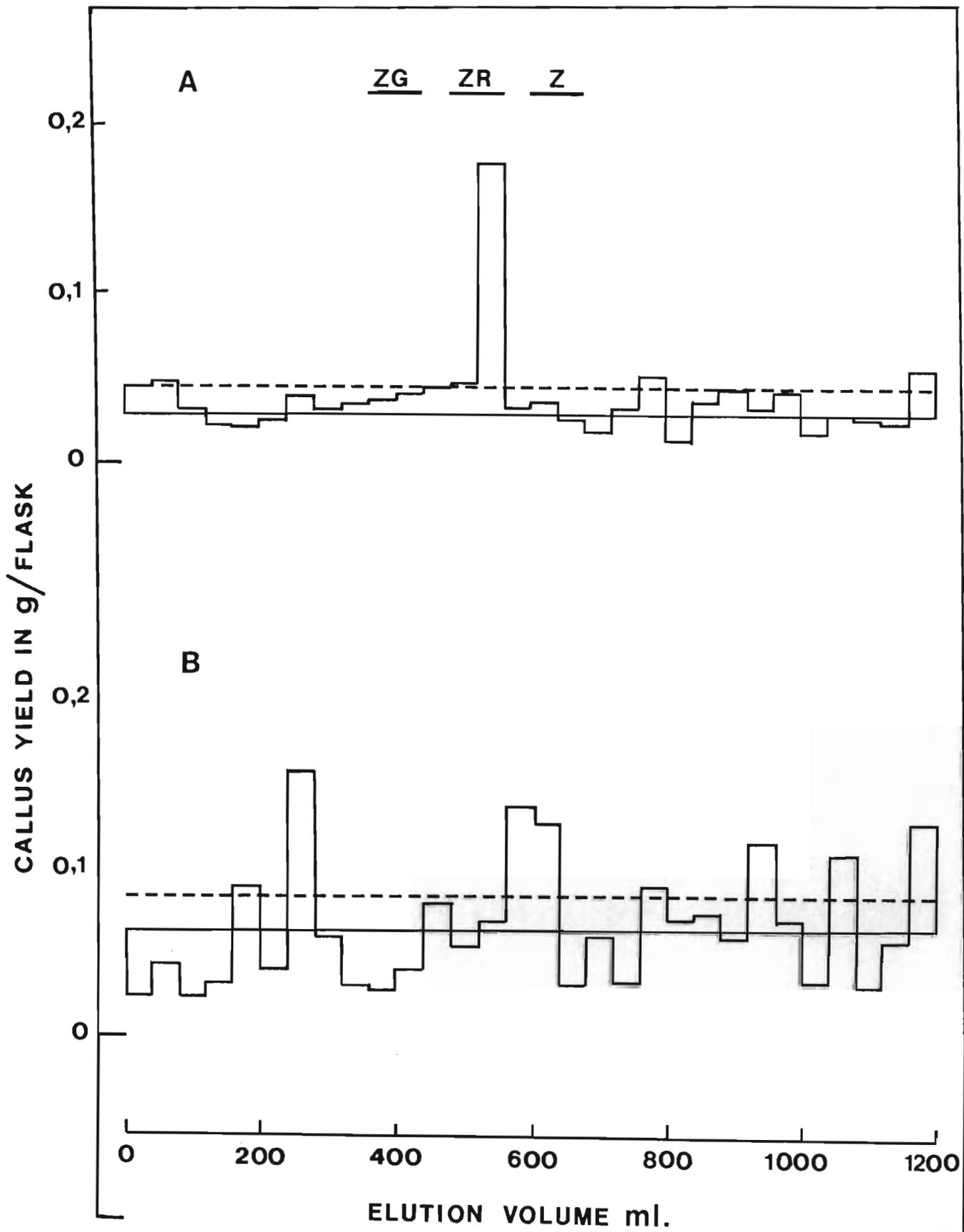


Figure 5.7. The cytokinin activity in 0,25 grammes of dry embryo sporophyte tissue sampled on day 6 of incubation. The cytokinins were eluted from Rf's 0,1-0,5 (fraction A) and Rf's 0,6-0,9 (fraction B) of paper chromatograms and then fractionated separately on a Sephadex LH-20 column eluted with 35 per cent ethanol. Authentic Z = zeatin, ZR = ribosylzeatin and ZG = glucosylzeatin co-eluted in the volumes indicated by the bars. The broken line indicates the confidence limit at $P = 0,01$.

Fig.5.7



and GILLILAND (1980) reported increases in the numbers of polyribosomes in cultured internodal bark segments which were forming callus. This cell division and polyribosome formation was accompanied by high cytokinin levels. It is thought that the polyribosomes are necessary for protein synthesis which is in turn required for enzymatic activity leading to mitosis. There is evidence to support this from the work of FOSKET, VOLK and GOLDSMITH (1977), and MUREN and FOSKET (1977), with cytokinin-induced cell division and control of protein synthesis in the cultured cells of *Glycine max*. Six days after the start of incubation, the high levels of cytokinin and observed increases in polyribosome numbers in the embryo sporophyte were in agreement with the aforementioned data.

