DORMANCY AND GERMINATION OF THE MANKETTI NUT, *RICINODENDRON RAUTANENII*

By

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This thesis is dedicated to my wife,

Julia.

CONTENTS

PREFACE

The research described in this thesis was carried out in the laboratories of the Department of Botany of the University of Natal, Pietermaritzburg under the supervision of Professor Johannes van Staden. This thesis, except where it is specifically indicated to the contrary in the text, is the result of my own investigation.

Arthur Brian Keegan

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ABSTRACT

The distribution of *Ricinodendron rautanenii* trees is confined to a fairly distinct band across southern Africa. This stretches from South West Africa in the west, through Botswana and Zimbabwe, to Mozambique in the east. These plants are a potential source of both timber and food. In this respect, the fruits and seeds of this species are highly nutritious and the latter, by virtue of their high lipid content, represent an excellent source of oils for both domestic and industrial uses. For these reasons consideration is being given to cultivating this species on a commercial scale. One problem, however, is that the seeds of these plants are dormant and in the first part of this study an attempt was made to establish the cause of this dormancy and how it could be overcome.

A thorough investigation of all the possible causes of dormancy revealed that ethylene was the only naturally occurring stimulus that could relieve this condition. Exogenously applied gibberellin (GA_3) and ethrel were found to be equally as effective as ethylene, but such treatments cannot be regarded as natural. It was also found that all the dormancy breaking treatments were only effective once the endocarp had been removed. This indicated that *R. rautanenii* seeds had a combined coat imposed-physiological dormancy where ethylene was only able to stimulate germination once the endocarp had been

removed. Scarification treatments showed that the endocarp most probably had its effect by restricting embryo enlargement since this structure did not inhibit water uptake or gaseous exchange. Once the germination requirements of these seeds had been established a more detailed investigation was carried out to determine their general sensitivity to ethylene, as well as the actual role this gas played in breaking dormancy.

Ricinodendron rautanenii seeds were found to exhibit a high degree of sensitivity to ethylene. The threshold concentration at which a response was obtained was approximately 10^{-3} microlitres per litre and this is the lowest yet recorded for any species prior to any additional seed treatments. At concentrations above this, the response was saturated indicating that the seeds are well adapted to the ethylene concentrations most likely to occur in the field. In addition to this, manketti seeds also responded to ethylene after only very brief exposures to the gas and optimum germination was recorded after 30 minutes incubation in an ethylene saturated atmosphere. The temperature range over which an optimum response to this phytohormone was obtained was found to be between 25 and 35°C.

One of the most striking features regarding the sensitivity of these seeds was the apparent ability of dry and partially imbibed seeds to perceive the dormancy

breaking stimulus. Furthermore, once treated, the seeds retained the dormancy breaking effect of ethylene even when subjected to almost complete re-dehydration. In this instance, 50 per cent germination was recorded for ethylene treated seeds which had lost approximately 97 per cent of their moisture content between dormancy breaking and re-incubation. It was thus concluded that, not only could *Ricinodendron rautanenii* seeds respond to very low ethylene concentrations but could probably also retain the effects of this gas during adverse environmental conditions.

The effects of imbibition and dormancy breaking were followed separately at the ultrastructural and biochemical level. The ultrastructure of dry embryonic axes of these seeds was characterized by massive stores of food reserves in the form of lipid and protein. Upon imbibition the number and size of spherosomes decreased and protein and globoid hydrolysis was clearly evident. Polysomes and microbodies (including mitochondria) were also visible prior to dormancy breaking but there was no evidence of any endoplasmic reticulum or dictyosomes. Imbibition also resulted in the expansion of the nuclei and there were indications of an increase in the granular content of the associated nucleoli. The number of nucleolar vacuoles, however, remained unchanged. These features indicated that nuclear activity had commenced albeit limited. The ultrastructure of untreated seeds which were maintained

in the imbibed state for an extended period of time (six days) was also examined. Cells of the embryonic axes of these seeds showed no further changes with regard to their nuclei and protein hydrolysis appeared to have ceased. At this time spherosomes resembled those in freshly imbibed tissue in terms of their size and numbers, suggesting that the lipid reserves had been resynthesized.

No immediate ultrastructural changes were observed after ethylene treatments. However, 24 and 48 hours after dormancy breaking further expansion of the nuclei was noted. At the same time the nucleolar vacuoles disappeared and the granular content of this region increased markedly. This suggested that an increase in the synthesis of various RNA fractions was taking place. Vigorous protein hydrolysis was also observed after the ethylene treatment whereas spherosome numbers increased.

Three days after the dormancy breaking treatment, the first signs of germination were visible. Externally this was characterized by a splitting of the testa in the region of the radicle. At this time, endoplasmic reticulum and dictyosomes were still not visible but from this point onwards the ultrastructural changes observed were typical of those recorded during the germination of other species. Thus, no single ultrastructural feature could be associated with the breaking of dormancy and the most notable changes which occurred during this period

took place in the nucleus.

Biochemical changes occurring during imbibition resulted in an overall decrease in the levels of extractable food reserves present in the embryonic axes. During this period, lipid levels were found to decrease by 44 per cent, protein levels by 12 per cent, sucrose levels by 68 per cent and glucose, fructose and starch levels by 100 per cent. These levels were found to return to their original values when seeds were incubated under moist conditions in the absence of ethylene for extended periods of time. Ethylene treatments, on the other hand caused a further, marked decrease in sucrose levels, whereas protein and lipid levels increased. Hydrolysis of the endosperm reserves commenced three days after the application of ethylene and this was characterized by a decrease in lipid levels and an overall increase in soluble carbohydrates. The timing of this event suggested that the endosperm was not involved in the actual process of dormancy breaking.

The importance of protein synthesis in dormancy breaking was also investigated. It was found that seeds incubated with a protein synthesis inhibitor, cycloheximide, failed to germinate, confirming the view that protein synthesis is an essential pre-requisite for germination. Inhibition of RNA synthesis with actinomycin D, on the other hand, did not prevent germination. This suggested that the materials necessary for early protein synthesis

were already present in the dry seeds. Actual measurements of protein synthesis showed that this process took place in the embryonic axis, cotyledons and endosperm of seeds imbibed for as little as two hours. Protein synthetic abilities increased considerably in most instances after 48 hours imbibition but then decreased upon application of ethylene. At the same time, however, a marked increase in the uptake of ^{14}C-leucine was noted in ethylene treated axes. This may indirectly reflect an effect of ethylene on membrane permeability. Protein synthesis in cycloheximide and actinomycin D treated embryonic axes was also measured. No consistent trends were evident but it was found that after ethylene treatments, protein synthesis was generally lowest in those seeds which were destined to germinate. In addition, these seeds also exhibited the greatest uptake of ^{14}C-leucine.

Ricinodendron rautanenii seeds incubated with compounds known to stimulate the pentose phosphate pathway failed to germinate. This indicated that dormancy in this species was probably not the result of a block in alternate respiration.

The possible involvement of endogenous phytohormones in the overall process of dormancy breaking was also investigated. In this regard, the role of gibberellic acid appeared to be enigmatic. This is based on the observation that applied gibberellins could stimulate ger-

mination whereas inhibitors of endogenous gibberellin synthesis applied to ethylene treated seeds had no effect. It was concluded from this that the effects of ethylene are not mediated via an enhancement of endogenous gibberellin synthesis.

A preliminary investigation carried out on the endogenous cytokinins showed that this hormone was absent from dry and imbibed seeds. A transient increase in zeatin levels was observed 24 hours after the ethylene treatment. A similar transient increase was noted in non-induced seeds maintained under moist conditions for six days. In this latter instance, however, the peak co-chromatographed with the biologically less active cytokinin, zeatin glucoside.

A basal level of endogenous ethylene production was recorded in all imbibed *Ricinodendron rautanenii* seeds. Ethrel, ethylene and gibberellin treatments caused an initial, transient increase in this ethylene production after which no further significant changes were recorded. It is suggested that dormancy breaking in this species is not related to enhanced endogenous ethylene synthesis.

The results of the biochemical and ultrastructural studies are discussed in relation to what is known regarding features associated with dormancy and its removal and on the known effects of ethylene on seed tissues.

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CHAPTER 1

GERMINATION REQUIREMENTS OF *RICINODENDRON RAUTANENII* SEEDS

INTRODUCTION

Ricinodendron rautanenii Schinz belongs to the family Euphorbiaceae. The genus is exclusively African and all member species are trees. The distribution range of *R. rautanenii* is somewhat limited (Figure 1.1) and plants may be found growing in the northern regions of South West Africa (Namibia), southern Angola, Botswana, Zambia, Zimbabwe and Mozambique. Only a single stand has been recorded in the Republic of South Africa and this is located in the northwestern region of the Transvaal (VAHRMEIJER, 1976).

The generic name, *Ricinodendron* is derived from two greek words; *Ricinus*, meaning tick (an obvious reference to the tick-like appearance of the seeds) and *dendron* meaning tree. The species was first collected in South West Africa by the German botanist Dinter in 1885 and was named after a Finnish missionary, the Reverend Rautanen who worked in Damaraland (PALMER and PITMAN, 1973). Over the years the plant acquired a number of common names including; the manketti nut tree, featherweight tree, mankettiboom and wilde akkerneut (VAHRMEIJER, 1976). The vernacular names, however, are somewhat more colourful and include

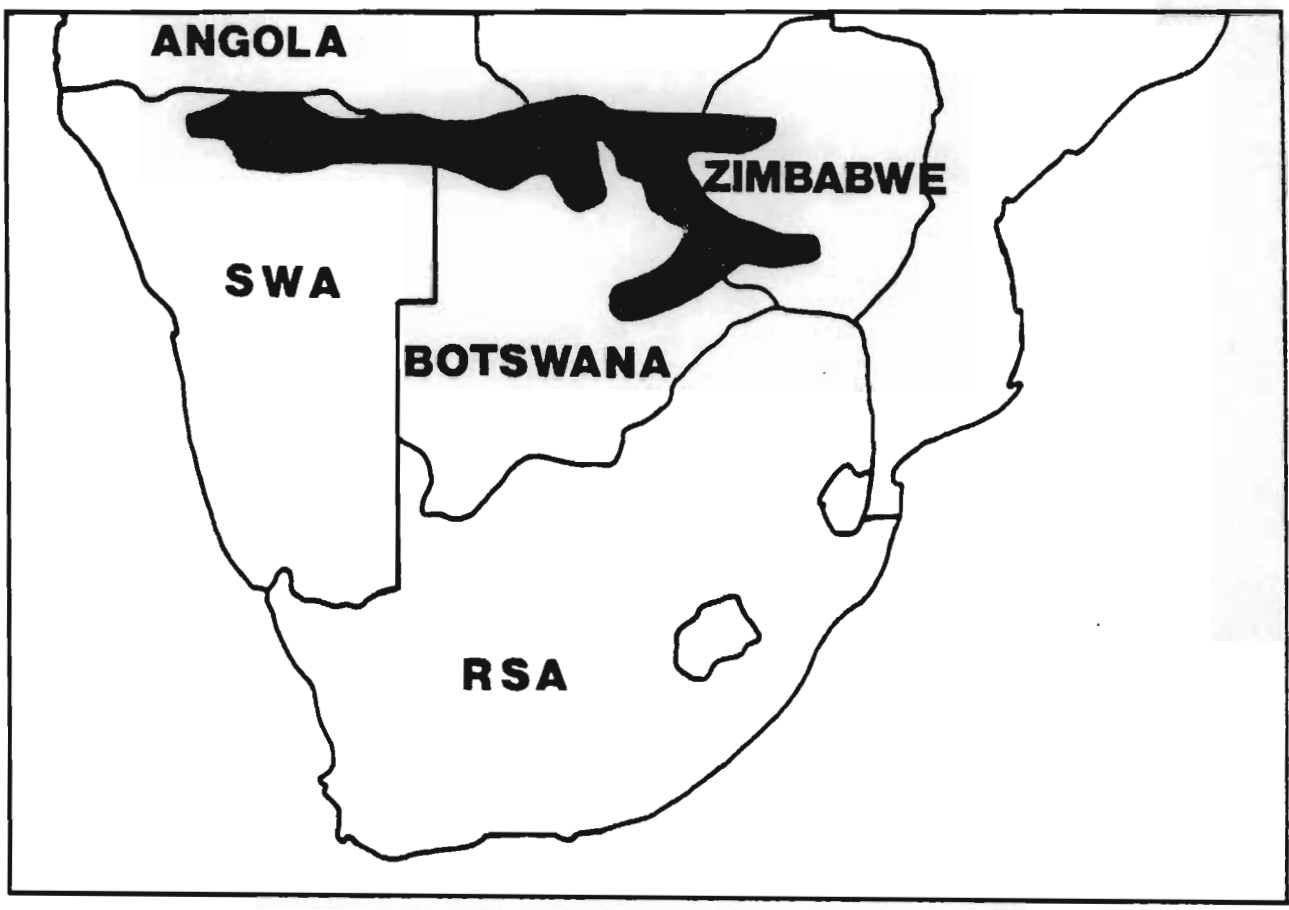

Fig. 1.1. The distribution range of *Ricinodendron rautanenii* Schinz

mugongo (Owambo, Western Caprivi and Deiriku), omungeta (Herero) and ngongo (Kawango and Deiriku).

In general appearance these large, deciduous trees have been likened to the marula (*Sclerocarya caffra* Sond.) and when bare are said to resemble the well known baobab trees (*Adansonia digitata* L.) (PITMAN and PALMER, 1972; STORY, 1958). Mature trees range from 7 to 20 metres in height. They usually have a straight bole of up to one metre in diameter which is topped by a characteristically broad crown. Young branchlets are covered with soft, short, white or red hairs which disappear with age. Thus, the mature branches are glabrous and have a smooth, grey to light golden brown bark. A characteristic feature of this bark is that it has a tendency to peel in thin strips (PALMER and PITMAN, 1972; VAHRMEIJER, 1976).

Leaves are borne at the ends of young twigs and have petioles of up to 15 centimetres in length. At the tips of each petiole there are usually two very prominent glands the function of which is not clear. The leaves themselves are digitately compound, having three to seven leaflets. Each leaflet is approximately 5 to 12 centimetres long and is oval in shape. The margins of these leaflets are characteristically beset with small, green, glandular teeth. Leaf colour is distinctive and adaxial surfaces are usually dark green, whereas the abaxial surfaces tend to be pale grey (STORY, 1958; PALMER and PITMAN, 1972; VAHRMEIJER, 1976; COATS PALGRAVE, 1977). These features

are illustrated in Figure 1.2.

In common with many members of the Euphorbiaceae, *R. rautanenii* is dioecious. The small pale yellow flowers (ten millimetres in diameter) are borne at the ends of long furry pedicels and appear before the rains from October to December. Female flowers are single-ovuled and both sexes are imperfect. The inflorescence is best described as a loose panicle with the panicle of female trees having fewer flowers than those of the male (PALMER and PITMAN, 1972; VAHRMEIJER, 1976). Once fertilized, the pistilate flowers give rise to large, reddish-brown fruits which ripen from February onwards. These fruits, also illustrated in Figure 1.2, are drupaceous and ellipsoidal. They are usually about 35 millimetres long and 25 millimetres in diameter and have an average mass of ten grammes. Covering these fruits is a thin leathery exocarp which constitutes about 10 per cent of the whole fruit. Beneath this, and comprising about 20 per cent of the fruit mass, is a layer of edible tissue, the mesocarp. This mesocarp surrounds a finely pitted but extremely hard endocarp which resembles the 'stone' of peach (*Prunus persica* (L.) Batsch) fruits. One or sometimes two fairly large seeds are firmly enclosed within this endocarp (PALMER and PITMAN, 1972; COATS PALGRAVE, 1977). These seeds have an average diameter of ten millimetres and constitute approximately 10 per cent of the entire fruit. Each has a tough, woody testa enclosing a copious endosperm and a small, two millimetre long embryo with thin, papery cotyledons. These cotyledons lie

embedded in the endosperm and are barely distinguishable from it (Figure 1.2). Most of the food reserves are thus contained in the endosperm and are comprised mainly of lipid (57 per cent) and protein (26 per cent) (WEHMEYER, 1976).