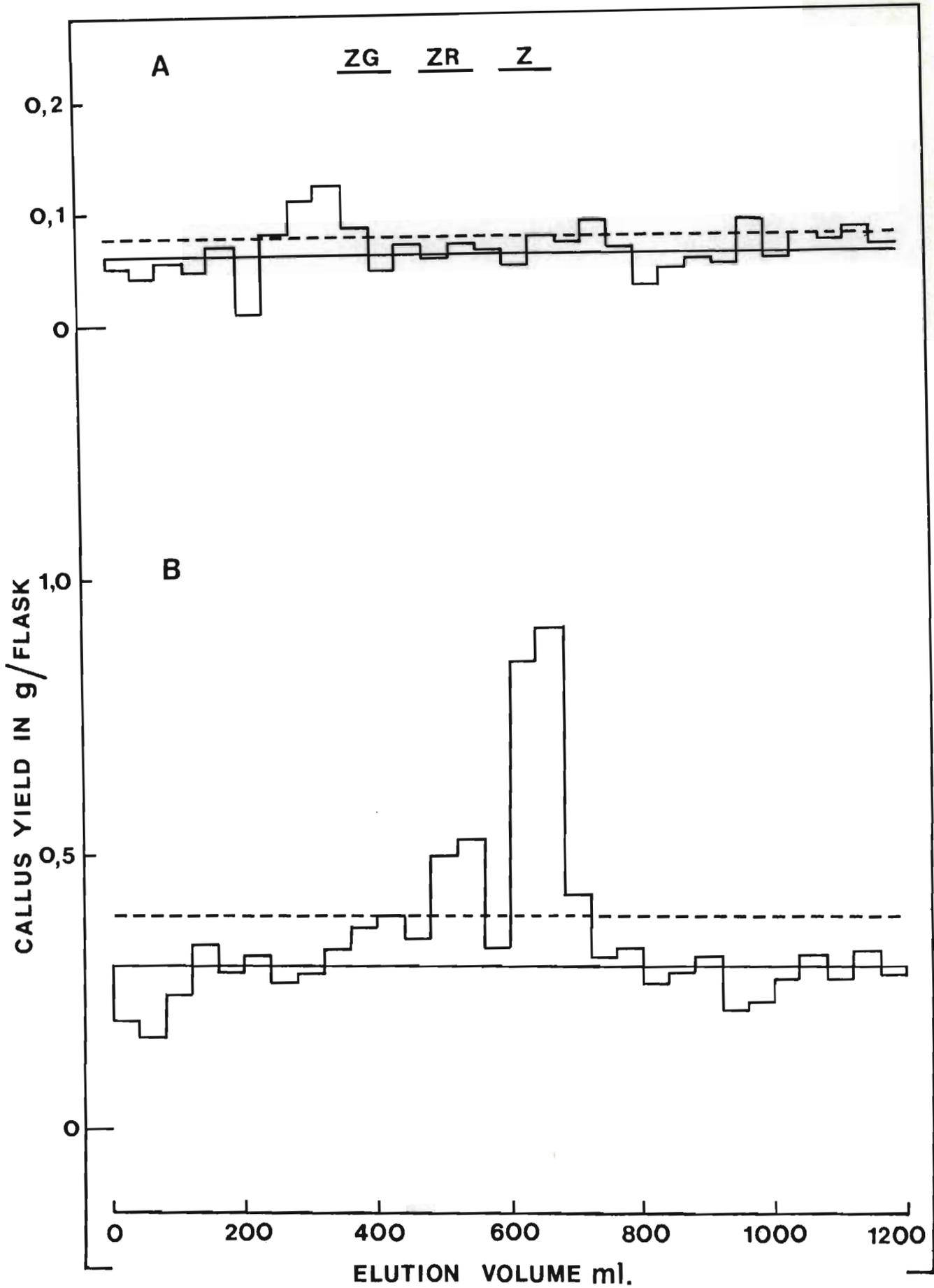
The cytokinins have been shown to be involved in the regulation of both proteolytic and amylolytic activity in germinating *Phaseolus vulgaris* seeds (GEPSTEIN and ILAN, 1979; 1980). The presence of the embryo axis was necessary to promote the activity of these two enzyme systems in the bean cotyledons. In both cases the effect of the embryo axis could be replaced by kinetin. ASHTON (1976), PENNER and ASHTON (1967b) and TSAI and ASHTON (1974) have demonstrated the effect of cytokinin in regulating protein reserve breakdown by endopeptidases and dipeptidases in *Cucurbita maxima* L. seeds. Some of this work is difficult to interpret as applied hormones have been utilized. This however, does not detract from the fact that cytokinins do play some role in the regulation of seed food reserve breakdown during germination (DIMALLA and VAN STADEN, 1977). In the germinating

seeds of *Podocarpus henkelii* the levels of protein and lipid decrease during the first nine days of incubation (Figs. 4.2 and 4.5). The levels of amino acids increase during this time (Table 4.4). Six days after the start of incubation the very high levels of cytokinin in the seed could be associated with the mobilization of some of the food reserves through the regulation of enzyme systems. The only way to verify such an hypothesis would be to monitor the levels of amylases, peptidases and lipases in relation to the cytokinin levels during germination.

In the female gametophyte there was also a large increase in the cytokinin activity recorded after six days of incubation (Table 5.1). The cytokinins detected co-chromatographed with ribosylzeatin and zeatin (Fig. 5.3). Column chromatography confirmed this, showing that the peaks displaying cytokinin activity co-eluted with ribosylzeatin and zeatin respectively (Fig. 5.8). This increased cytokinin activity was not accompanied by the same large increase in polyribosome formation noted in the embryo sporophyte (Plate 4.3). The slight increase in endoplasmic reticulum and ribosomes was only noted in this seed component nine days after incubation (Plate 4.3). The levels of protein and lipid had decreased markedly by this time. The enzymes for food reserve catabolism could have been formed in the seed component during maturation, only to be activated during germination. There would thus be no need for new protein synthesis and consequently polyribosome formation. The higher cytokinin levels could be associated solely with activation of the enzymes (ILAN and GEPSTEIN, 1981).

Figure 5.8. The cytokinin activity in 10,0 grammes of dry female gametophyte tissue sampled on day 6 of incubation. The cytokinins were eluted from Rf's 0,1-0,5 (fraction A) and Rf's 0,6-0,9 (fraction B) of paper chromatograms and then fractionated separately on a Sephadex LH-20 column eluted with 35 per cent ethanol. Authentic Z = zeatin, ZG = glucosylzeatin and ZR = ribosylzeatin co-eluted in the volumes indicated by the bars. The broken line indicates the confidence limit at $P = 0,01$.

Fig. 5.8



After nine days of incubation 45 per cent of the *Podocarpus henkelii* seeds had germinated. The levels of the cytokinins had decreased in all three seed components by this time. The largest decrease was recorded in the embryo sporophyte (Fig. 5.1, Table 5.1). Paper chromatography revealed that there was no change in the types of cytokinin present in all three seed components at this time. Once the seeds had germinated, the decline in cytokinin levels concurred with the data of BROWN and VAN STADEN (1973) for *Protea compacta* and *Leucadendron daphnoides* seed. In these seeds the levels of cytokinin increased during treatment with high oxygen tensions and decreased to a very low level once the seeds had germinated. This suggests that the cytokinins were no longer required for the subsequent growth of the seedling, or that their absence was due to their having been used as metabolites for further growth and development. The latter suggestion is feasible since there is no evidence of glucosylzeatin, which is thought to be a storage form and synthesized when excess free cytokinin is present (HENSON and WAREING, 1976; PARKER and LETHAM, 1973). The subsequent cell division and cell differentiation which accompanies seedling development does not appear to require cytokinin. The low levels of cytokinin which were present could be sufficient to maintain cell division and subsequent differentiation.

Many other seeds have a requirement for some form of environmental stimuli before they commence germination. The stimuli are often accompanied by a change in the cytokinin levels in the seeds before germination occurs (VAN STADEN and BROWN, 1973; VAN STADEN, 1973). The seeds of *Podocarpus*

henkelii are surrounded by a thick, tough epimatium which acts as a barrier to water uptake and also prevents growth of the radicle. When these two restrictions are removed by scarification the seed germinates readily. The high cytokinin levels which were recorded in both the embryo sporophyte and female gametophyte six days after scarification did not appear to be a response to this treatment. In this case it appears that they were required by the seed for germination, as the levels increased during the early stages of germination. These high levels of cytokinin could be associated with cell division (SHORT and TORREY, 1972) or food reserve metabolism. The exact role cytokinins play in the germination of *Podocarpus henkelii* seeds requires further investigation.

The high levels of cytokinin detected in the germinating seeds of *Podocarpus henkelii* indicate that this hormone must be synthesized during germination. The high levels of cytokinin in the embryo sporophyte six days after the start of incubation could have been provided by the female gametophyte. The latter seed component is ca.30 times larger than the embryo sporophyte on a dry mass basis at this time. Thus only a small quantity of cytokinin from the female gametophyte could provide sufficient cytokinins for the embryo sporophyte. This however, does not explain in which seed component the cytokinins associated with germination are synthesized. Use was made of applied {8-¹⁴C} zeatin to attempt an understanding of the transport and metabolism of cytokinins within the germinating seed of *Podocarpus henkelii*.

Seeds of *Podocarpus henkelii* were scarified and {8-¹⁴C} zeatin was applied in some of the seeds to a cut on the surface

of the epimatium, and in others it was injected into the female gametophyte tissue through a previously made hole in the epimatium. In both cases three microlitres of labelled zeatin (\pm 54 000 dpm) were applied. After application, the hole in the epimatium through which the zeatin was applied to the female gametophyte was sealed with lanolin. The seeds were placed in moist vermiculite and incubated at 25°C for 3, 6, and 9 days. Thereafter they were dissected into the epimatium, female gametophyte and embryo sporophyte respectively. These tissues were immediately extracted for cytokinins and the extracts separated with paper chromatography. The developed paper chromatograms were divided into ten equal Rf zones and each zone was assayed separately for radioactivity as previously described. The results of this study are presented in Tables 5.3 and 5.4. The data from Rf's 0,1 and 0,2 (fraction A) are combined, as are the data for Rf's 0,3-0,5 (fraction B) and Rf's 0,6-0,9 (fraction C) (Fig. 5.9).

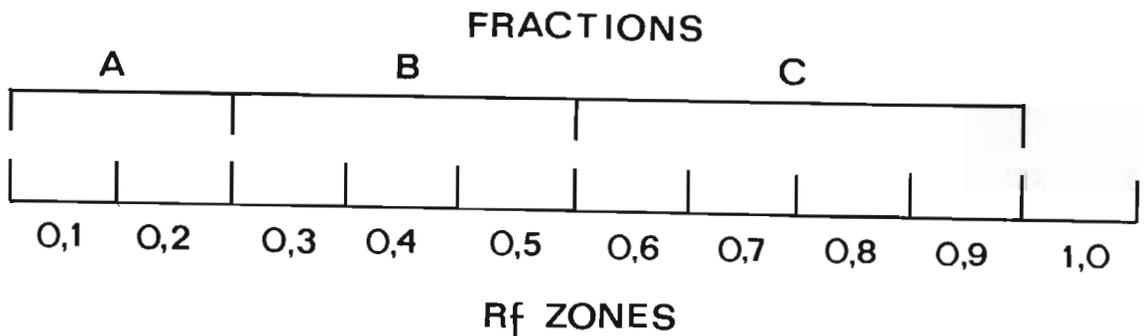


Figure 5.9. Line diagram showing how the Rf zones of paper chromatograms were divided into three fractions for subsequent column chromatography.

The results are presented as percentages of total radioactivity recovered from each of the three fractions associated with the three seed components. The total radioactivity recovered from

the seeds at each sample date is also presented as a percentage of activity in the seed components.

When {8-¹⁴C} zeatin was applied to the epimatium there was very little transport of this compound to the female gametophyte, and none to the embryo sporophyte. Nine days after the application of the labelled zeatin to the epimatium, only 1,32 per cent of the recovered radioactivity was detected in the female gametophyte (Table 5.3). The {8-¹⁴C} zeatin was metabolized within the epimatium over the nine days of incubation. Glucosylzeatin is known to run at Rf's 0,3-0,5. It is thus thought that the radioactivity in this zone was associated with glucosylated cytokinins. The compound with which the recovered radioactivity was associated in fraction A (Rf 0,1-0,2) is unknown (Table 5.3). It is not clear from the data which cytokinin is translocated from the epimatium to the female gametophyte. The initial high levels of radioactivity in fraction C (Rf's 0,6-0,9) co-chromatograph with zeatin. This tends to indicate that zeatin is translocated to the female gametophyte. The metabolism of the labelled zeatin to a polar form and the compound which runs at Rf's 0,1-0,2 apparently occurs subsequently within the female gametophyte (Table 5.3).

The {8-¹⁴C} zeatin which was applied to the female gametophyte was also metabolized to compounds which chromatographed at Rf's 0,1-0,2 and 0,3-0,5 respectively. After nine days of incubation only 22 per cent of the radioactivity remained in the Rf's which co-chromatographed with zeatin (Table 5.4). In the embryo sporophyte sample no radioactivity

Table 5.3. Radioactivity recovered from the three seed components of *Podocarpus henkelii* seeds after the application of three microlitres of {8-¹⁴C} zeatin (54 000 dpm) to a cut on the epimatium. The seeds were scarified, incubated on a moist substrate at 25°C and sampled after 3, 6 and 9 days of incubation. The seed components were then dissected out and extracted for cytokinins. The radioactivity associated with cytokinin-like compounds was assayed from the paper chromatograms. The results are expressed as percentage total recovered radioactivity in each of the three regions of the chromatogram for each seed component.

Incubation (days)	Region of chromatogram	Percentage of total radioactivity recovered		
		Epimatium	Female Gametophyte	Embryo Sporophyte
3	Fraction A Rf 0,1-0,2	10,04	3,03	0
	-			
	Fraction B Rf 0,3-0,5	12,80	8,91	0
	-			
	Fraction C Rf 0,6-0,9	77,16	88,06	0
	Percentage of total radioactivity re- covered in each seed component	98,95	1,05	0
6	Fraction A Rf 0,1-0,2	17,33	19,71	0
	-			
	Fraction B Rf 0,3-0,5	18,10	37,10	0
	-			
	Fraction C Rf 0,6-0,9	64,56	42,78	0
	Percentage of total radioactivity re- covered in each seed component	99,06	0,94	0
9	Fraction A Rf 0,1-0,2	26,42	26,35	0
	-			
	Fraction B Rf 0,3-0,5	24,61	27,39	0
	-			
	Fraction C Rf 0,6-0,9	48,96	46,25	0
	Percentage of total radioactivity re- covered in each seed component	98,68	1,32	0

Table 5.4. Radioactivity recovered from the three seed components of *Podocarpus henkelii* seeds after the injection of three microlitres of {8-¹⁴C} zeatin (54000 dpm) into the female gametophyte. The seeds were scarified, incubated on a moist substrate at 25°C and sampled after 3, 6 and 9 days of incubation. The seed components were then dissected out and extracted for cytokinins. The radioactivity associated with the cytokinin-like compounds was assayed from the paper chromatograms. The results are expressed as percentage total recovered radioactivity in each of the three regions of the chromatograms for each seed component.