Ecologically speaking the species is gregarious and is often found growing in groves or forests forming stands of up to 60 000 hectares in extent. In open woodlands they are frequently found in association with *Terminalia sericea* Burch. ex DC., *Baikiaea plurijuga* Harms, *Burkea africana* Hook., *Pterocarpus angolensis* DC., *Giubourtia coleosperma* (Benth.) J. Léonard, *Strychnos cocculoides* Baker, *Afzelia quanzensis* Welw. and some species of *Combretum* Loefl. Wherever this plant forms the dominant tree species they are usually spaced about 20 metres apart (VAHRMEIJER, 1976). The trees grow in a summer rainfall region and receive approximately 524 millimetres precipitation per annum. During this wet period the daily maximum temperatures are often in excess of 30°C (South African Weather Bureau, Pretoria) suggesting that summer growth is vigorous. Probably one of the major factors contributing to the limited distribution range of this species is their apparent requirement for specific soil conditions. They grow almost exclusively in deep Kalahari sands or limestone outcrops. The only recorded forest of these trees in South Africa was in fact found growing in an isolated patch of Kalahari sand (VAHRMEIJER, 1976).

Wherever they grow, extensive use is made of these trees

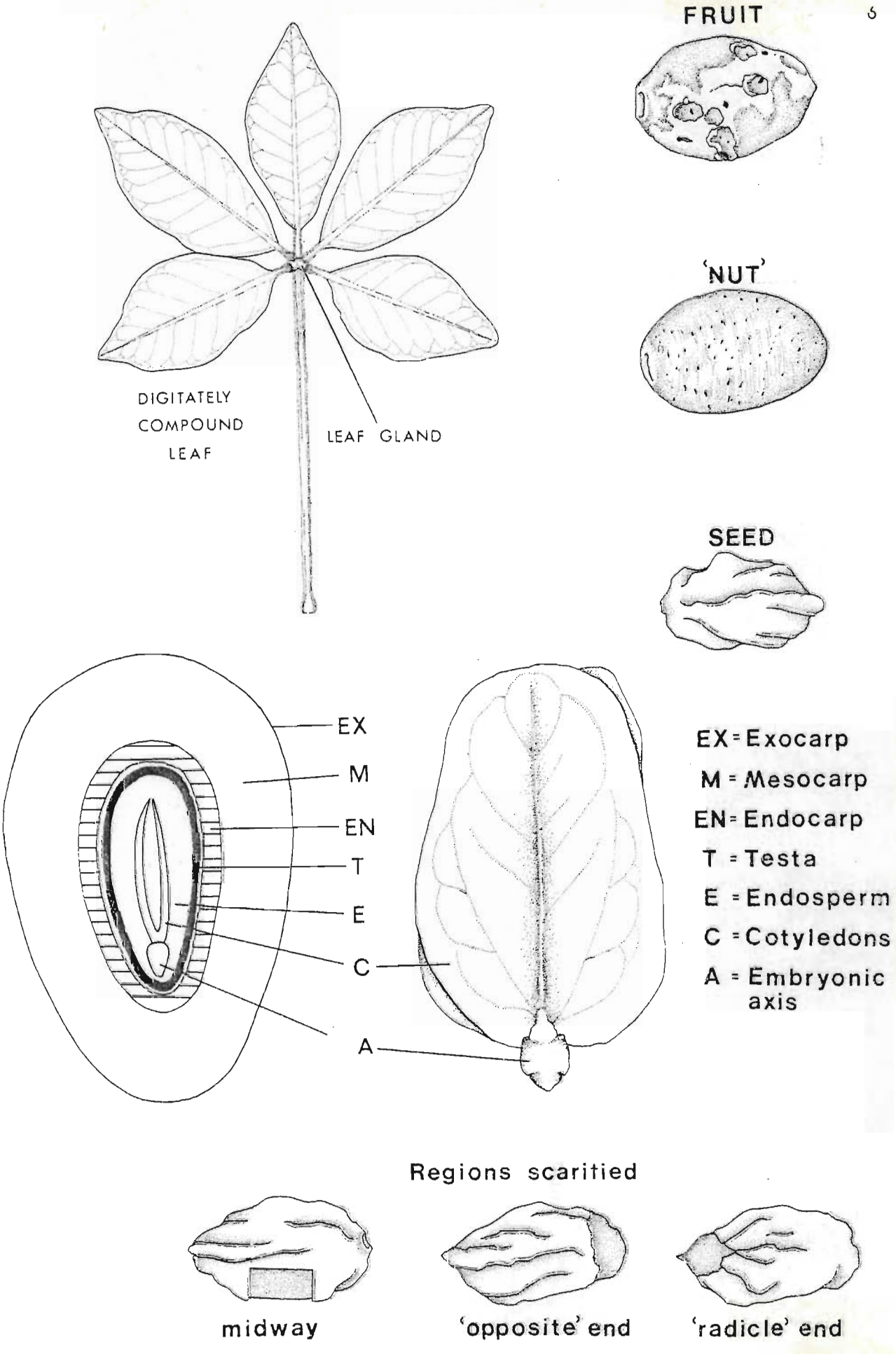

Figure 1.2 Diagrams showing the general morphology of the leaves, fruits and seeds of *Rhizophora rautanenii*. Also illustrated are the various regions of testa which were scarified in

by local bantu and bushmen tribes. Like the baobab (*Adansonia digitata*) these trees tend to trap rain water and thus may serve as a supply of drinking water during times of scarcity (STORY, 1958). Felled trees provide a timber which has a number of unique properties. This makes it useful not only to the local inhabitants, but also for a wide range of goods should the trees be commercially exploited. The wood is pale yellowish-white, has a wavy grain and is coarse textured. However, the most notable feature of this timber is its light weight (560 kilogrammes per cubic metre green and 240 kilogrammes per cubic metre air dry) and comparative strength. This would make it a satisfactory substitute for South American balsa wood. Commercially it has already been used for pattern making, light packing cases, furniture backing, insulating material, floats, toys and drawing boards. In a somewhat more primitive sense it is also the material much favoured by West African tribes for coffin making! (WATT and BREYER-BRANDWIJK, 1962). One drawback, however, is that the timber discolours easily and to obtain satisfactory results must be sawn immediately after cutting and dried as rapidly as possible (COATS PALGRAVE, 1977).

No portion of this plant is reported to have any medicinal properties, nor does it appear to contain any toxic compounds. However, the fruits and seeds are much sought after because of their nutritive value. In areas where the trees grow prolifically, these fruits and seeds may form the staple diet of the local inhabitants, particularly

during dry years. For example, it is reputed that these nuts form the mainstay of the vegetable diet of the !Kung bushmen of Botswana who consume between 100 and 300 of these nuts per day (VAHRMEIJER, 1976; PITMAN and PALMER, 1972).

Preparation of the fruits for human consumption is simple. After cooking, the exocarp is usually removed and discarded since it is tough and indigestible. A porridge or soup is then made from the remaining pulp (mesocarp) and this has an astringent but not unpleasant taste due to its fairly high sugar content (WEHMEYER, 1976). This sweetish flavour is in fact retained for a long period of time after the fruits are shed and the tissue does not readily decompose. Thus, the pulp from fresh fruits may be dried and stored for later use and even the dry crumbly flesh from old fruits is still edible. A rather potent but refreshing beer is also sometimes brewed from this pulp. However, it is not only man who is partial to these fruits, and elephants are also reported to relish them (VAHRMEIJER, 1976; WATT and BREYER-BRANDWIJK, 1962).

It is undoubtedly the nut or seed which is the most popular and nutritious part of these fruits. They are eaten raw or dried but are most pleasant when roasted. A meal or paste is sometimes made by pounding the nuts and this is often added to meat, roots or baobab pulp (PITMAN and PALMER, 1972). The nutritional value of both the seeds and fruit pulp was investigated by the Food Chemistry Division of the National Food Research Institute (Council

for Scientific and Industrial Research, Pretoria). Their data is reproduced in Table 1.1. The report clearly indicates that the dry flesh (mesocarp) is a good source of minerals and contains proteins and B group vitamins in about the same proportion as a cereal such as wheat. The endosperm is extremely rich in protein, oil and minerals. The extractable lipid has a high percentage of linoleic acid, (an essential fatty acid) and the eleostearic acid content is also fairly high. WEHMEYER (1976) concluded that assuming all these nutrients are available, an adult man eating 100 fruits daily (i.e. 300 grammes flesh and 140 grammes nuts) would receive the following percentages of his recommended daily allowance :

Energy	71
Protein	115
Vitamin E activity (α-tocopherol)	400
Vitamin C	98
Nicotinic acid	82
Riboflavin	50
Thiamine	136
Calcium	72
Magnesium	438
Iron	100
Zinc	74
Phosphorus	148

These fruits can thus make a significant contribution to an individual's daily nutritional requirements.

Table 1.1 Chemical composition of the mesocarp and seed tissues of manketti fruits (Adapted from WEHMEYER, 1976).

Chemical composition of the fruit	Mesocarp	Seed	
Moisture	8,4	4,2	
Ash	5,6	4,1	
Protein (N x 6,25)	9,4	26,0	
Fat (petroleum ether extract)	1,2	57,3	
Fibre	2,5	2,5	g/100g
Sucrose	29,8		
Glucose	0,2		
Fructose	0,5		
Total carbohydrate (by difference)	72,9	5,9	
Energy value (kJ per 100 g)	1 424	2 692	
Calcium	104	193	
Magnesium	266	527	
Iron	4,30	3,70	
Copper	1,6	2,82	
Zinc	1,79	4,09	
Sodium	1,86	3,10	
Potassium	2 666	673	mg/100g
Phosphorus	62,0	845	
Thiamine	0,49	0,31	
Riboflavin	0,21	0,21	
Nicotinic acid	4,79	0,31	
Vitamin C	14,7	-	
α-Tocopherol)		29	
γ-Tocopherol) Vitamin E		536	
Amino acids			
Aspartic acid	0,4	2,4	
Threonine	0,2	1,0	
Serine	0,3	1,3	
Glutamic acid	0,7	4,2	
Proline	0,2	1,2	
Glycine	0,2	1,2	
Alanine	0,4	1,0	
Half cystine	-	0,1	
Valine	0,2	1,8	g/100g
Methionine	trace	0,4	
Isoleucine	0,2	0,7	
Leucine	0,2	1,4	
Tyrosine	0,1	0,5	
Phenylalanine	0,2	1,3	
Lysine	0,2	0,7	
Histidine	0,1	0,7	
Arginine	0,7	3,5	
Fatty acids (percentage of total acid content)			
Palmitic acid		9,5	
Stearic acid		7,6	
Oleic acid		17,7	
Linoleic acid		42,9	
Arachidic acid		0,6	
α + β eleostearic acid		21,7	

An additional feature of this species is that the fruits are produced in extremely large numbers. Some reports have it that the fruits may lie knee-deep under the trees after shedding (PITMAN and PALMER, 1972). In 1914 it was estimated that the Tsumeb forest in South West Africa produced approximately one ton of nuts per hectare per annum (VAHRMEIJER, 1976). Private exploitation of these nuts was permitted by the German government between 1911 and 1914 and this resulted in the annual export of 2 000 tons of nuts to Germany. After the region was recaptured by troops of the(then)Union of South Africa in 1916, a similar quantity of nuts was shipped to Britain where they were used for the production of margarine. The nuts were also at one time exploited on a small scale in neighbouring Angola where the oil was extracted and used for domestic purposes (VAHRMEIJER, 1976; PITMAN and PALMER, 1972).

It is clear, therefore, that these plants have considerable economic potential especially in view of the steady increase in demand for vegetable oils. The extraction of manketti seed oils could be achieved with very little additional investment since adequate machinery is presently available. This oil could be used for both domestic purposes and as a drying oil in the paint industry due to its high unsaturated fatty acid content. (Industrial use, however, would require the removal of the high levels of vitamin E since this compound renders the oil stable against oxidation). The suggestion has also been made that,because the nuts are so palatable, they could be marketed as a luxury item similar

to cashew nuts. This, however, would require the development of suitable machinery that would crack the nuts without damaging the kernel (WEHMEYER, 1976).

Any future scheme to harvest these nuts on a commercial scale from existing trees would have to take into consideration their importance as a natural food source for both man and beast. For the same reason, indiscriminate felling of trees for their timber would also be undesirable. The scarcity of these plants in South Africa has, in fact, led to their protection by the law. Thus, in order to reach an effective compromise between conservation on the one hand, and exploitation on the other, serious consideration should be given to establishing cultivated stands of this species. It appears that this would not be difficult since vegetative propagation has already been achieved using truncheon cuttings (WHITE, 1962). However, any future breeding and development programme would require additional genetic variability which could only be derived from seed propagation (STRAUSS, MICHAUD and ARDITTI, 1979). At present this would not be possible for the manketti since too little is known regarding the germination characteristics of this species.

In a preliminary study on this aspect, KUMAR (1978) reported that manketti seeds are dormant and that germination could only be induced by applications of gibberellic acid. The actual dormancy mechanism itself was not ascertained, and no other data regarding these seeds is known to exist.

Thus, the present study was undertaken to obtain more information regarding the cause of dormancy in these seeds. In addition, although the study was not commercially orientated, it was hoped that some practical benefits would also result from it.

In a seed such as *R. rautanenii* where the actual dormancy mechanism is unknown, every existing possibility has to be examined. Notable examples of such studies are those which were carried out on seeds of Velvetleaf, *Abutilon theophrasti* Medic. (LA CROIX and STANIFORTH, 1964); Sugar Maple, *Acer pseudoplatanus* L. (WEBB and WAREING, 1972); Witchweed, *Striga asiatica* (L.) Knutze (= *S. lutea* L.) (EGLEY, 1972); Reed Canary Grass, *Phalaris arundinacea* L. (LANDGRAFF and JUNTILLA, 1979) and Sickle-leaved Hares Ear, *Beupleurum falcatum* L. (MOMONOKI, 1979). Any such study on seed dormancy must attempt to establish both the reasons for the phenomenon and the mechanisms by which it is achieved. To assist workers in this field there is now a large and constantly increasing volume of literature on the topic of seed dormancy and germination. To illustrate this, no fewer than 1 300 articles, ten reviews and three books were published on this subject between 1973 and 1977 (TAYLORSON and HENDRICKS, 1977). In the discussion which follows, brief consideration will be given to some of the major concepts and theories.

Seeds generally achieve two major purposes in the life cycle of plants. One is to aid in the dispersal of the species

and the other is to maintain the survival of the species during adverse environmental conditions (OSBORNE, 1981). Two unique features of seeds equip them for this. Firstly, seeds of most angiosperm species are well adapted to survive desiccation as their moisture content can drop to less than 10 per cent without losing the potential for full cellular function. In this dehydrated state, the embryos are particularly resistant to environmental extremes and diseases. The other unique feature of seeds is the ability of some to imbibe water, commence metabolic activity but not germinate. This is the phenomenon referred to as dormancy and is defined by VILLIERS (1972) as 'the failure of otherwise viable seeds to recommence development immediately when supplied with water and oxygen at temperatures recognized as normally favourable for plant growth. This delay may last for varying lengths of time under constant conditions and in some cases may even continue indefinitely until some special condition is fulfilled'. As a long-term survival stratagem the evolution of seed dormancy has been highly successful. One of the major benefits is that all the different mechanisms tend to inhibit seeds from germinating at the wrong time and in the wrong place. This in turn ensures that when germination does occur there is a far greater likelihood of the seedling reaching maturity. As an example of how effective this phenomenon may be, some seeds have been reported to survive as long as 600 years without any loss of viability (LERMAN and CIGLIANO, 1971).

Different species have adopted different dormancy mechanisms

and the depth of this dormancy may vary considerably between individuals of the same species. In ecological terms this appears to be of considerable adaptive significance and based on this, GRIME (1981) has recognized two broad categories of seed banks in the British Isles. The first of these was the transient seed bank characterized by many grass species with large seeds and no pronounced after-ripening requirements. Also included in this group were some tree, shrub and herb species which had mild after-ripening requirements but which, nevertheless, still germinated rapidly. The second group was the persistent seed bank which, as the name implies, could remain in the soil for long periods. Here the seeds were generally small (facilitating rapid soil penetration) and had fairly specific germination requirements. This persistent seed bank represents the truly dormant seeds and probably functions to ensure against population extinction during adverse environmental conditions. The adaptive significance of dormancy has never actually been proved in the field (MAYER and SHAIN, 1974) but the different dormancy mechanisms do appear to enable species which share a common habitat, to occupy different ecological niches. Furthermore, a mathematical model proposed by COHEN (1968) suggested that germination must be controlled and that uniform germination in wild species was undesirable. Thus, the phenomenon of dormancy is of immeasurable value to plants.

In broad terms, germination may be restricted in two ways.