Incubation (days)	Region of chromatogram	Percentage of total radioactivity recovered		
		Epimatium	Female Gametophyte	Embryo Sporophyte
3	Fraction A Rf 0,1-0,2	9,75	15,92	4,34
	-			
	Fraction B Rf 0,3-0,5	12,99	19,30	95,66
	-			
	Fraction C Rf 0,6-0,9	77,25	64,77	0
	Percentage of total radioactivity re- covered in each seed component	58,51	41,40	0,09
6	Fraction A Rf 0,1-0,2	20,98	29,23	34,42
	-			
	Fraction B Rf 0,3-0,5	20,04	38,45	63,84
	-			
	Fraction C Rf 0,6-0,9	58,97	32,31	1,72
	Percentage of total radioactivity re- covered in each seed component	32,52	65,79	1,68
9	Fraction A Rf 0,1-0,2	28,47	34,47	39,35
	-			
	Fraction B Rf 0,3-0,5	30,80	40,41	57,40
	-			
	Fraction C Rf 0,6-0,9	40,71	22,11	3,24
	Percentage of total radioactivity re- covered in each seed component	54,38	44,95	0,65

was detected in fraction C (Rf's 0,6-0,9) after three days of incubation. The radioactivity recovered was detected mainly in the Rf's 0,3-0,5. This indicates that the metabolites of zeatin were translocated from the female gametophyte to the embryo sporophyte. After six and nine days of incubation the percentage radioactivity recovered in Rf's 0,3-0,5 decreased with a corresponding increase in the amount of metabolite which chromatographed in Rf's 0,1-0,2. There does appear to be an increase in the amount of total radioactivity recovered (1,68 per cent) from the embryo sporophyte on day six (Table 5.4). This tends to suggest that the embryo sporophyte might draw on the excess of zeatin applied to the female gametophyte at this time, particularly as this was the period when the peak of endogenous cytokinin activity was recorded in this seed component (Fig. 5.1). However, the fact that most of the endogenous cytokinin activity co-eluted with zeatin and ribosylzeatin precludes this suggestion, as only 1,72 per cent of the radioactivity recovered at this time chromatographed at the Rf's 0,6-0,9. The majority of the radioactivity (63,84 per cent) chromatographed in the Rf's 0,3-0,5, which is where glucosylzeatin co-chromatographs. Another factor nullifying the above suggestion was that when $\{8-^{14}\text{C}\}$ zeatin was applied to the epimatium, none was transported to the embryo sporophyte. This indicates that the embryo sporophyte is probably self-supporting with regards to cytokinin requirements during germination.

A substantial amount of the radioactivity (up to 58 per cent) applied to the female gametophyte was recovered in the epimatium (Table 5.4). Some of this could have arisen

from cross contamination during the application of $\{8-^{14}\text{C}\}$ zeatin. It is noticeable that most of the transport of the label from the female gametophyte to the epimatium and vice versa, was in the form of zeatin. After three days of incubation 77 per cent of the radioactivity detected in the epimatium chromatographed at the region where zeatin is known to occur (Table 5.4). The actual percentages of radioactivity recovered in each seed component did not change much during the nine days of incubation. The metabolism of the labelled zeatin was not however, as static. The amount of radioactivity detected in the C fraction (Rf's 0,6-0,9) of the epimatium had decreased to 41 per cent after nine days of incubation. There was an accompanying increase in the amount of radioactivity detected in Rf's 0,1-0,2 and 0,3-0,5 at the same time. The movement of radioactivity between the female gametophyte and embryo sporophyte thus appears to be in the form of a metabolite of $\{8-^{14}\text{C}\}$ zeatin. The transport of labelled zeatin between the female gametophyte and epimatium and vice versa appears to be in the form of $\{8-^{14}\text{C}\}$ zeatin.

In order to try and elucidate the metabolites of the applied $\{8-^{14}\text{C}\}$ zeatin a new column chromatographic technique was undertaken utilizing Sephadex LH-20. This was swollen and eluted with ten per cent methanol (HUTTON and VAN STADEN, 1981). The sample studied was taken from the female gametophyte component of seeds to which $\{8-^{14}\text{C}\}$ zeatin had been applied to the female gametophyte. After incubation for three days the seeds were sampled and extracted for cytokinins as previously described. The paper chromatogram from this extract was divided into three regions, fractions A, B

and C as indicated previously. These three regions were separated from each other and the radioactivity eluted off the paper with increasing concentrations of ethanol. After concentrating the eluates they were applied separately to the Sephadex column. Ten millilitre fractions were collected off the column, and four of these were combined together. Two millilitres of each sample were assayed for radioactivity and the remaining 38 millilitres were tested for biological activity using the soybean callus bioassay. The biological- and radioactivity of the three fractions are shown in Figs. 5.10, 5.11 and 5.12.

The radioactivity detected in fraction A (Rf's 0,1-0,2) appears to be made up of two compounds (Fig. 5.10). The one metabolite ($8\text{-}^{14}\text{C}$) zeatin elutes from the column from 200-240 millilitres. This metabolite does not appear to have any biological activity as there was no growth of the soybean bioassay in this region. The second peak of both biological- and radioactivity eluted from the column at 640-720 millilitres. Authentic adenosine co-elutes in this volume from the same column. There was a peak of only biological activity which eluted between 920-960 millilitres. This is the region in which authentic adenine co-elutes. The seeds of *Podocarpus henkelii* had thus metabolized the applied ($8\text{-}^{14}\text{C}$) zeatin to compounds which co-eluted with adenine and adenosine and an unknown compound. The compound which co-eluted with adenine, and displayed biological activity could possibly have been part of the complement of endogenous cytokinins present within the seed. There is little possibility that the compound could have been adenine, as MILLER (1965) has shown that this compound is not active in the soybean callus bioassay.

Figure 5.10. The cytokinin activity (histogram) and radioactivity ●-● detected in extracts of the female gametophyte tissue on Rf's 0,1-0,2 (fraction A) of paper chromatograms following fractionation on a Sephadex LH-20 column eluted with 10 per cent methanol. $\{8-^{14}\text{C}\}$ zeatin was injected into the female gametophyte and the scarified seeds sampled after three days of incubation at 25°C. ADO = adenosine and ADE = adenine. The broken line indicates the confidence limit at $P = 0,01$ for the soybean bioassay.