The first is enforced or imposed dormancy where the reluctance to germinate is due to the absence of suitable environmental conditions. This condition is perhaps more aptly described as quiescence and may include those seeds experiencing hardseededness. The other form of germination restriction is organic dormancy and is related to the innate inability of the seed to germinate, sometimes also referred to as physiological incompetence. Such seeds are truly dormant and here it is often possible to separate, in time, the rehydration of the seed tissues and application of the dormancy breaking treatment. After a systematic investigation of this phenomenon CROCKER (1916) suggested the first classification of the various dormancy types. Based on this, NIKOLAEVA (1977) produced a modern classification which incorporated information from more recent examples. This has been reproduced in Table 1.2. Here it can be seen that dormancy may be divided into three broad groups. These are the exogenous, endogenous and combined forms of dormancy and each will be considered in more detail.

Exogenous dormancy results from the effect(s) of structures covering the embryo. This includes the endosperm, testa, pericarp and various other fruit tissues. These enveloping structures usually exert their effect in either a physical or chemical manner. The physical effect can be broken down even further into impermeability of the seed coat or hardseededness and a purely mechanical restriction of radicle protrusion.

Table 1.2 A classification of organic seed dormancy patterns (NIKOLAEVA, 1977)

Types of dormancy	Factors causing dormancy	Conditions breaking dormancy	Example
Types of exogenous dormancy			
Physical	Impermeability of seed coat to water	Scarification	*Gledichia*
Chemical	Inhibitors of pericarp	Removal of pericarp or leaching of the fruits	*Fraxinus rhinchophylla*
Mechanical	Mechanical resistance of covers to embryo growth	Various methods for destroying the covers	*Elaeagnus angustifolia*
Types of endogenous dormancy			
Morphological	Underdevelopment of embryo (UE)	Warm stratification	*Elaeis guineensis*
Physiological	Physiological inhibiting mechanism (PIM) of germination		
Nondeep	PIM weak	Short cold stratification, light, dry-storage, injury of covers, growth stimulators	*Triticum* spp. *Impatiens balsamina* *Lactuca sativa*
Intermediate	PIM intermediate	Long cold stratification and several other influences	*Acer negundo*
Deep	PIM strong	Long cold stratification only	*Acer tataricum* *Malus sylvestris*
Morpho-physiological	Combination of UE and PIM		
Intermediate, simple	Combination of UE and intermediate PIM of germination	First warm, then cold stratification, growth stimulators	*Aralia mandshurica*
Deep, simple	Combination of UE and strong PIM of germination	First warm, then cold stratification	*Panax ginseng*
Deep, simple, epicotyl	Combination of UE and strong PIM of epicotyl growth	Same	*Virburnum opulus* *Trillium* spp.
Deep, simple, double	Combination of UE and strong PIM of hypocotyl and epicotyl growth	Warm followed by cold stratification for hypocotyl growth then another combination of warm and cold stratification for epicotyl growth	
Intermediate, complex	Combination of UE and intermediate PIM of post-development and germination	Cold stratification or growth stimulators	*Aralia continentalis*
Deep, complex	Combination of UE and strong PIM of post-development and germination	Cold stratification only	*Tulipa tarda*

Hardseededness is most widespread in the Leguminoseae. Of 260 legume species examined, 85 per cent had some or all impermeable seeds (GUPPY, 1912). This feature also occurs in some species of the Cannaceae, Chenopodiaceae, Convallariaceae, Convolvulariaceae, Geraniaceae, Malvaceae, Solanaceae, Anacardiaceae and Rhamnaceae (ROLSTON, 1978). The main region responsible for seed coat impermeability is made up of a prismatic, radially elongated, compactly arranged palisade layer of macrosclereids (ROLSTON, 1978; WERKER, 1981). In the Leguminoseae, workers have recognized various parts and features of these macrosclereids which may be responsible for impermeability and these include;

(1) a waxy layer over the palisade cells,
(2) the cuticle,
(3) suberized or cuticularized outer periclinal walls,
(4) a suberized lamella around all the cell walls and intercellular spaces and/or upon the inner side of the outer periclinal wall,
(5) a cork-like filling at the strophiolar cleft,
(6) the so-called 'light line',
(7) changes in the micellar structure of the cellulose during dehydration - an irreversible change manifested by changes in the swelling potential of gels,
(8) the subcuticular mucilaginous layer of the outer periclinal wall composed of pectic in-

soluble materials which shrink upon desiccation and undergo chemical changes that make them less pervious to water,

(9) contraction of the palisade cells as a whole, and

(10) water-proof substances in the walls of the palisade layer including lignin, tannins and quinones (WERKER, 1981).

WATSON (1948), however, could find no specific characteristics responsible for the impermeability of hard seeds as compared with soft ones and this observation still appears to be valid (NIKOLAEVA, 1977). Furthermore, there does not appear to be any single factor responsible for seed coat impermeability.

It is generally assumed that under natural conditions, hardseededness is gradually lost by the action of external, mechanical and biological forces. Environmental factors such as frost and fire probably induce permeability in some cases (MARKS, 1979), and mechanical scarification may occur when seeds pass through the digestive tracts of birds and animals. Nevertheless, it is the action of soil micro-organisms which is regarded as the most common factor responsible for inducing permeability. However, it should be noted that there is very little direct evidence to support this (BARTON, 1965).

There are also a number of artificial treatments which can be applied to seeds to overcome hardseededness. These in-

clude;

(1) chemical treatments using concentrated sulphuric acid to break down the coat,

(2) soaking in organic solvents such as ethyl alcohol, acetone and petroleum ether to remove any waxy substances,

(3) treatment with hydrolytic enzymes such as hemicellulase and pectinase,

(4) high pressure treatments of 500 to 2 000 atmospheres,

(5) physical reduction of impermeability by impaction or percussion as caused by vigorous shaking,

(6) mechanical scarification by abrasion - the most common commercial method of reducing impermeability,

(7) freezing treatments with liquid air, nitrogen or oxygen which can result in seed coat fracturing during subsequent thawing,

(8) brief high temperature treatments using boiling water and dry heat, and

(9) radiation treatments using infra-red, various radio frequencies, gas plasma and microwaves (ROLSTON, 1978).

Seeds germinating after any one or combination of these treatments often indicates that dormancy is due to an impermeable seed coat.

This phenomenon of hardseededness, like most other dormancy mechanisms, is an inherited characteristic and may be determined by the effects of the environment on the parent plant (EVENARI, KOLLER and GUTTERMAN, 1966). In some species it can be artifically manipulated during seed ripening by changes in the day length or oxygen availability (GUTTERMAN and HEYDECKER, 1973; MARBACH and MAYER, 1974).

Compared with hardseededness, mechanical resistance to embryo growth is probably a rare occurrence. The suggested principle involved is that the embryo simply cannot develop sufficient thrust to penetrate the covering structure. Dormant seeds of cocklebur (*Xanthium pensylvanicum* Wallr.) are thought to exhibit this mechanism but actual measurements of the physical thrust showed that the germinating seed embryos developed sufficient force to overcome the effect of the testa (ESASHI and LEOPOLD, 1968). Seeds of *Elaegnus angustifolia* Blanco deserve special mention since, although they are often cited as classic examples of this type of dormancy, there is a serious lack of experimental evidence to support this. The only justification for this, in fact, has been the finding that the presence of the endocarp notably inhibits germination (NIKOLAEVA, 1977). Seed coat effects on the light sensitivity of certain seeds are also cometimes related to the exertion of a mechanical restriction. Dormancy breaking light treatments are thus thought to affect the ability of the radicle to overcome this resistance. This may either be by actually weakening the barrier or by increasing the expansive force of the em-

bryo (CHEN, 1970). However, in many of the examples which invoke this mechanism, dormancy could be attributed to factors other than, or in addition to, mechanical constraint. Furthermore, when one considers the tremendous pressures generated by growing plant organs, it is only when the seed is covered by exceptionally hard structures that one would suspect this mechanism to be operative (VILLIERS, 1972).

It would be expected that those characteristics which cause impermeability to water would probably also cause gaseous impermeability since the molecular size of oxygen,carbon dioxide and water is very similar (MAYER and POLJAKOFF-MAYBER, 1975). Despite this, it has been found that in some species oxygen is selectively excluded by seed coats which are otherwise freely permeable to water. This represents a form of so-called 'chemical exogenous dormancy'. There are two mechanisms by which oxygen can be prevented from reaching the embryo. The first of these is where water acts as a physical hindrance to oxygen diffusion. In this case, the presence of water in the imbibed seed results in oxygen having to diffuse over relatively long distances. This may be exacerbated by the fact that, in water, the rate of oxygen diffusion is generally very low. In addition to this, excess water in and around the integuments may also encourage the development of a large, mixed micro-organism population which could compete with the embryo for oxygen (HEYDECKER and CHETRAM, 1971). Mucilaginous substances which are present in some seeds (e.g. *Herschfeldiana incana* Moench), could also significantly reduce the amount of

available oxygen (NEGBI, RUSHKIN and KOLLER, 1966). Such compounds swell considerably but contain very little free water resulting in less medium for the oxygen to dissolve in (WERKER, 1981). The other means of reducing oxygen availability is by the 'chemical consumption' of the gas during its passage through the seed coat towards the embryo. This may be due to substances present in the seed envelopes or in the embryo itself. A classic example of such a mechanism is found in seeds of *Malus domestica* L., the apple. Here the seed coats are rich in phenolic compounds which,in moist conditions,are enzymatically oxidized to quinones. This 'scavenging' of oxygen is thought to reduce the amount available for the embryo thereby preventing germination (COMÈ, 1967). Similarly, the glumes of *Aegilops kotschyi* Boiss. appear to contain an oxygen scavenging oxidase and *Oryza sativa* L. seed coats show a high level of redox enzyme activity (WURZBURGER, 1974; ROBERTS, 1964). In contrast to this fairly straightforward chemical utilization of oxygen, a truly 'live' control mechanism may exist in the seed coats of *Ambrosia trifida* L. (DAVIS, 1930) and *Cucurbita pepo* L. (BROWN, 1940). In these examples the metabolic activity of an inner layer of living cells was thought to significantly reduce the amount of available oxygen. Other seed structures such as the endosperm may also limit the supply of oxygen to the embryo but few studies have been carried out on this aspect (WERKER, 1981).

Dormancy may also be imposed by the selective impermeability

of seed coats to carbon dioxide. Such a mechanism is thought to exist in some species of *Trifolium*, *Medicago* and *Trigonella* where an increase in the ambient CO_2 concentration was found to stimulate germination (MAYER and POLJAKOFF-MAYBER, 1975). However, it is not clear how CO_2 impermeability is actually achieved.

The remaining form of chemical endogenous dormancy is that brought about by inhibitory substances present in the seed coats. The occurrence of such compounds is fairly common and seems to occur more frequently in plants from tropical and sub-tropical regions. In those species, such as the tomato (*Lycopersicon esculentum* L.), where the dispersal unit consists of seeds enclosed in the maternal tissues, the latter very often contains inhibitory compounds (WAREING, 1965). These serve to prevent any premature germination since the seeds themselves are usually not dormant. In truly dormant seeds the most common group of coat inhibitors are the aromatic acids. These include salicylic-, o- and n-coumaric-, cinnamic- and m-oxygenzoic acids (BARTON, 1965). In addition, abscisic acid has also recently been located in seed envelopes (MAYER and POLJAKOFF-MAYBER, 1976). A group of potent germination inhibitors which occur in the testa of peach (*P. persica*) seeds are thought to maintain dormancy by their effect on the embryo. In this species the condition is compounded by the fact that the endocarp interferes with the outward leaching of these inhibitors from the testa and it is only once this structure is removed that germination proceeds

(DU TOIT, JACOBS and STRYDOM, 1979). In those species where such inhibitors occur it is generally assumed that, under natural conditions,they are removed by the percolating action of rain water. In some desert species this requirement is in fact absolute since even sub-soil irrigation could not substitute for the effect of rain and germination would only proceed once a certain quantity of rain had fallen (SORIANO, 1953). This serves to illustrate that great care should be taken in the design of any leaching experiments to determine the role of coat inhibitors. Another point which must constantly be borne in mind, is the fact that inhibitors are widespread in plant tissues and are even found in actively growing regions. Thus, their mere presence in dormant seeds would not necessarily imply that they are responsible for maintaining that condition (WAREING, 1965).

At present there is still too much speculation and too many hypotheses regarding the overall classification of the various types of coat imposed dormancy. To clarify the issue, more detailed information regarding the histochemistry and structure of these envelopes is required.

The second major category of dormancy types includes those situations where dormancy is primarily determined by specific anatomical, morphological or physiological peculiarities of the embryo itself. In these instances it is invariably those factors which bring about physiological changes that stimulate germination.

Considering these individually, dormancy due to rudimentary or underdeveloped embryos (i.e. morphological dormancy) is found in 46 Angiosperm genera (MARTIN, 1946) but is rare in the dicotyledons (NIKOLAEVA, 1977). In instances where it does occur, it appears that the seeds are shed when the embryo has not yet reached its complete morphological maturity and remain as such until the dormancy breaking treatment is experienced. The most common of these dormancy breaking treatments is a three to five month period of warm after-ripening. An extreme example of this is found in seeds of the Brazilian tree, *Anona crassiflora* Mart. which require up to ten months after-ripening. Here the seeds are shed when the embryo is little more than a hyaline mass of cells with no recognizeable parts. Differentiation only commences after the onset of the after-ripening treatment (RIZZINI, 1973). *Heracleum sphondylium* L. and *Ilex opaca* Ait. also deserve special mention here, since both have been thoroughly investigated and are classical examples of this mechanism. At shedding, the seeds of *H. sphondylium* contain only small, heart-shaped embryos which constitute about 0,4 per cent of the dry mass. During after-ripening this value changes to 30 per cent as a result of embryo growth (STOKES, 1952). The embryos of *I. opaca* exhibit a form of morpho-physiological dormancy. In this species, embryo growth is arrested at the heart-shaped stage due to the presence of inhibitors. Germination only occurs once the physiological requirements for dormancy breaking have been satisfied, i.e. the disappearance of the inhibitors during after-ripening (HU, ROGALSKI and WARD, 1979). This combination of growth

inhibition and embryo development actually occurs in the majority of cases and is prevalent in seeds of tropical species (NIKOLAEVA, 1977).

The remaining form of endogenous dormancy is usually found in seeds with fully developed embryos but which have considerably reduced physiological activity. Varying degrees of this physiological dormancy have been recognized and these are broadly divided into non-deep, intermediate and deep physiological dormancy (Table 1.2). However, irrespective of the depth of dormancy, the most common treatments which effectively overcome it are after-ripening at certain temperatures, periods of dry storage or exposure to light. These will be considered individually.

Many seeds will not germinate unless they are exposed to some particular temperature prior to incubation at 'normal' germination temperatures. The most common of these treatments is pre-incubation at low temperatures or, more correctly, after-ripening at low temperatures (LEWAK and RUDNICKI, 1977). This requirement appears to be associated with plants from temperate regions and is prevalent in species of the Rosaceae and Coniferae (STOKES, 1965). In these climates the low temperatures and presence of moisture during the winter months would provide the after-ripening treatment necessary to permit germination in the spring. This mechanism seems to ensure that seeds do not germinate during the unfavourable winter period.

Cold after-ripening usually occurs most rapidly between 2° and 5°C over a period of two to five months depending on the species (STOKES, 1965). Two factors suggest that considerable metabolic activity takes place during these cold treatments. Firstly, seeds must be imbibed for after-ripening to be effective. The second indication lies in the fact that seeds are able to germinate over an increased range of temperatures as after-ripening proceeds. A variety of metabolic changes have, in fact, been measured under these conditions. In cold after-ripening cherry seeds (*Prunus cerasus* L.), for example, the embryonic axis was found to increase in cell number, dry mass and total length (POLLOCK and OLNEY, 1959). Important hormonal changes also occur during this period in some species and in earlier studies on this particular aspect, it was suggested that dormancy resulted from the presence of inhibitors. This was based on the finding that excised embryos would often germinate once they had been rinsed in water. However, the seedlings which resulted from this treatment were very often physiologically dwarfed and closely resembled the seedlings obtained from unchilled or inadequately chilled intact seeds (BASKIN and BASKIN, 1975). These dwarf seedlings would resume normal growth only after a brief period at low temperatures or after applications of gibberellic acid. It was also found that in many species the entire chilling requirement could, in fact, be replaced by exogenously applied gibberellins. Furthermore, recent studies on the endogenous hormone levels in after-ripening *Acer saccharinum* L. and *Leucodendron daphnoides* Meissn.

seeds showed considerable increases in gibberellin and cytokinin concentrations. This provided evidence that gibberellic acid was intimately involved in the process of after-ripening. It probably also explained the phenomenon of physiological dwarfing in that it is the synthesis of these gibberellins during after-ripening that provides the essential stimulus for normal seedling growth. However, increases in gibberellic acid during chilling is not a universal phenomenon and in some cases cold after-ripening served only to remove a block to gibberellin biosynthesis. In *Corylus avellana* L. seeds, for example, synthesis itself only took place after the seeds were transferred to the 'normal' germinating temperature. Nevertheless, it appeared that irrespective of when the actual synthesis took place, this hormone was essential for germination and normal seedling growth.