CALLUS YIELD IN g/FLASK

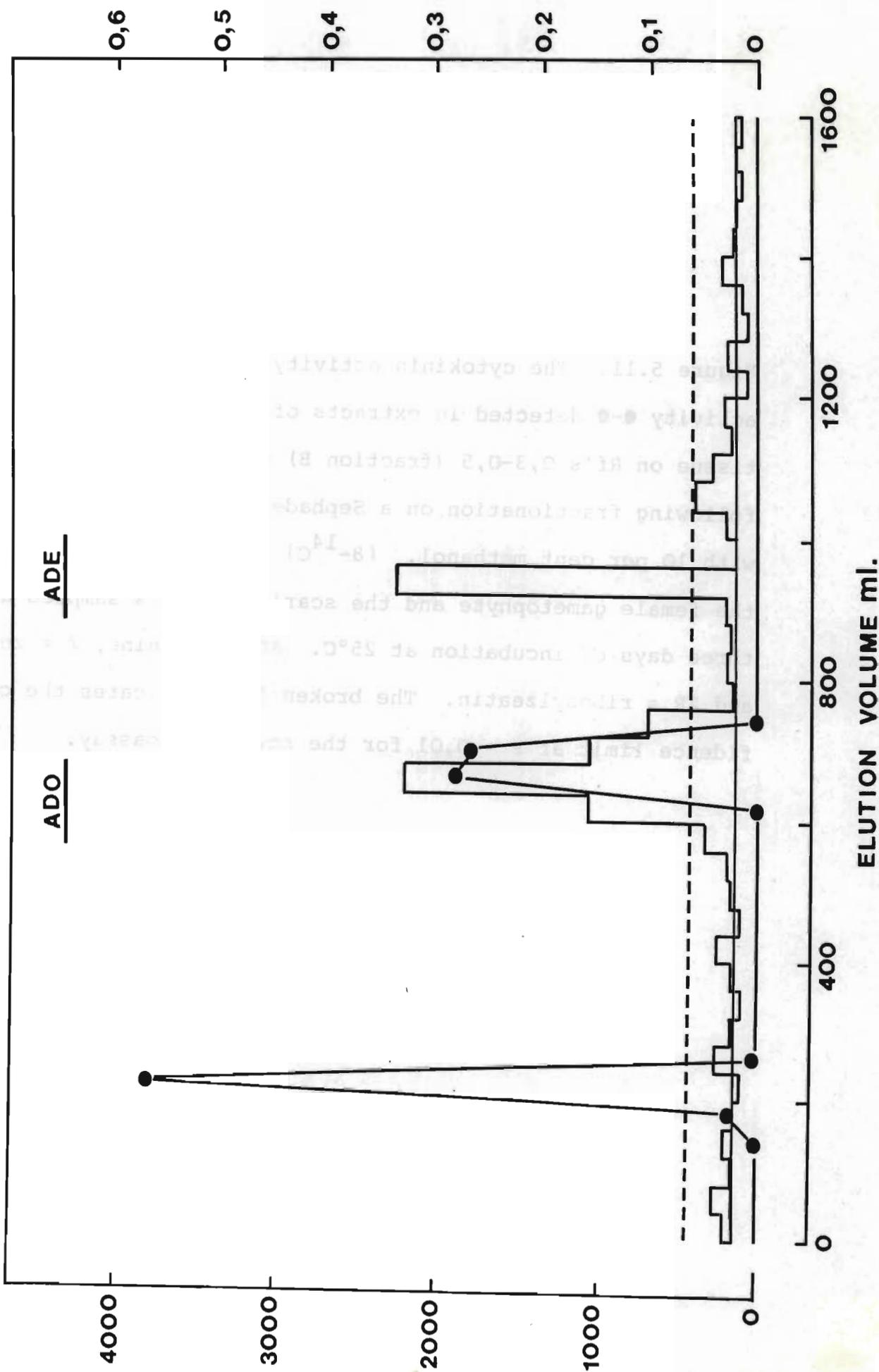


Fig. 5.10

incubation at 25°C. The broken...

Figure 5.11. The cytokinin activity... detected in extracts of... on RL's 0.3-0.5 (fraction B) and fractionation on a Sephadex... (8-14C)...

САГЛОНА ЛИБРО ИИ А\ЕРУАК

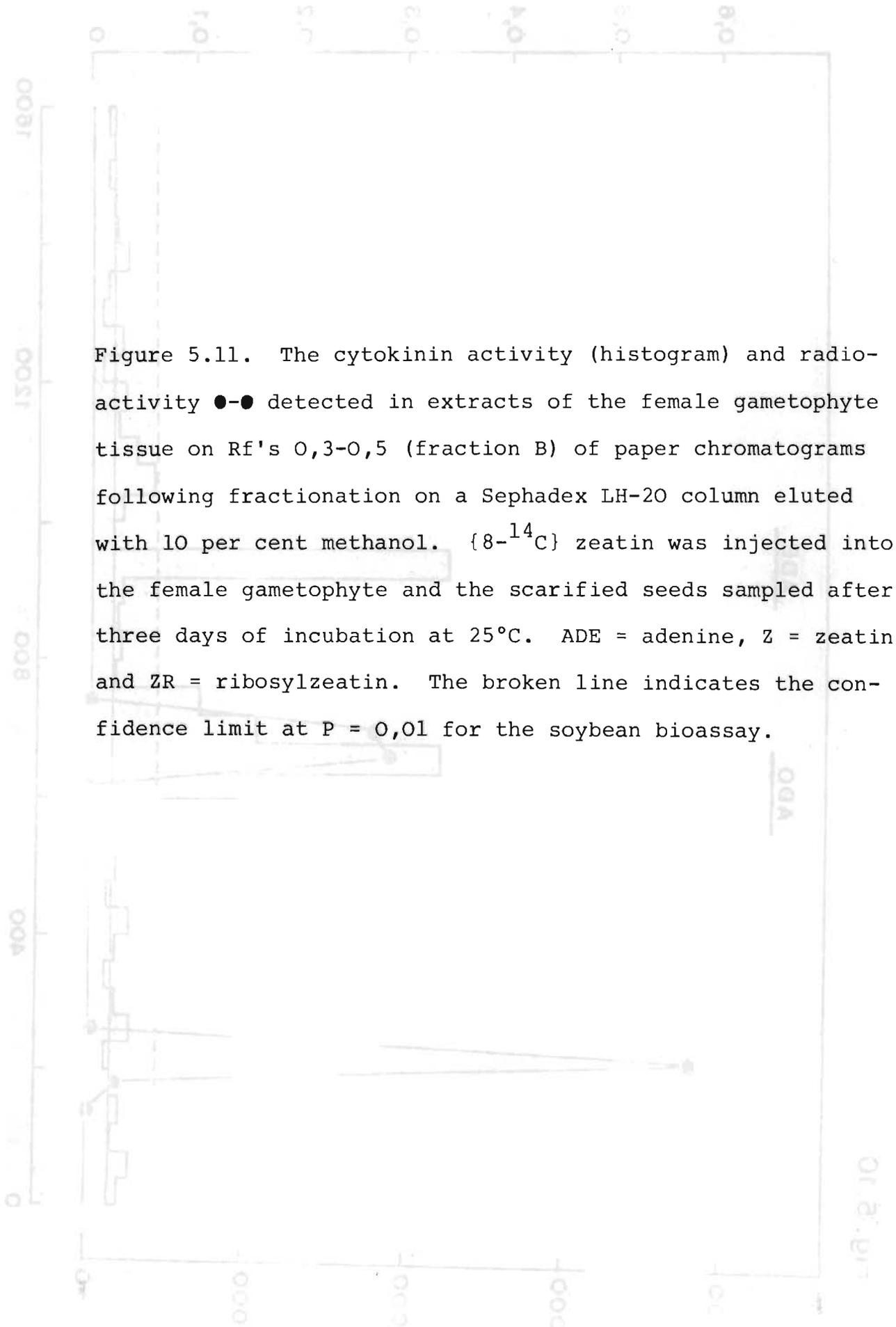


Figure 5.11. The cytokinin activity (histogram) and radioactivity ●-● detected in extracts of the female gametophyte tissue on Rf's 0,3-0,5 (fraction B) of paper chromatograms following fractionation on a Sephadex LH-20 column eluted with 10 per cent methanol. $\{8-^{14}\text{C}\}$ zeatin was injected into the female gametophyte and the scarified seeds sampled after three days of incubation at 25°C. ADE = adenine, Z = zeatin and ZR = ribosylzeatin. The broken line indicates the confidence limit at $P = 0,01$ for the soybean bioassay.