Adaptions to certain local conditions have given rise to some interesting variations on the straightforward cold temperature requirement described (NIKOLAEVA, 1977). Seeds of *Aralia mandshurica* Seem require two separate periods of after-ripening; one at 18-20°C followed by another of similar duration at 0-3°C. *Virburnum opulus* L. seeds, on the other hand, germinate readily at 'normal' temperatures but subsequent outgrowth of the epicotyl will only take place after seedlings with sufficiently developed root systems are subjected to a chilling treatment. In extreme cases some seeds require more than a year to germinate and are termed biennial. Certain species of *Trillium* exhibit

this and their first requirement for embryo development is a period of warm temperatures followed by a period of low temperatures to stimulate hypocotyl growth and root formation. Further growth of the root system only occurs after another warm spell and proper growth of these now partially germinated seedlings takes place after a final exposure to low temperatures.

In many cereals and grass species (such as those of *Digitaria* and *Festuca*) the cold after-ripening requirement may be completely or partially replaced by dry storage at ambient temperatures (STOKES, 1965). Attempts to relate this loss of dormancy in dry storage to changes in inhibitor levels have met with mixed success. For example, no correlation was found to exist in the dry after-ripening seeds of *Xanthium pensylvanicum* (WAREING and FODA, 1957). On the other hand, extracts from fresh glumes of *A. kotschyi* seeds inhibited the germination of lettuce, whereas those from one year old glumes did not (WURZBURGER and LESHEM, 1969). This suggested the disappearance of an inhibitor during storage. In some cereals this disappearance is attributed to the volatile nature of the inhibitor, whilst in others, the inhibitors are thought to undergo auto-oxidation. Enzymes, however, do not appear to be involved in this decline of inhibitory activity in dry seeds (STOKES, 1965; WAREING and SAUNDERS, 1971). Gibberellins are also intimately involved in dormancy loss during dry after-ripening. As in the case of cold treatments, however, the levels of gibberellins do not always increase

during dry storage. In such cases (e.g. *Avena fatua* L.) it is usually the ability to synthesize this hormone which is enhanced by the treatment (TAYLOR and SIMPSON, 1980).

No form of after-ripening is known to occur at high temperatures and there is little evidence to suggest that such temperatures can break dormancy in any manner whatsoever. An exception to this, of course, are the previously mentioned seeds in which high temperatures induce changes in the coat structure. Some desert species also show a clear response to elevated temperatures, and storage at 50°C promotes their germination and enhances seedling growth (CAPRON and VAN ASDALL, 1967).

Another factor which is known to play a role in the dormancy and germination of numerous species, is light (or the absence of it). Those seeds which require light to germinate are termed positively photoblastic, whereas those which only germinate in the dark are referred to as negatively photoblastic. There is yet another group of seeds which are completely indifferent to light although some of these may acquire a light requirement as a result of certain environmental conditions.

The precise mechanism by which light (or dark) overcomes seed dormancy is unclear but the role of the blue chromoprotein, phytochrome, in almost all light mediated germination responses is axiomatic (KHAN, 1977). The presence of phytochrome in seeds is well documented and its effect

may be promotive or suppressive, depending on the species. It does not appear to be active in dry seeds but is fully hydrated when 17 to 19 per cent of the seeds' uptake has occurred (TAYLORSON and HENDRICKS, 1977). Phytochrome exists in two forms and these are P_{FR}, the active form, and P_R the inactive form. The transformation of these can take place in a number of ways and these include;

(1) the activation of phytochrome from an inactive pre-existing form,
(2) the synthesis of phytochrome from precursors,
(3) the transformation of P_R to P_{FR} by red light or less effectively, by blue light,
(4) the transformation of P_{FR} to P_R by far-red light, which requires higher energies than the reverse conversion,
(5) the reversion of P_{FR} to P_R in the dark,
(6) the inverse dark reversion, detected in some dry seeds, causing a partial conversion of P_R to P_{FR}, and
(7) the destruction of P_{FR} by enzymes or some other means (MAYER and POLJAKOFF-MAYBER, 1975).

These transformations are summarized in Figure 1.3. The figure shows that phytochrome is normally in a state of equilibrium between its two forms and that this may be changed at any time by a large number of different factors. For example, in those species where light inhibits germination (e.g. *Nemophila insignis* Benth.) the effect would

be due to the far-red component causing all the active P_{FR} to be converted back to inactive P_R. Once a phytochrome effect such as this has been induced, it may subsequently persist in the dry seed for up to 12 months (VIDAVER, HSIAO and HSIUNG, 1972).

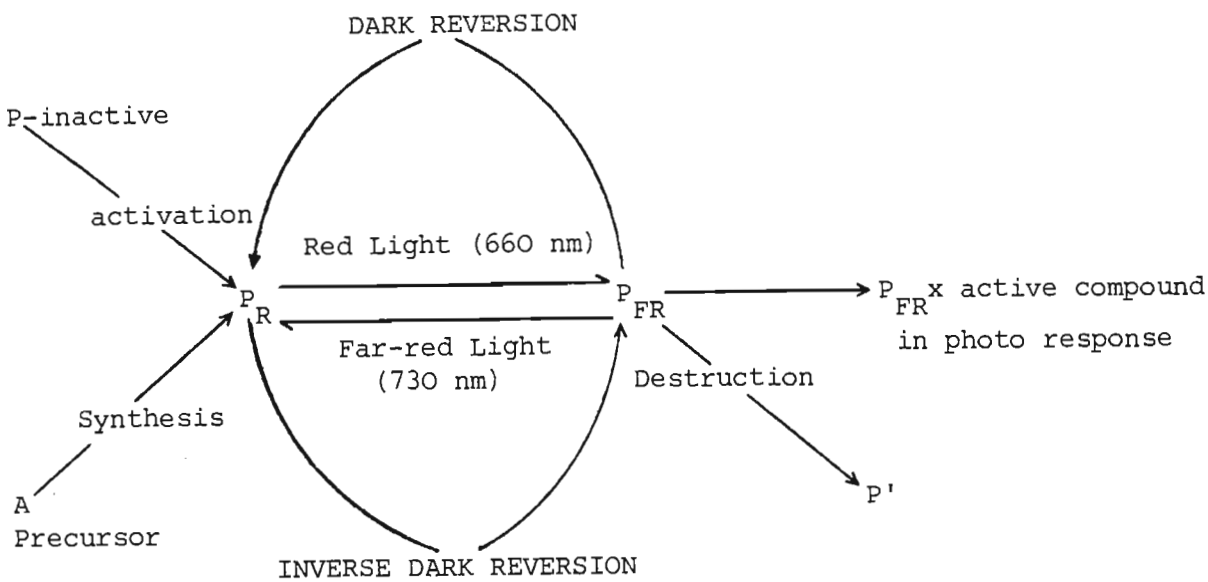

Fig. 1.3 The transformations undergone by phytochrome (MAYER and POLJAKOFF-MAYBER, 1975)

The requirement for P_{FR} has been associated with such factors as seed coats, membranes, various gases and more particularly with plant growth regulators. As with cold after-ripening, earlier studies suggested a close relationship between inhibitors and the photo-mechanism of seeds. It is now known that gibberellins and cytokinins are also involved in light mediated responses. KOHLER (1966) reported increases in the endogenous levels of gibberellin-like substances in illuminated lettuce seeds. Increased cytokinin synthesis has also been observed in seeds of *Lactuca sativa* L. after red light treatments (VAN STADEN,

1973). It is still not clear whether or not light has its primary effect on these hormones, nor has the actual site of phytochrome action been determined. Nevertheless, a number of hypotheses and models have been proposed to explain the action of red light. One of these, that of SMITH (1970), is outlined in Figure 1.4.

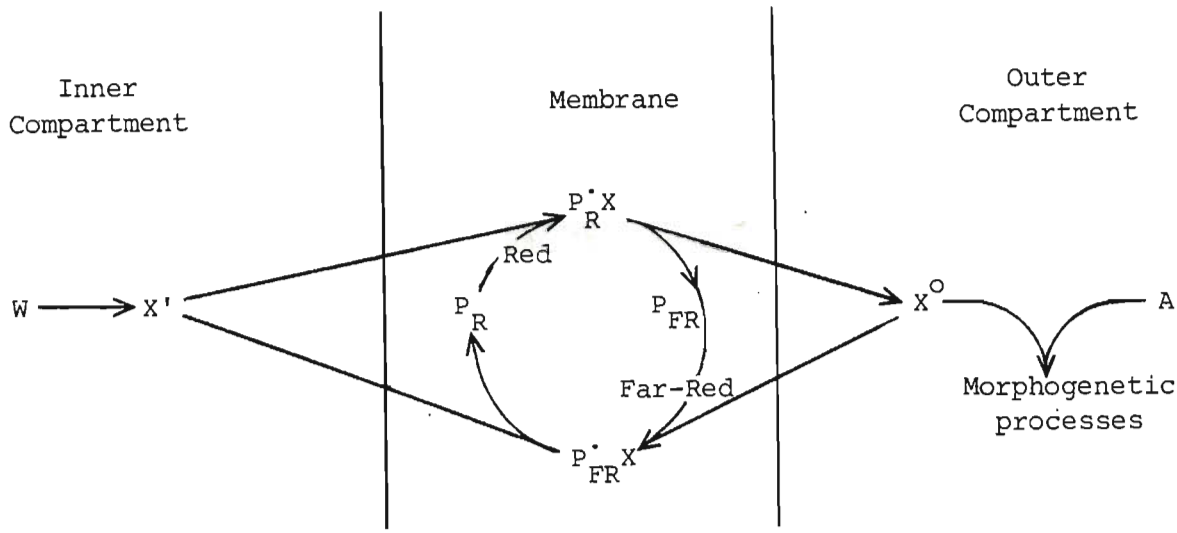

Fig. 1.4 A speculative model of phytochrome action (SMITH, 1970)

In this model SMITH proposed that two pools of a key compound (of unknown identity) exists in the seed. X' represented the pool inside a membrane bound compartment and X^O the pool outside of it. $P_R^{\cdot}$ and $P_{FR}^{\cdot}$ represent the immediate photoproducts of P_R and P_{FR} which form complexes with compound X. Thus, for example, red light treatments would favour the production of X^O and in this form X could interact with one or more metabolites present outside the membrane (represented by A). This in turn would initiate the sequence of reactions leading to some or other morphogenetic process. The action of phytochrome is thus likened to that of a permease enzyme specific for an important

metabolite (perhaps a hormone) and this action is directly driven by photoconversions. Cell membranes are known to be affected by phytochrome transformations but so also are many other aspects of metabolism and the difficulty lies in determining which of these are the cause of stimulated germination and which occur as a result of it (MAYER and POLJAKOFF-MAYBER, 1975).

The actual distribution of red and far-red light has been found to vary under natural conditions. At dusk and dawn, incident radiation becomes enriched with the far-red component. Furthermore, the spectral composition of radiation changes as it penetrates through a leaf canopy where leaves selectively absorb the red light component, resulting in a relative increase in the far-red light (SINCLAIR and LEMON, 1974). STOUTJESDYK (1972) demonstrated that these natural increases in far-red light could inhibit the germination of positively photoblastic seeds. The change in light quality as it passes through various seed and fruit structures has been used by CRESSWELL and GRIME (1981) to explain the tremendous variation in light response even in seeds from the same inflorescence. They propose that this germination polymorphism is probably related to the fact that as seeds mature, phytochrome is arrested in various photostationary states. These states are determined by the quality of light received by the seeds immediately before drying out. This in turn depends on the rate of chlorophyll loss in the investing structures since green tissues tend to selectively absorb red light. Therefore, seeds which de-

velop within green structures will almost certainly have light requirements since all their phytochrome will be in the inactive P_R form. In contrast, those seeds exposed to unfiltered light before drying will have most of their phytochrome in the active form making them indifferent to light. This theory may account for the vast differences in light requirement of members of the same species but other factors which may also contribute to this are;

(1) the genetic constitution of the parent plant,
(2) growth conditions experienced by the parent plant,
(3) post-harvest treatment of the seed, and
(4) conditions prevailing at the time of germination.

Thus, the modern view, in ecological terms, is that it is no longer sufficient to consider whether or not seeds are exposed to light. Both the spectral composition and the phytochrome reaction within the seeds must also be taken into consideration. In addition, genetic factors are also of considerable importance in this phenomenon.

The dormancy mechanisms mentioned in this general discussion are by no means mutually exclusive. On the contrary, various combinations of exogenous and endogenous dormancy are known to exist. For example, seeds of some *Tilia* species which are physiologically dormant also have impermeable seed coats. Only once the seed coat has de-

graded sufficiently to permit water uptake, will the metabolic changes associated with cold after-ripening commence (NIKOLAEVA, 1977). Another example already mentioned, is found in seeds of *Ilex opaca* which exhibit a form of morpho-physiological dormancy (HU *et al.*, 1979).

Secondary or acquired dormancy is a phenomenon which occurs in only a few species. It refers mainly to the condition where seeds will no longer germinate in an environment which previously supported their germination. Secondary dormancy has been induced in what are normally non-dormant *Xanthium* seeds by reducing the availability of oxygen. Seeds of certain *Lactuca sativa* cultivars also exhibit an acquired thermodormancy when imbibed in the dark at 33°C and this can only be relieved by fairly drastic treatments such as chilling or applied gibberellic acid (KHAN, 1981). However, secondary dormancy is generally overcome by normal dormancy breaking treatments and no special conditions are required. This suggests that the mechanisms underlying secondary dormancy are the same as those for primary dormancy. Furthermore, recent studies have shown few differences in hormonal action and interaction between seeds with primary and secondary dormancy (KHAN, 1981).

This then concludes the general discussion on the various dormancy mechanisms. Prior to commencing any experimental work on *Ricinodendron rautanenii* seeds, a brief survey was made of the germination requirements of a number of other well known oil-rich seeds. The results of this are

presented in Table 1.3. The data clearly demonstrates that there is no single dormancy mechanism common to all oil-seeds. Consequently, for the manketti each possibility had to be thoroughly investigated and this constitutes the first part of this study.

Table 1.3 The dormancy mechanisms of a number of well known oil-rich seeds and the treatments best known to promote their germination.

Scientific Name	Common Name	Oil Content %	Dormancy Mechanism	Applied Treatments	References
Macadamia ternifolia	Macadamia nut	75 - 79			
Bertholettia excelsia	Brazil nut	65 - 68	Insufficient detail†		WOODROOF, 1967
Carya illinoensis	Pecan nut	65	Coat - Physical	High or low temperature incubation	VAN STADEN and DIMALLA, 1977
Corylus avellana	Hazel nut	60 - 68	Cold after-ripening	Gibberellin	JARVIS *et al.*, 1968
Pistacia sp.	Pistachio nut	65	Coat - Chemical	Coat removal	WOODROOF, 1967
Ricinus communis	Castor bean	35 - 57	Coat	Coat removal	PURSEGLOVE, 1968
Arachis hypogaea	Peanut	45 - 56	Hormonal - Ethylene	Applied Ethylene	KETRING and MORGAN, 1969
Junglans nigra	Black walnut	55	Cold after-ripening	Artificial cold after-ripening	WOODROOF, 1967
Sesamum nigra	Sesame	45 - 55	Dry after-ripening	Insufficient detail†	ASHRI and PALEVITCH, 1979
Helianthus annuus	Sunflower	32 - 46	Cold after-ripening	Gibberellin	CSERESNYES, 1979
Protea compacta	Botrivier protea	18	Coat - Physical	Increased O_2	BROWN AND VAN STADEN, 1973

† Little work appears to have been carried out on this aspect.