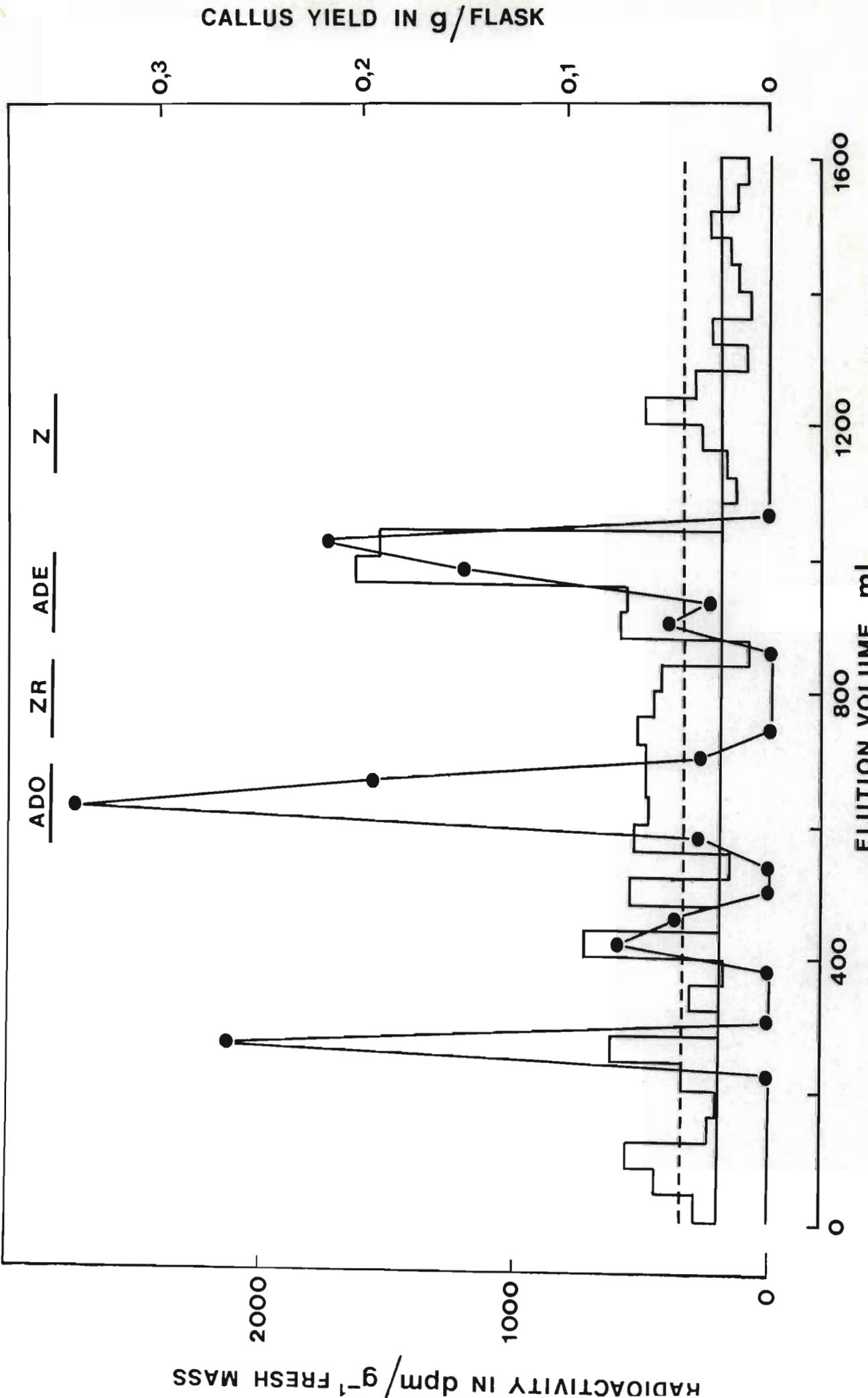


Fig. 5.11

In the B fraction (Rf's 0,3-0,5) Fig. 5.11 which was eluted through the column, several peaks were recorded which displayed both biological and radiochemical activity. The peak which eluted between 240-280 millilitres is thought to be the same peak recorded in the A fraction which ran between 200-240 millilitres. There was a small amount of biological activity associated with this peak. The second peak which displayed both biological- and radioactivity eluted between 400-440 millilitres. It is known that authentic glucosylzeatin runs on paper chromatograms between Rf's 0,3 and 0,5. Glucosylzeatin could thus be expected to have run in this fraction. No authentic glucosylzeatin was available at the time of study to run through this chromatographic system, so its elution volume is unknown. Hence it cannot be stated whether or not this metabolite would co-elute with glucosylzeatin. The third peak of radioactivity co-eluted with adenosine. Associated with this peak was a broad flat peak of biological activity which is thought to be the result of an endogenous cytokinin which co-elutes with ribosylzeatin at 720-840 millilitres. The fourth peak of both biological- and radioactivity elutes off the column between 960-1040 millilitres which corresponds partly to the elution volume for adenine. There was one further small peak exhibiting biological activity only. This peak co-eluted with authentic zeatin between 1240-1280 millilitres. The B fraction off paper thus appears to contain the same compounds as the A fraction except for a compound which co-elutes with adenine and the presence of one further unknown compound.

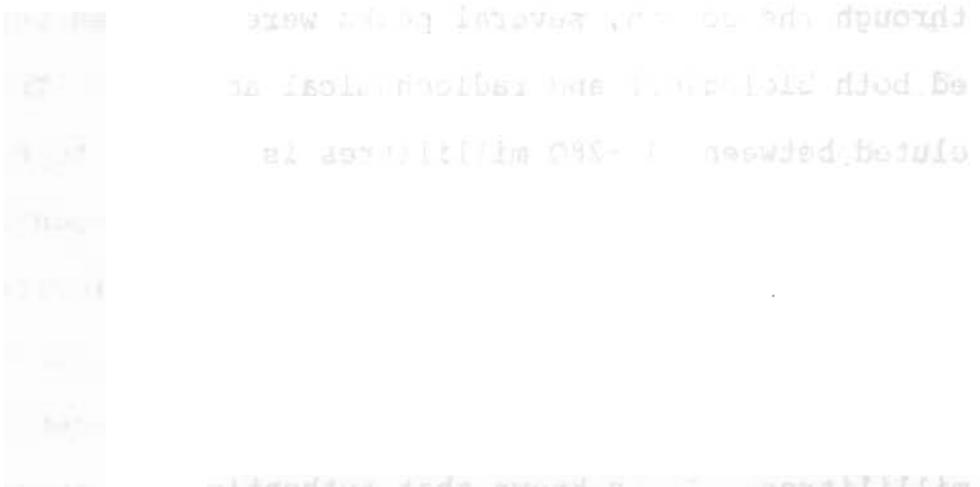


Figure 5.12. The cytokinin activity (histogram) and radioactivity ●-● detected in extracts of the female gametophyte tissue on Rf's 0,6-0,9 (fraction C) of paper chromatograms, following fractionation on a Sephadex LH-20 column eluted with 10 per cent methanol. {8-¹⁴C} zeatin was injected into the female gametophyte and the scarified seeds sampled after three days of incubation at 25°C. ZR = ribosylzeatin and Z = zeatin. The broken line indicates the confidence limit at P = 0,01 for the soybean bioassay.