MATERIALS AND METHODS

1.0 General

Mature fruits of *Ricinodendron rautanenii* Schinz were supplied by the Owambo Development Corporation of South West Africa. These were collected from the Tsumeb forests in Owamboland and batches from both the 1977 and 1978 harvest were received. The two batches were kept separate and stored dry at 5°C. This temperature was regarded as suitable since it had previously been reported that seeds remained viable for up to two years when stored at 10°C (KUMAR, 1978). All the experiments described in this section were carried out on 'nuts' where the exocarp and mesocarp tissues had been removed (Figure 1.2). Thus, in all further discussion the word 'nut' will be used to describe seeds which were still enclosed in their woody endocarp but where the fleshy tissues had been removed. Removal of the endocarp was achieved by first sawing a two millimetre deep ridge around the long axis of the 'nut'. The 'nut' was then split using a pair of modified pliers, with care taken not to damage the seed inside. This operation was time consuming and thus placed a constraint on the number of replicates used in the experiments. In most instances four replicates of 20 'nuts' or seeds were used and all experiments were repeated at least once using samples from each year's harvest. The selection of 'nuts' and seeds for use in the various experiments was done by placing them in beakers of water and discarding those which

remained floating after 15 minutes. The remaining 'nuts' and seeds were then immersed in a 0,1 per cent solution of Kaptan ({N-)trichloromethylthio)-4-cyclohexene-1,2 carboximide}) for 30 minutes in an attempt to reduce the incidence of fungal attack. An additional wash of 1 per cent sodium hypochlorite was given to those seeds which, for experimental purposes, had any portion of the endosperm exposed. This was done because experience had shown that exposed seeds were highly susceptible to fungal and bacterial infection.

Unless otherwise indicated, the 'nuts' and seeds were incubated on either autoclaved vermiculite or acid washed sand (both of which are inert) which were usually moistened with glass distilled water. The containers used were either autoclaved glass petri dishes or plastic seed trays sterilized with 80 per cent alcohol. Where plastic seed trays were used, a high relative humidity was maintained by placing them in polythene bags. All incubators used were accurate to within 0,5°C. Chemicals used were reagent grade. Germination was recorded daily and in accordance with normal practice, germination was regarded as completed when the radicle had emerged from the covering structures and was sufficiently elongated to show positive geotropic curvature (WEBB and WAREING, 1972; BROWN and VAN STADEN, 1973). In all those experiments where no significant differences were detected between 'nuts' and seeds of the 1977 and 1978 harvests, the results were pooled and are presented in this report as a single result.

2.0 Effect of the covering structures

The presence of the hard, woody seed coats in *R. rantanenii* seeds suggested that they may play some role in the dormancy of this species. As already discussed, the covering structures of a seed may prevent germination in a number of different ways which may include;

(1) impermeability of the envelope to water uptake or gas exchange,
(2) presence of an inhibitor in the seed coat,
(3) retention of an inhibitor within the envelope, and
(4) mechanical restraint of radicle elongation (EGLEY, 1972).

When dormancy is imposed by the covering structures of a seed their complete or partial removal very often results in increased germination (BARTON, 1965). Thus, in the first experiment carried out, an attempt was made to establish the overall effects of these structures. At the same time, the optimum temperature for germination was also investigated. In this latter investigation, 'nuts', seeds and excised seeds (testa removed) were incubated at temperatures of 5, 10, 15, 20, 25, 30 and 35°C. In addition, some 'nuts', seeds and excised seeds were subjected to an alternating temperature treatment using 5 and 25°C. This was combined with an eight hour light, 16 hour dark

photoperiod with the lower temperature occurring during the dark period. The entire experiment was terminated after 21 days and those 'nuts' and seeds which had not germinated at 5, 10, 15 and 20°C were removed and then incubated at 30°C for a further 14 days.

2.1 Experiments examining the possible physical effects of the covering structures

2.1.1 *Structure*

The structure of both the endocarp and testa was examined under the scanning electron microscope. For this purpose, portions of each coat were mounted on specimen stubs and coated with an 80 angstrom thick layer of gold using a Polaron spluttrer. These were then viewed with a Jeol JSM-T200 scanning electron microscope.

2.1.2 *Water uptake*

Two experiments were carried out to determine whether or not the endocarp and/or testa of *R. rautanenii* seeds restricted water uptake. In the first of these the amount of water imbibed by 'nuts', seeds and excised seeds of known mass was determined gravimetrically on a daily basis. Incubation was at 30°C and the experiment was terminated after 21 days. In the second experiment the uptake and distribution of tritiated water (obtained from Amersham Radiochemicals, United Kingdom) was investigated. Two replicates of ten 'nuts' and seeds were used and these were placed in 100 millilitres of water containing 20 µℓ of ^3H_2O

and incubated at 30°C for 48 hours. The specific activity of this solution was 0,05 µCi. After the incubation period the seeds and 'nuts' were given three washes in distilled water to remove any residual activity. The coats were then removed and the radioactivity present in the embryonic axes, cotyledons and endosperm, determined separately. This was achieved by digesting 20 milligrams of the fresh tissue in 0,5 millilitres digestion fluid made up of hydrogen peroxide and perchloric acid (1:1 v/v). Digestion was carried out in scintillation vials and was enhanced by incubating these at 60°C for three hours. After this period the vials were left to cool whereupon 10 millilitres of 'Redy-Solv' EP scintillation fluid was added to each vial (BREVEDAN and HODGES, 1978). The vials were then left for 12 hours after which the radioactivity was assessed by counting in a Beckman LS8100 spectrometer. Correction for quenching was achieved by the channels ratio method.

2.1.3 *Scarification*

A number of experiments were carried out to distinguish between coat impermeability and any physical restriction of radicle elongation. For this 'nuts' and seeds were mechanically scarified by filing away small portions of the endocarp or testa. The scarification treatments applied included;

(1) endocarp or testa scarified at the 'radicle end',

(2) endocarp or testa scarified at the end opposite the radicle,

(3) endocarp or testa scarified on the side of the seed midway between the two ends and incubated with the scarified surface facing upwards, and

(4) same as (3) but incubated with the scarified portion in contact with the substrate (Figure 1.2) (BROWN and VAN STADEN, 1973).

It is also thought that water uptake can be improved, and mechanical restriction reduced, when seed coats are hydrolyzed with strong acids (ROLSTON, 1978). The effects of this treatment on *R. rautanenii* 'nuts' and seeds were assessed by immersing these in concentrated sulphuric acid. Samples were taken after 2, 6, 12, 24 and 96 hours respectively. These were then incubated at 30°C after washing in running water for 60 minutes to remove all traces of the acid.

Representative samples of 'nuts' and seeds were also subjected to a number of additional treatments which are known to modify coat structures and reduce impermeability. These included:

(1) Heat treatments where 'nuts' and seeds were placed in boiling water for 30 and 60 seconds or incubated dry at 100°C for 5 and 10 minutes prior to incubation at 30°C (LA CROIX and STANIFORTH, 1964).

(2) Freezing and thawing treatments where 'nuts' and seeds were kept at -20°C for 60 minutes. After gradual thawing these were then incubated at 30°C (ROLSTON, 1978).

(3) Removal of any waxy substances by immersing 'nuts' and seeds in absolute ethyl alcohol or acetone for 12, 24 and 48 hour periods (ROLSTON, 1978). Subsequent incubation was at 30°C.

2.2 Experiments examining the possible chemical effects of the covering structures

2.2.1 *Leaching treatments*

Coat imposed dormancy may also be due to the presence of inhibitors in the seed coat as in the case of *Corylus avellana* (BRADBEER, 1968). Two leaching experiments were carried out to determine whether or not such compounds were responsible for dormancy in *R. rautanenii* seeds. In the first leaching treatment an attempt was made to simulate the effects of rain. To achieve this, water from an overhead spray mechanism was passed over intact 'nuts' and seeds at a rate of approximately three litres per minute for six hours each day. Samples were removed after 5 and 10 days and incubated at 30°C.

The second leaching experiment was based on that of BROWN and VAN STADEN (1973) where intact 'nuts' and seeds were shaken in 200 millilitres of distilled water for 24, 48 and 72 hours respectively. The water in each beaker was

replaced every six hours and the leachates from each of these was retained. Subsequent incubation was at 30°C. The bulked leachates from each treatment were then concentrated under vacuum to 100 millilitres. To determine whether these leachates contained any germination inhibitors, five replicates of 20 excised seeds were incubated in petri dishes containing acid washed sand moistened with 10 millilitres of each concentrate. Distilled water was used as a control and all petri dishes were incubated at 30°C.

2.2.2 *Gas treatments*

The possibility also existed that certain compounds present in the covering structures could reduce the amount of oxygen available to the embryo. Some indication of this would have been provided by the leaching treatments already described. Nevertheless, an additional experiment was carried out where 'nuts' and seeds were incubated in gas tight 500 millilitre ehrlenmeyer flasks which were daily flushed with either oxygen, nitrogen or air. Subsequent incubation was at 30°C. Oxygen diffusion to the embryo may also be retarded by the presence of excess water accumulating in the seed coats. To examine this possibility mid-scarified 'nuts' and seeds, placed with the scarified region facing upwards, were incubated at 30°C in atmospheres enriched with either oxygen, nitrogen or air. Care was taken in both experiments to ensure that the pressure in the flasks did not exceed atmospheric pressure since this alone may retard germination (BROWN

and VAN STADEN, 1973).

2.2.3 *Light treatments*

In many seeds where the covering structures play a significant role in dormancy, germination is under phytochrome control (EVENARI, 1965). However, the woody nature of *R. rautanenii* seed coats suggested that it was unlikely that such a mechanism existed in this instance. Nevertheless, intact and mid-scarified (with exposed portion facing upwards) 'nuts' and seeds were subjected to various light treatments. These included;

(1) continuous light,
(2) continuous dark,
(3) continuous dark with a daily two hour red-light treatment, and
(4) continuous dark with a daily two hour far-red light treatment. Incubation was at 30°C.

3.0 Endogenous dormancy factors

It is possible that, in spite of their robust appearance, the covering structures of *R. rautanenii* seeds may only play a limited role (if any) in the dormancy phenomenon. In such instances dormancy would most probably be due to the physiological incompetence or morphological under-development of the embryo. It is unlikely that dormancy in *R. rautanenii* can be ascribed to morphological under-development since, although the embryos of this species are

small, all the regions are fully differentiated. However, the possibility that these embryos exhibited physiological incompetence was investigated in considerable detail.

3.1 Low temperature after-ripening

It is well known that both embryo and coat imposed dormancy may be overcome by periods of storage at low temperatures (WAREING and SAUNDERS, 1971). This possibility was investigated in the manketti by incubating intact and mid-scarified 'nuts' and seeds, as well as a representative number of excised seeds, under moist conditions at 5°C. The mid-scarified seeds were placed such that the exposed portion was in contact with the substrate. Samples were removed after 30, 60, 90 and 120 days and subsequently incubated at 25, 30 and 35°C respectively. An additional experiment was carried out using acid scarified 'nuts' and seeds. These were also incubated at 5°C but samples were removed after only 14, 28 and 42 days since seeds were found to succumb to fungal attack when kept for periods longer than this. In addition, subsequent incubation was only at 30°C in this experiment.

3.2 Dry after-ripening

To determine whether or not the seeds of this species, like those of many cereals and grasses, have a dry after-ripening requirement, intact 'nuts' and seeds were placed in open trays and stored dry at 10, 20, 25 and 30°C for 30 days.

Thereafter they were placed on moist substrates and incubated at 30°C.

3.3 Leaching

An additional leaching experiment was carried out to establish whether or not water-soluble inhibitors present within the seed tissues themselves, were responsible for dormancy. Excised seeds were used in this instance and these were subjected to the same leaching treatments as described in section 2.2.1 except that samples were taken after 48 and 96 hours.

3.4 Sterile embryo culture

The *in vitro* culture of dormant embryos has long been recognized as a successful means of obtaining early germination (SHARMA, 1979). This technique has also been included in the International Rules for Seed Testing (1976) as a means of establishing the viability of dormant seeds. Germination under these conditions would suggest that *in vivo*, the embryos lack certain key compounds or that dormancy is due to inhibitors, present in the surrounding tissues. For this type of study, no single nutrient medium is regarded as ideal and different media have been used for different species (HENNY, 1980; JANERETTE, 1978). Two culture media were selected for this experiment.

The first of these was MURASHIGE and SKOOGS' (1962) basal

medium. One litre of this medium was prepared by placing 500 millilitres of glass-distilled water into a one litre volumetric flask and then adding the appropriate amounts of stock solutions and vitamins (Table 1.4). No hormones were added. Following the addition of 30 grammes of sucrose, the volume was made up to one litre with more distilled water and the pH was adjusted to 5,8. Ten grammes of Difco Bacto agar (i.e. 1 per cent w/v) were then added and dissolved in the solution by autoclaving at 1,05 bars for five minutes. Using a syringe, ten millilitres were dispensed into 2,5 x 8 centimetre specimen tubes. Each tube was capped and autoclaved at a pressure of 1,05 bars for 20 minutes.

The other medium used was a modified MILLERS (1963; 1965) basal medium. The stock solutions composing this medium are outlined in Table 1.5. These were combined in the proportions indicated and all further preparations were the same as those described above. A one per cent agar solution containing no nutrients or hormones was used as a control.

In this experiment only embryonic axes were used and these were excised from either dry, untreated dormant seeds or seeds which had been mid-scarified and incubated at 5°C for 30 days. Initial preparation for sterile culture was carried out by washing whole, excised seeds in a 3 per cent solution of sodium hypochlorite (NaOCℓ) for three minutes (JANERETTE, 1978). This was followed by three, ten minute

Table 1.4 Basal medium for the sterile culture of excised *Ricinodendron rautanenii* embryos (Adapted from MURASHIGE and SKOOG, 1962)

Stock Solution	Chemical	Mass g 500 mℓ$^{-1}$	mℓ Stock Solution per litre Medium
I	NH_4NO_3	82,5	10
II	KNO_3	47,5	20
III	$CaCl_2$	17,2	10
IV	$MgSO_4.7H_2O$	18,5	10
V	NaFeEDTA	2,0	10
VI	KH_2PO_4	8,5	10
VII	H_3BO_3	0,310	10
	$MnSO_4.4H_2O$	1,115	
	$ZnSO_4.7H_2O$	0,430	
	KI	0,042	
VIII	$NaMoO_4.2H_2O$	0,013	10
	$CuSO_4.5H_2O$	0,0013	
	$CoCl_2.6H_2O$	0,0013	
IX	Thiamine HCl	0,005	10
	Niacin	0,025	
	Pyridoxine HCl	0,025	
	Glycine	0,010	
Additional Sugar			30 g 1^{-1} medium
	Agar		10 g 1^{-1} medium
pH adjusted to 5,8 with NaOH			

Table 1.5 Basal medium for the sterile culture of excised *Ricinodendron rautanenii* embryos (Adapted from MILLER, 1962; 1963)

Stock Solution	Chemical	Mass g l^{-1}	mℓ Stock Solution per litre Medium
I	KH_2PO_4	3,0	100
	KNO_3	10,0	
	NH_4NO_3	10,0	
	$Ca(NO_3)_2.4H_2O$	5,0	
	$MgSO_4.7H_2O$	0,715	
	KCl	0,65	
	$MnSO_4.4H_2O$	0,14	
II	NaFeEDTA	1,32	10
	$ZnSO_4.7H_2O$	0,38	
	H_3BO_3	0,16	
	Kl	0,08	
	$Cu(NO_3)_2.3H_2O$	0,035	
	$(NH_4)_6Mo_7O_4.4H_2O$	0,01	
III	Myo-inositol	10,0	10
	Nicotinic acid	0,2	
	Pyrodoxine HCl	0,08	
	Thiamine	0,08	
IV	NAA	0,02	10
Additional	Sucrose		30 g l^{-1} medium
	Agar		10 g l^{-1} medium
pH adjusted to 5,8 with NaOH			

washes in sterile glass-distilled water. The embryonic axes were then excised and transferred to the culture media on a laminar flow bench. These were subsequently incubated at 25 ($\pm$ 1)°C under continuous light and the experiment was terminated after 28 days.

3.5 Applied plant hormones

Applied hormones are effective in overcoming many different types of dormancy. For example, growth promoters such as kinetin and gibberellic acid can substitute for the chilling requirement of certain seeds (WEBB and DUMBROFF, 1969; BASKIN and BASKIN, 1970). In addition, the results obtained from such treatments often provide useful information regarding the possible endogenous hormone levels. To observe the effects of applied hormones on *R. rautanenii* germination, 'nuts', seeds and mid-scarified seeds (exposed portion facing downwards) were incubated on acid washed sand moistened with the various hormone solutions listed in Table 1.6.

For each treatment, three grammes of acid washed sand were placed in each of five petri dishes. The substrate was then moistened with five millilitres of the respective hormone solutions. Throughout the experiment both 'nuts' and seeds were prevented from drying out by continued reapplication of the various solutions.

Table 1.6 Plant growth regulators applied to dormant *R. rautanenii* 'nuts' and seeds. Concentrations used were based on those which appear most frequently in the literature.