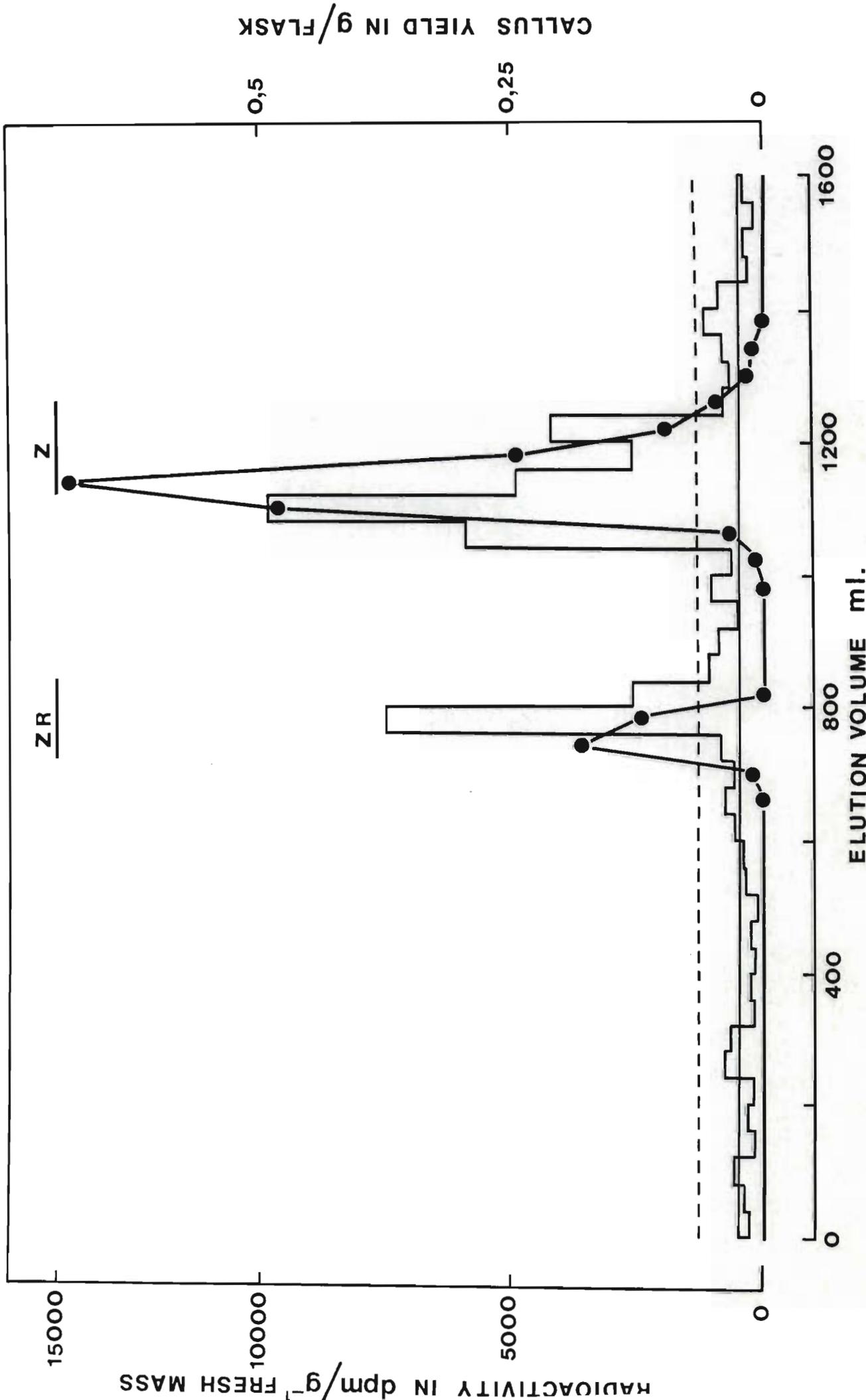


Fig. 5.12

CALLUS YIELD IN g/FLASK

RADIOACTIVITY IN dpm/g FRESH MASS

ELUTION VOLUME ml.

After fractionation on the Sephadex column the C fraction (Rf's 0,6-0,9) Fig. 5.12 displayed two peaks of activity which contained both biological- and radioactivity. The first peak co-eluted with ribosylzeatin and the second with zeatin. These results indicate that the {8-¹⁴C} zeatin was metabolized within the female gametophyte of *P. henkelii* seeds to several metabolites. These data concur with the results of studies with {8-¹⁴C} zeatin in other plant systems. In studies where labelled zeatin was applied to the xylem of fruiting *Lupinus albus* plants DAVEY and VAN STADEN (1981) have shown that several metabolites were produced. The principal biologically active metabolites were shown to be ribosylzeatin and glycosylzeatin. Several other metabolites were also produced which did not display biological activity. The data also agree with that of HENSON (1978) who applied labelled zeatin to detached *Alnus glutinosa* (L.) Gaertn. leaves. It was noticeable in the aforementioned studies that cytokinin did not appear to be readily translocated between leaves and organs in the free form. In the seeds of *P. henkelii* the labelled zeatin appeared to be transported between the epimatium and female gametophyte and vice versa. The transport of radioactivity between the female gametophyte and embryo sporophyte was however apparently mainly in the form of metabolites of zeatin. It is understood that this chromatographic system does not provide an identification of the metabolites present, particularly as the two compounds adenosine and adenine have been shown to be inactive in the soybean callus bioassay (MILLER, 1965). The metabolites of the applied zeatin show similar chromatographic properties to the known compounds with which they co-elute.

The Sephadex chromatographic system revealed that the metabolism of applied zeatin went beyond conversion to components that co-eluted with ribosylzeatin and glucosylzeatin. Two peaks of radioactivity which co-eluted with adenine and adenosine were also present, as were two further unidentified peaks. All these peaks of radioactivity were apparently formed from the applied {8-¹⁴C} zeatin which did not contain any spurious peaks of radioactivity prior to application.

The studies of cytokinins in the germinating *Podocarpus henkelii* seeds show that there is a definite interrelationship between cytokinins and the metabolic events associated with germination. ILAN and GEPSTEIN (1981) in their review of food reserve breakdown in germinating seeds suggest that cytokinins play a very important role in the control of the regulating influence the embryo axis exerts over the seed as a whole. This, combined with the observation of cytokinin activity associated with polyribosome formation at the commencement of germination in *Podocarpus henkelii* seeds, points to a close association of cytokinins in the metabolism of germination. Very little is known about the sites of action of cytokinins in germinating seeds. An understanding of this relationship will only result from further detailed metabolic studies.

CONCLUSION

True to most gymnospermous seeds, those of *Podocarpus henkelii* have a very long embryogenesis lasting some nine months from August through to April. Despite the many differences in seed structure and length of embryogenesis, parallels may be drawn between both the monocotyledonous and dicotyledonous seeds, and those of *Podocarpus henkelii*.