Compound	Concentration in mg ℓ^{-1}
Zeatin	10
	1
Gibberellin-A_3	500
	50
	20
Indole acetic acid	20
	10
Ethrel*	250
	75

* Effective ethylene concentrations calculated from manufacturers specifications. Actual ethrel concentration applied = 0,5 and 0,2 millilitres per litre respectively.

RESULTS AND DISCUSSION

Role of the covering structures

The first experiment carried out on manketti 'nuts' and seeds attempted to establish whether the seed coats were responsible for dormancy. In addition the experiment was also designed to establish the optimum temperature for germination since species differ considerably in their temperature requirements. (To illustrate this, optimum germination of *Carya illinoensis* (Wang) K. Koch cv. Curtis seeds occurs at between 30 and 35°C (VAN STADEN and DIMALLA, 1975), whereas *Acer pseudoplatanus* seeds will not germinate at temperatures above 12°C (WEBB and WAREING, 1972)). The results of this experiment are presented in Table 1.7.

Table 1.7 Percentage germination of 'nuts' and seeds incubated for 21 days at various temperatures. Figures in parentheses represent the confidence limits where P = 0,05.

	Temperature						
	5	10	15	20	25	30	35
Nuts	0	0	0	0	0	0	0
Seeds	0	0	0	0	0	0	0
Excised seeds	0	0	0	0	23 ($\pm$4)	28 ($\pm$5)	30 ($\pm$2)

These data show that some germination does occur once the testa has been removed but only at temperatures greater than

20°C. Two deductions were made from this. The first was that the temperature requirements for the germination of this species are well defined. (From this a germination temperature of 30°C was selected for use in subsequent experiments based on the fact that fewer seeds than those incubated at 35°C were infected with fungus and that germination occurred more rapidly at this temperature than at 25°C). The second, and probably the most important deduction,was that the testa in some way contributed to the dormant condition of these seeds. With regard to the latter, blocks to germination eliminated by the removal of seed envelopes are often considered to include one or more of the following factors;

(1) impermeability of the envelopes to water uptake or gas exchange,
(2) presence of an inhibitor in the seed coat,
(3) retention of an inhibitor within the envelope, and
(4) mechanical restraint of radicle elongation.

Each of these was examined in the experiments carried out. The results of the investigations conducted to determine the extent of water impermeability are presented in Figure 1.5. From this it appeared that the water uptake curve for excised seeds resembled the typical triphasic water uptake pattern of most germinating seeds (BEWLEY and BLACK, 1978) although only 35 per cent germination was recorded in this instance. When the covering structures were left intact,

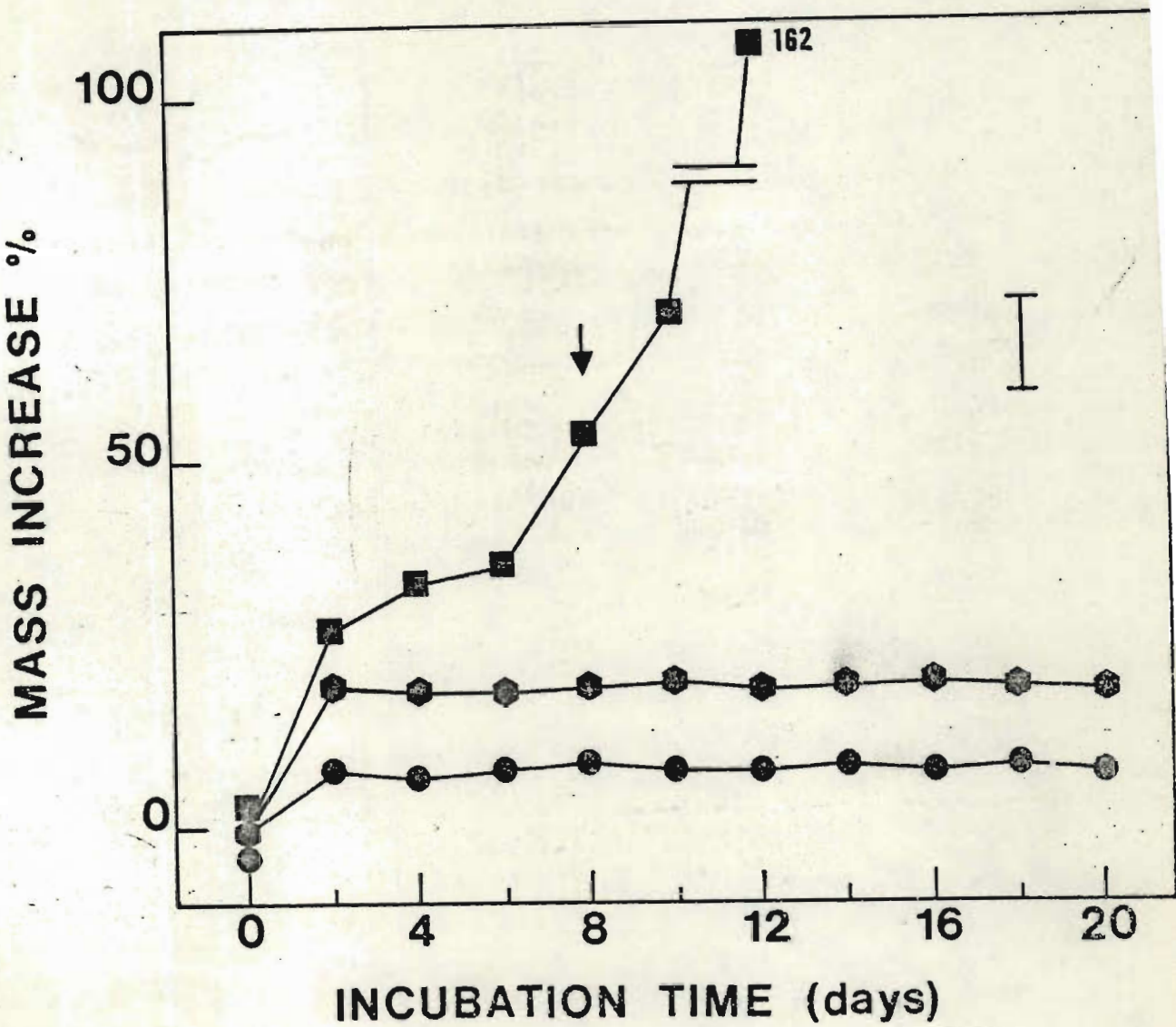

Figure 1.5 The effect of the seed coats on water uptake. Intact 'nuts' (●), intact seeds (⬢) and excised seeds (■) were incubated in the dark at 30°C on moist vermiculite. The figures represent the mean of four replicates of 20 seeds or 'nuts'. The bar represents the maximum 95 per cent confidence limit. Arrow denotes the time at which germinat

however, the water uptake curve was found to reach an asymptote after approximately 48 hours. It was not clear from this data alone whether the water imbibed by the intact 'nuts' and seeds was in fact reaching the seed tissues themselves or whether it was simply being held in the coat matrix. In this respect, the results of the tritiated water uptake experiment were more conclusive and these are presented in Figure 1.6.

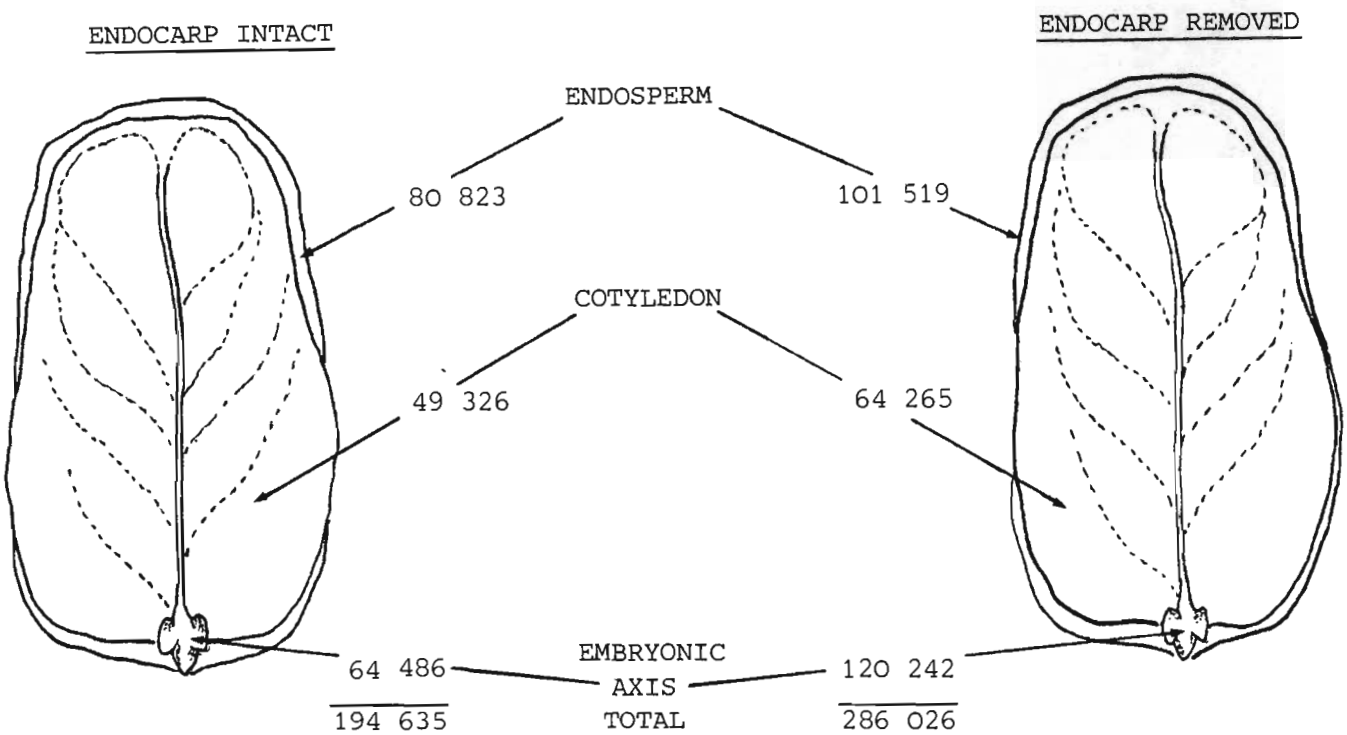

Fig. 1.6 Uptake and internal distribution of tritiated water determined in seeds where the endocarp was either present or absent. Radioactivity is expressed as DPM's per gramme fresh mass.

It is clear from this that water does indeed penetrate both the endocarp and testa and is imbibed by all the internal seed tissues. The results also show that 47 per cent more water was imbibed by those seeds where the endocarp was removed. This suggests that in such instances water is more

accessible and probably results from the fact that the fluid simply has a shorter distance to travel. From these data it was concluded that restriction of water uptake was not the means by which the testa inhibited germination.

An examination of the covering structures under the scanning electron microscope supported this view. For example, the large pores visible on the endocarp surface were found to extend through to the testa (Plate 1.1A). In addition, the lignified cells comprising the endocarp are not arranged compactly, resulting in the presence of large air spaces. The testa on the other hand, has no visible pores and is made up of a pallisade layer of tightly compressed macrosclereids (Plate 1.1B). Plate 1.1B also shows that although the ends of these macrosclereids are imperforated and that a compact layer of cells is clearly visible on the inner surface of the testa, none of the cellular structures or cuticular materials often associated with impermeability, are visible. In addition, numerous pits are located on the surface of the macrosclereids. These features alone suggest that the endocarp and testa are not water impermeable.

In many earlier studies it was suggested that the covering structures of some seeds mechanically restricted radicle extension (WEBB and WAREING, 1972). More recently, WILSON, HIBBS and FISCHER (1979) proposed that although the dormancy of *Acer pensylvanicum* L. seeds is essentially endogenous, the testa exerts a mechanical restriction on embryo growth after the cold after-ripening requirement has been satisfied.

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Plate 1.1

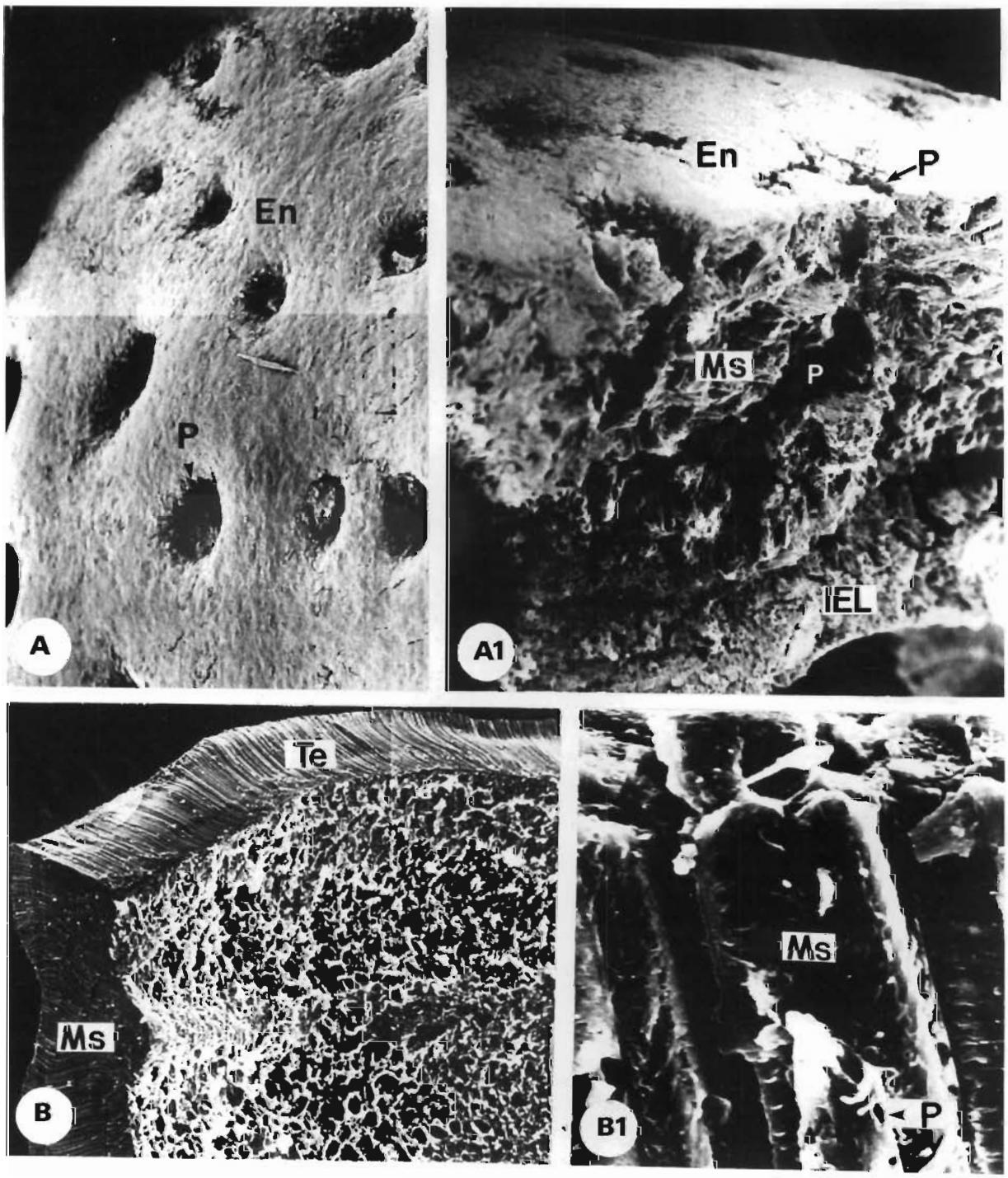
SCANNING ELECTRON MICROGRAPHS OF THE SEED COATS OF *RICINODENDRON RAUTANENII* SEEDS.

A: A surface view of the endocarp (EN) showing the numerous large pores (P). X 100.

A1: A magnified view of the endocarp (EN) showing the disorganized arrangement of the macrosclereids (MS) and how the pores (P) are continuous throughout this structure. A more compact internal endocarp layer (IEL) can also be seen. X 500.

B: A general view of the testa (Te) showing the more compact arrangement of the macrosclereids (Ms). On the inner surface of the testa a compact internal layer of macrosclereids (IMS) is also visible. The cellular debri (CD) visible on the surface of the testa is probably the remnants of the nucellus. X 200.

B1: The ends of the macrosclereids (MS) comprising the testa are imperforate but numerous pores (P) can be found on the surface of these lignified cells. X 100.


En
P
A
En
P
Ms
P
IEL
A1
Te
Ms
B
Ms
P
B1

On the basis of coat structure it is feasible that this mechanism operates in *R. rautanenii* seeds especially in the light of VILLIERS' (1972) statement that such a mechanism should only be sought amoung those seeds which remain enclosed within extremely hard covering structures. However, no germination was recorded in any of the scarification treatments applied. This indicated that the testa and endocarp did not restrict radicle extension in manketti seeds, in spite of their hard, woody nature. In addition, treatments such as acid scarification, heating and cooling, freezing and thawing and soaking in organic solvents, (all of which may modify the coat structure in some way), did not remove dormancy. This supported the earlier data and served to confirm the proposal that both mechanical restriction of the embryo and water impermeability are not primarily responsible for dormancy in these seeds.

Another possible effect of the covering structures is the restriction of oxygen diffusion to the embryo as a result of 'chemical impermeability' (COME, 1967; WAREING and FODA, 1957). The presence of large amounts of darkly coloured substances in the leachates of manketti seeds (endocarp removed) suggested that the testa of this species may impose dormancy in this manner. These compounds, it was initially thought, might play a significant role in reducing the amount of oxygen available to the embryonic axis. However, the results from the gas treatments showed that the seed coats were not impermeable to oxygen since raising the partial pressure of this gas did not stimulate germination.

(Furthermore, the scarification treatments mentioned earlier, which would also improve gas exchange should this be retarded by excess water, also had no effect on germination). Further evidence that these darkly coloured substances were not responsible for dormancy was provided by the fact that leaching treatments did not relieve dormancy. This also indicated that these compounds are not growth inhibitors which impose dormancy by their inward diffusion. Furthermore, only 10 per cent germination was recorded in both the control and leachate treated excised seeds. This then provided strong evidence against the existence of any coat inhibitors.

The lack of any significant response from leaching and scarification treatments also suggested that neither the testa nor the endocarp restricts the outward diffusion of endogenous inhibitors as was found to be the case in *Acer pseudoplatanus* seeds (WEBB and WAREING, 1972).

Thus, the results obtained so far showed that those treatments usually associated with the breaking of coat imposed dormancy had little or no effect on *R. rautanenii* seeds. Furthermore, the germination of manketti seeds could not be stimulated by light treatments, thereby eliminating the possibility that the germination of this species is under phytochrome control. It must, therefore, be concluded at this stage, that in spite of the fact that removal of the testa did result in some germination, the covering structures of these seeds only play a very limited role in imposing the

dormant condition.

Endogenous dormancy

The alternative to coat imposed dormancy is the so-called endogenous dormancy, although the two are by no means mutually exclusive. Embryo dormancy appears to be most prevalent amoung species which have some form of after-ripening requirement. In some such species (e.g. *Acer pensylvanicum*) it has been found that during after-ripening the embryo develops sufficient thrust to overcome the restraint placed on it by the testa (WILSON *et al.*, 1979). The results of the various low temperature after-ripening experiments carried out on *R. rautanenii* seeds are presented in Figure 1.7.

In this experiment, only side-scarified seeds responded to the low temperature treatment. The results also showed that after-ripening for less than 30 days was insufficient to break dormancy, whereas periods longer than this were not effective due to bacterial infection. As in previous experiments, higher germination percentages were obtained at 30 and 35°C. It is difficult to comment on the relevance of these results since no germination occurred in intact 'nuts' or seeds subjected to these low temperatures. The effect of scarification, it appears, was not to improve water uptake since no germination resulted from incubating acid scarified seeds at 5°C for varying lengths of time. Nevertheless, scarification may still be an important factor

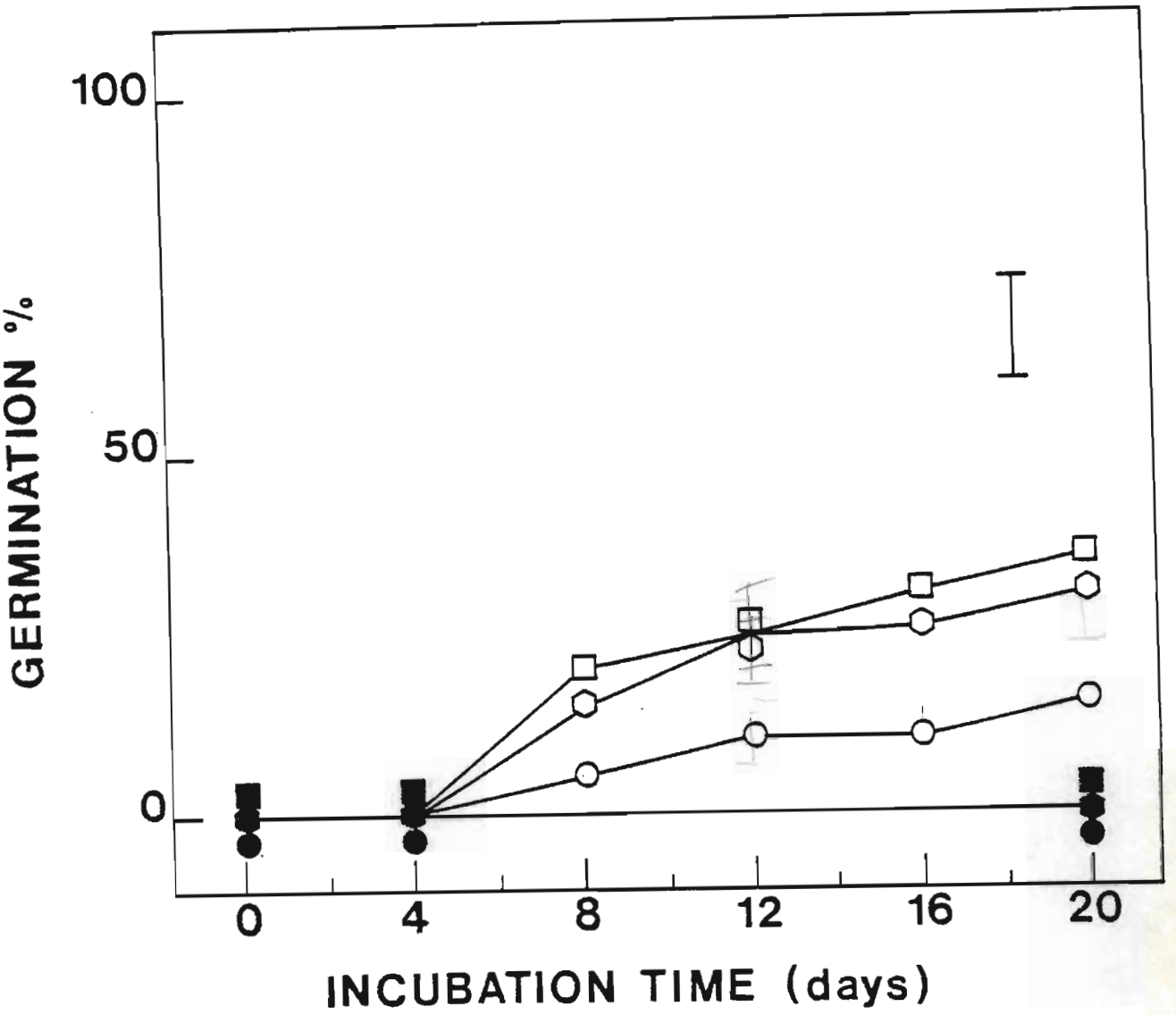


Figure 1.7 Germination of intact (shaded symbols) and side scarified (open symbols) seeds after 30 days stratification at 5°C. Subsequent incubation was at 25 (● , ○), 30 (⬢ , ⬡) or 35°C (■ , □). The bar represents the maximum 95 per cent confidence limit. No response was obtained from 'nuts' treated

under natural conditions since it has been reported that germination of manketti seeds will not take place unless the 'nuts' (ingested as fruits) have spent some time inside the alimentary canal of elephants (MENNINGER, 1977)! Unfortunately, the scientific validity of this report is questionable.

Unlike cold after-ripening, dry storage at various temperatures had no effect on germination whatsoever. This may indicate that the seeds need to be fully imbibed befor dormancy can be broken. However, this water would not be required to leach out any endogenous inhibitors since none of the excised seeds subjected to the various leaching treatments germinated.

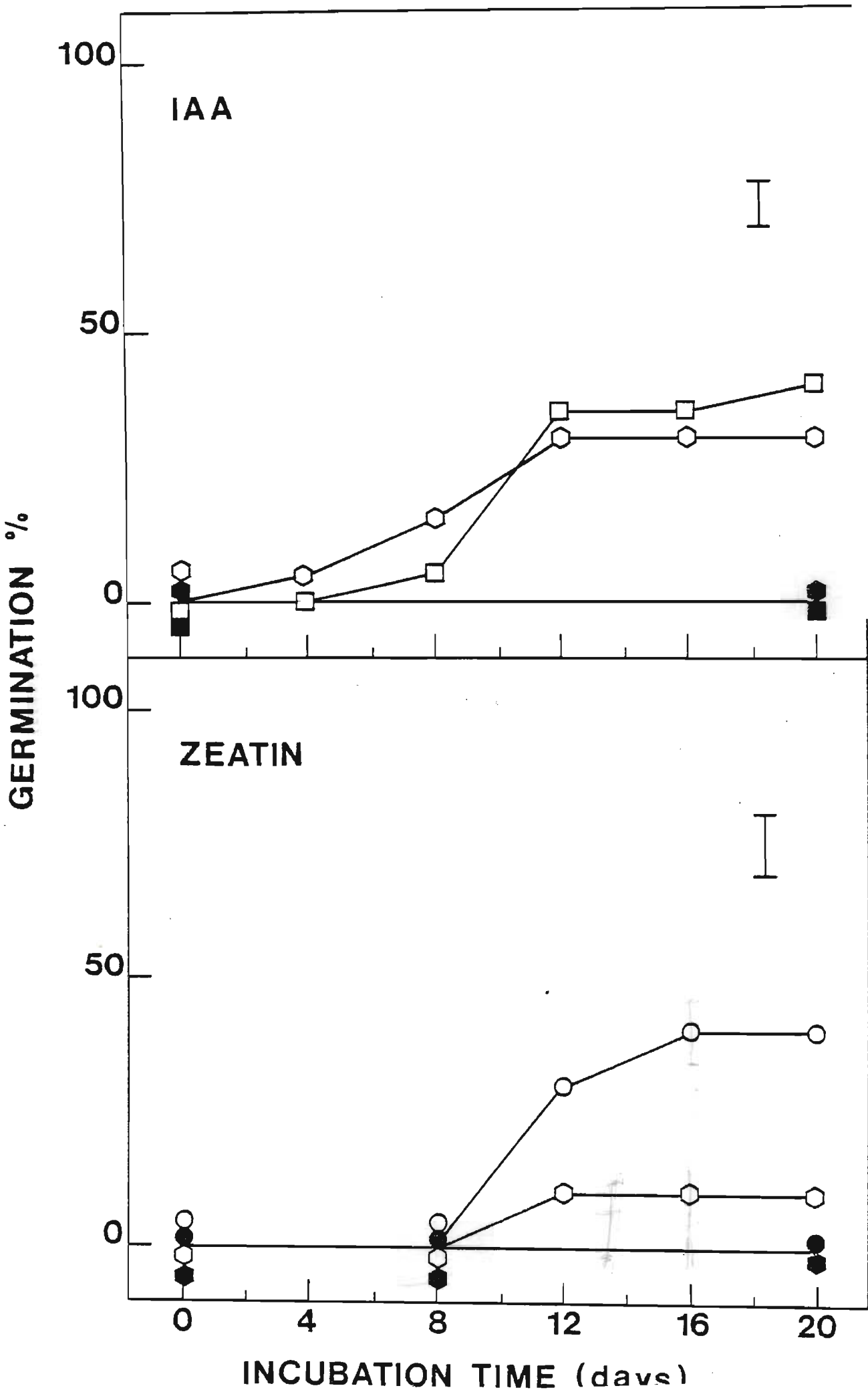
The results of the sterile embryo culture experiment are presented in Table 1.8.

Table 1.8 Germination of excised embryonic axes incubated for 28 days under sterile conditions on two different culture media. Sterilization was achieved using 3 per cent NaOCℓ. Figures in parentheses represent confidence limits where p = 0,05.

Culture Medium	Percentage Germination	
	Fresh Seed	After-ripened
MURASHIGE and SKOOG basal Medium	15 ($\pm$3)	0
MILLERS' Basal Medium	0	0
Agar	0	0

From these results it seems that manketti embryos, unlike those of many other species (such as *Abutilon theophrasti* and *Calyopsis basilis* Dietr which readily germinate on semi-solid culture media (LA CROIX and STANIFORTH, 1964; SHARMA, 1979)), are deeply dormant. These data also show that this dormancy cannot be attributed to the presence of inhibitors in the endosperm since removal of the embryos from this environment did not enhance germination. Similarly, the lack of germination in the presence of sucrose suggests that dormancy does not result from the inability of the embryo to hydrolyse more complex food reserves. This is further supported by the fact that embryos excised from cold after-ripened seeds, a treatment which often leads to increased reserve mobilization (LEWAK and RUDNICKI, 1977), did not germinate. Thus, much of the evidence points to the requirement for a highly specific external stimulus.

The results obtained from the experiments using exogenous hormone applications provided valuable information regarding the elusive germination stimulus. These are presented in Figures 1.8, 1.9 and 1.10. From these data it was clear that *R. rautanenii* seeds showed a marked response to plant growth regulators. Auxins, which have largely been ignored as agents for dormancy release (KHAN, 1977), did induce some germination but only in the case of scarified seeds (Figure 1.8). However, no seedlings resulted from this treatment. Zeatin also stimulated radicle elongation but its most marked effect was on the cotyledons. These organs were found to swell considerably and in some cases callus formation was