In the developing seeds of this genus, samples examined just after fertilization contained relatively high levels of lipid and protein. This was probably a reflection of the high levels of enzymes and structural proteins necessary at this stage of development (TAN and MORRISON, 1979a). In the early stages of development an unusual feature of the *Podocarpus henkelii* seed was the presence of storage protein, which disappeared with development and reappeared with maturity. The levels of soluble sugars increased in both the female gametophyte and embryo sporophyte during development. Maximum levels were recorded just prior to the increase in starch. A similar pattern of events with free amino acids and proteins was also noted. These events do not differ greatly from those described in developing *Zea Mays* kernels (INGLE, BEITZ and HAGEMAN, 1965) and *Pisum sativum* seeds (PATE, 1975).

The endogenous cytokinin levels detected in the seeds of *Podocarpus henkelii* fluctuated with development. At the

first sampling in December low quantities of cytokinin were detected in both the epimatium and female gametophyte. In *Ginkgo biloba* seeds, BANERJEE (1968) reported high levels of cytokinins in the early stages of development in the female gametophyte, associated with cell division. The ultra-structural studies showed that in December, cell division in the female gametophyte of *P. henkelii* seeds had ceased and cell expansion was taking place. This could account for the relatively low levels of cytokinin present. The first sample of the embryo sporophyte was collected in late January and the cytokinin levels detected were at a high peak, as were the levels in the female gametophyte. The peak of cytokinin activity recorded in the developing seeds of *P. henkelii* was coincident with the period of rapid increase in fresh and dry mass of both the embryo sporophyte and female gametophyte. These data conform to the suggestion of LUCKWILL (1977) that elevated levels of phytohormones are necessary in order to create a strong physiological sink capable of competing with the stem and root apices for metabolites and mineral nutrients.

The ultrastructural and biochemical studies confirmed that the peak levels of cytokinins were present at a time of high metabolic activity and the accumulation of food reserves in both the female gametophyte and embryo sporophyte. These observations lend support to the suggestion of BURROWS (1975) that cytokinins appear to exert their effect on plant metabolism as mediators, promoters or inhibitors of growth at a

level close to, though not necessarily at the genome. The exact role the cytokinins play in the aforementioned developmental processes is not clear. Detailed studies of endogenous cytokinins as well as applied hormones in relation to specific metabolic events are necessary to elucidate their precise function.

Paper and column chromatography both indicated that the high levels of cytokinin detected were associated with compounds which co-chromatographed and co-eluted with zeatin and ribosylzeatin. During the latter stages of development when the levels of cytokinin decreased, compounds which co-chromatographed and co-eluted with glucosylzeatin became more evident. Glucosylation is thought to be a method of inactivating cytokinins, and thus rendering them as storage forms (HENSON and WAREING, 1976). The occurrence of compounds which co-elute with glucosylzeatin when metabolic activity is decreasing as the seed matures, lends support to this storage form hypothesis.

An interesting feature of the maturing seeds of *P. henkelii* is that they are not subject to any desiccation. Consequently at maturity they have a very high moisture content of ca. 62 per cent. Most seeds dehydrate during maturation (HARRINGTON, 1972), and this process is thought to contribute to a low metabolic activity together with an enhanced resistance to adverse environmental conditions such as desiccation (THOMAS, 1972). These seeds are able to remain viable for varying lengths of time without utilizing much of the

large store of reserves necessary for germination. This reduction of seed moisture affects the biochemistry and cytology of the seeds by limiting the aqueous medium in which many of the enzymes and cellular metabolic events function (ROBERTS, 1972; THOMAS, 1972). Consequently seeds which do not dehydrate to a low moisture content at maturity do not appear to enjoy any of the advantages of such a condition. This could be one of the explanations for the inefficiency of the *P. henkeli* seeds as propagules.

The RQ values of the mature seeds were not measured, but it is hypothesized that they would be higher than the values obtained for dry seeds. The electron microscope studies indicated that the cellular organelles do not become indistinct at maturity as in the case of dry seeds (BAIN and MERCER, 1966a; ÖPIK, 1968). The utilization of protein and lipid reserves and the increase in size of the embryo sporophyte during six weeks of cold storage supports this suggestion. The ability of the seeds to fix atmospheric carbon is in all probability necessary to supplement energy utilized through metabolism before the seed germinates.

The epimatium imposes a 'coat imposed' dormancy on the seeds by limiting the growth of the embryo sporophyte. As soon as this restriction is lifted by scarification the seeds germinate, with or without additional water, depending upon the moisture content of the seed. Under natural conditions, the seeds of *Podocarpus henkeli* are subject to desiccation by the environment and appear to lose water at a rate which

exceeds imbibition. The latter process is made difficult because of the dry conditions which prevail during winter. The high moisture state of the seeds of *P. henkelii* appears to be the major limiting factor in their viability.

In 'dry' seeds the catabolism of stored reserves is regulated mainly by enzymes which are not present in the unimbibed seed. These enzymes are synthesized after imbibition *de novo* whilst the seed is germinating (BEWLEY and BLACK, 1978). Thus there is a requirement for new protein synthesis followed by increased activity of endoplasmic reticulum and ribosomes. In the 'wet' seeds of *P. henkelii* ultrastructural studies of the germinating embryo sporophyte indicated that after six days of incubation there was a rapid increase in the number of ribosomes and polyribosomes in the cotyledons and hypocotyl. This change in cellular activity was accompanied by a large increase in the levels of endogenous cytokinins. Similar observations of applied cytokinins increasing ribosome numbers in *Cucumis sativus* cotyledons have been reported by WOŹNY I GWÓŹDŹ (1980).

By the sixth day of incubation, there was a decrease in both the reserves of protein and starch in the embryo sporophyte, and in the lipid levels in the female gametophyte. LONGO, PEDRETTI, ROSSI and LONGO (1979) have reported the accelerated breakdown of reserves in excised cotyledons of *Citrullus vulgaris* Scrad. in the presence of benzyladenine. GEPSTEIN and ILAN (1979) have demonstrated that the embryo axis of *Phaseolus vulgaris* exerts a promotive influence over

the activity of α -amylase in the cotyledons. They suggest that this effect is mediated by cytokinins.

The high cytokinin levels detected in the embryo sporophyte and female gametophyte of *P. henkeii* seeds at a time when germination was first recorded and food reserves were being mobilized is in accordance with previous findings. However, there are no indications as to the role of cytokinins in food mobilization. It would be of considerable interest to dissect out the cotyledons, hypocotyl and radicle of the germinating embryo sporophyte, and analyse them separately for cytokinin activity and food reserve levels. Any trends regarding cytokinin levels and particular food reserve mobilization could be followed by further experimentation. Applied cytokinins could be utilized to assess their possible activity on specific enzymes. Studies regarding the synthesis of new protein to bring about the formation of the particular enzyme would prove useful. KHAN (1981) draws attention to the paucity in our understanding of cytokinin action in seeds. Advances in this field will only be achieved with detailed studies at the molecular level.

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