100
IAA
50
0
GERMINATION %
100
ZEATIN
50
0
0
4
8
12
16
20
INCUBATION TIME (days)

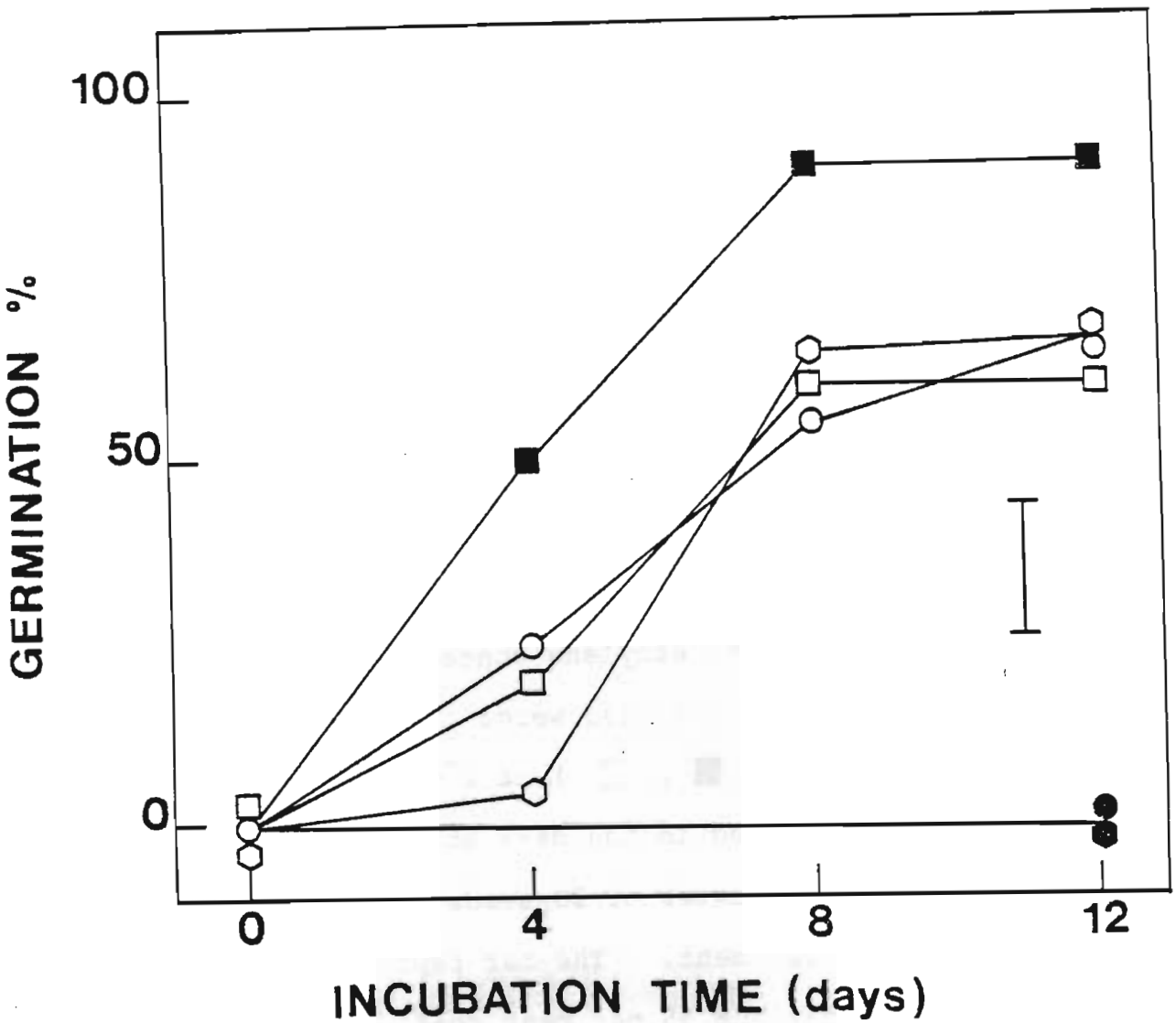
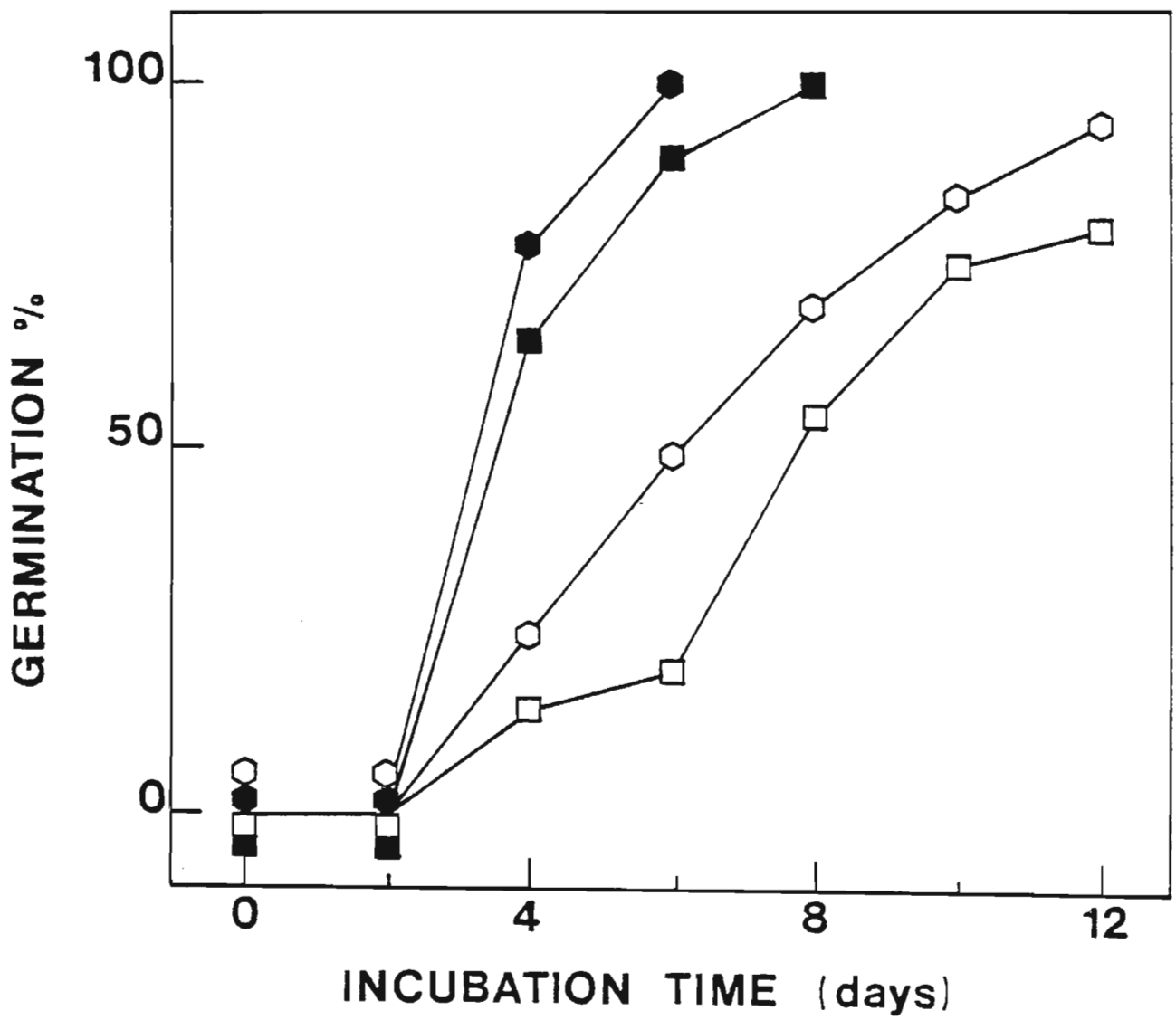


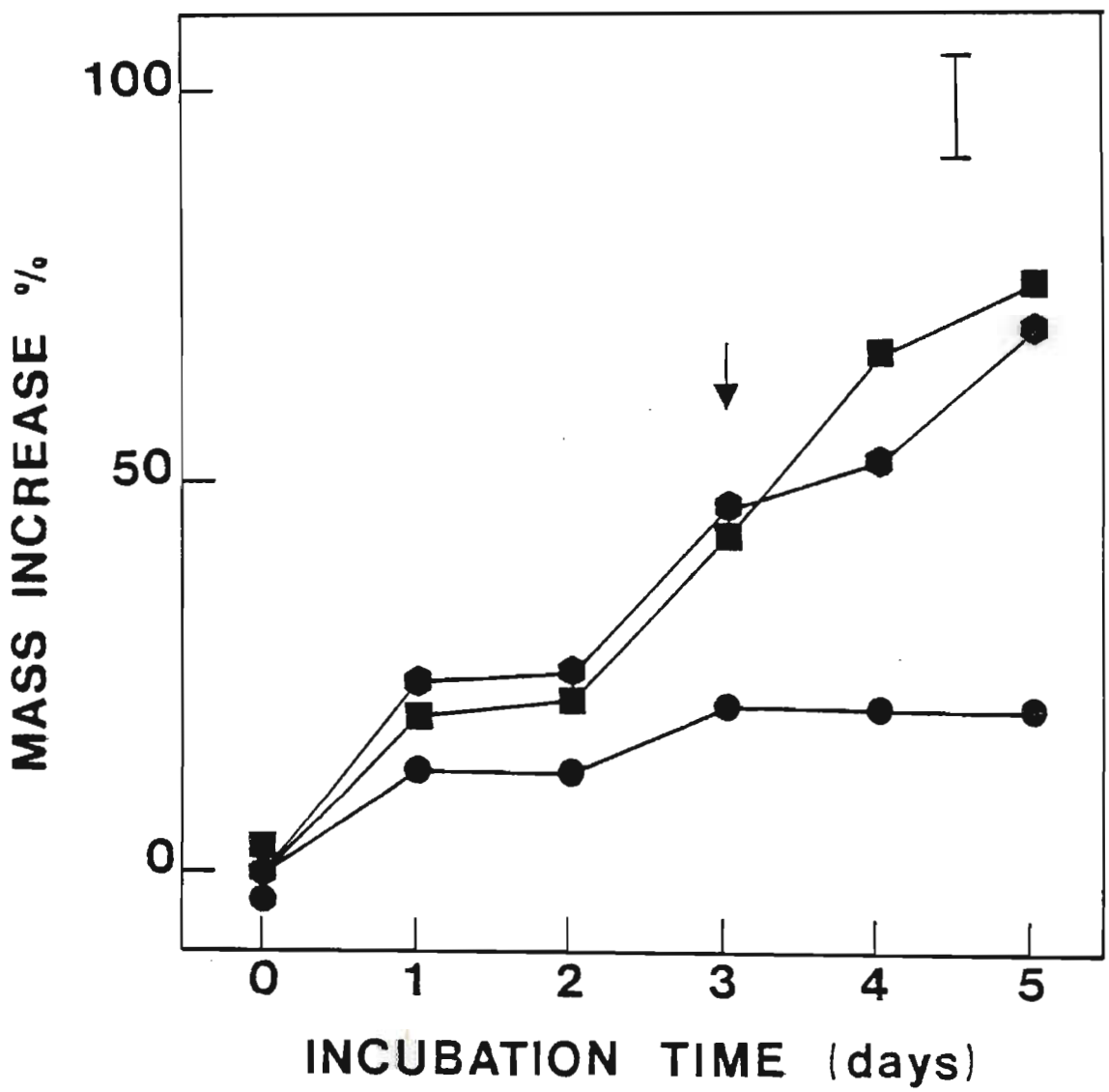
Figure 1.9 Germination of intact (shaded symbols) and side scarified (open symbols) manketti seeds treated with 20 (● , ○), 50 (⬢ , ⬡) or 500 (■ , □) mg ℓ^{-1} gibberellic acid (GA_3). Seeds were incubated in the dark at 30°C and four replicates of 20 seeds were used for each treatment. The bar represents the maximum 95 per cent confidence limit. No response was obtained from 'nuts' treated

Figure 1.10 Germination of intact (shaded symbols) and side-scarified (open symbols) *Ricinodendron rautanenii* seeds incubated on vermiculite moistened with solutions of 2-chloroethyl phosphonic acid (ethrel). The corresponding ethylene concentrations of the solutions used were: 75 (⬢ , ⬡) and 250 (■ , □) $\mu\ell\ \ell^{-1}$. Seeds were incubated in the dark at 30°C and four replicates of 20 seeds were used for each treatment. The bar represents the maximum 95 per cent confidence limit. No response was obtained from intact 'nuts' treated with this compound.


100
50
0
GERMINATION %
0
4
8
12
INCUBATION TIME (days)

also observed. KUMAR (1978) reported a similar finding. Normal germination was, however, noted in the gibberellin and ethrel treated seeds. Figure 1.9 indicates that the germination of mid-scarified seeds took place over a wide range of gibberellin concentrations, whereas intact seeds only responded to a relatively high gibberellin concentration of 500 milligrams per litre. Nevertheless, germination in the latter treatment was rapid and 90 per cent of the seeds had germinated within 80 days. The water uptake curve for this treatment, presented in Figure 1.11, shows that imbibition followed the typical triphasic pattern previously mentioned. No response was obtained from intact 'nuts'. These data provide further proof that the testa is not impermeable to water but the reason why gibberellin is only effective at high concentrations in intact seeds was not clear. It could be due to the fact that since so little water is actually imbibed during passive imbibition, the relative gibberellin concentration would have to be high in order to be effective. These findings are consistent with those of KUMAR (1978) who found that a concentration of 1 000 milligrams per litre GA_3 could overcome the dormancy of this species. When considering the effects of gibberellin, however, it must be borne in mind that although this hormone is sometimes regarded as playing a universal role in seed germination (AMEN, 1968), its effects could also be pharmacological, especially at such high concentrations. In such instances, the response could be unrelated to the endogenous mechanism for dormancy breaking (JONES and STODDART, 1979).

Figure 1.11 Water uptake by intact *Ricinodendron rautanenii* seeds incubated on vermiculite moistened with either 500 mg ℓ^{-1} gibberellic acid (GA_3) (⬢) or ethrel having a corresponding ethylene concentration of 75 $\mu\ell$ ℓ^{-1} (■). Distilled water was used in the controls (●). Seeds were incubated in the dark at 30°C and the results are the means of four replicates of 20 seeds each. The bar represents the maximum 95 per cent confidence limit. Arrow denotes the time at which germination was first recorded.


100
50
0
MASS INCREASE %
0
1
2
3
4
5
INCUBATION TIME (days)

Treatment of seeds with ethrel resulted in 100 per cent germination in a very short period of time (Figure 1.10). Figure 1.10 also indicates that intact seeds responded even more rapidly than those which had been scarified. This suggested that the active component of ethrel, ethylene, could be involved in the natural breaking of manketti seed dormancy. The water uptake curve for this treatment (Figure 1.11), also displayed the typical triphasic pattern indicating that under the correct conditions, the testa does not present itself as a barrier to germination. It is also of particular interest here that gibberellin and ethylene treatments were found to overcome the dormancy of witchweed (*Striga asiatica* (L.) Knutze) seeds (EGLEY, 1972) and that ethylene (or ethrel) has been found, in many cases, to have a marked influence on the effect of other hormones; particularly in the release of coat imposed dormancy of seeds kept in the dark or dormancy imposed by high temperatures (KHAN, 1977). Thus, to speculate, it is conceivable that deficiencies in endogenous promoters such as gibberellin are involved in the natural dormancy of manketti seeds and that these deficiencies are alleviated in the presence of ethylene. These findings may also indicate why no response was obtained from the sterile culture of excised embryos since neither gibberellin nor ethrel was included in the media used.

The results of this initial series of experiments, then, clearly indicated that the robust structures covering the seeds of *R. rautanenii* are deceptive in that they play only a limited role in maintaining the dormant condition. No

evidence was found to suggest that either the endocarp or testa restricts water uptake, inhibits oxygen movement or contains any inhibitors. However, there is some evidence to suggest that the testa exerts a mild restriction on embryo enlargement in the absence of the appropriate germination stimuli. The role of the endocarp, on the other hand, is not clear. Results show that this structure is not water impermeable but no response whatsoever was obtained from intact 'nuts' subjected to the dormancy breaking ethrel and gibberellic acid treatments.

The significance of the response of scarified manketti seeds to low temperature after-ripening is difficult to assess. However, in view of the effect of applied gibberellins and ethrel, it is possible that under certain conditions (*viz.* scarification), stratification at low temperatures may initiate important endogenous hormonal changes. Such changes have been observed in other species (KHAN, 1977). In addition, scarification may be an important factor under natural conditions in view of the report that these 'nuts' would not germinate until they had passed through the alimentary canal of elephants. This particular aspect deserves further investigation but was beyond the scope of the present study.

The results obtained from the applied hormone studies, indicated that dormancy in this species was primarily the result of physiologically incompetent embryos. In these investigations, the response obtained from the application of

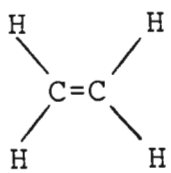
ethrel to intact seeds was of particular significance in that ethylene is a natural component of soil micro-environments. This gas may therefore be responsible for the natural release from dormancy. The role of gibberellins, on the other hand, is not as clear since this compound does not occur as a free agent in soils. Nevertheless, the main objectives of this first series of experiments (*viz.* to affect germination in this species) had been achieved. Further studies on the possible interactions between gibberellins and ethylene as well as more detailed aspects on the role of ethylene in the dormancy breaking process are discussed in Chapters 2 and 3.

CHAPTER 2

THE ROLE OF ETHYLENE IN THE DORMANCY BREAKING OF *RICINODENDRON RAUTANENII* SEEDS

INTRODUCTION

Ethylene is a simple hydrocarbon having the following structure :



This is often abbreviated as C_2H_4. It freezes at -181°C, melts at -169°C and boils at -103°C and is therefore a gas under normal conditions of temperature and pressure. Ethylene may be produced from practically any organic substance which is subjected to high temperatures or oxidation and is also a natural product of metabolism (ABELES, 1973). GANE (1934), working on apples, was the first worker to demonstrate that plant tissues produced ethylene although the ability of this gas to modify plant growth had already been recorded at the turn of the century by a Russian scientist Neljubov (ABELES, 1973). This gas has since been found to influence most major plant developmental processes and is today regarded as an integral component of the phytohormone complex. Processes affected by ethylene include not only seed germination, seedling development and growth of roots and leaves, but also phytogerontological phenomena such as flower fading, leaf senescence, fruit ripening and abscission which in some cases result from

various forms of environmental stress. Of these, it is the effects of ethylene on seed germination which will form the main topic of this discussion but for the sake of completeness, a brief consideration of the other phenomena follows.

The best known effect of ethylene on seedling growth is the so-called triple response exhibited by etiolated pea seedlings (CROCKER, KNIGHT and ROSE, 1913). This response is characterized by altered growth in the subhook regions where applied ethylene causes a reduction in stem elongation rate, an increase in stem diameter and diageotropism (LIEBERMAN, 1979). GOESCHL, RAPPAPORT and PRATT (1966) suggested that this response,when caused by endogenously produced ethylene, may represent a means for overcoming any physical resistance encountered in the soil by emerging seedlings.

With regard to root growth, ethylene was found to initiate their formation from the leaves, stems and even pre-existing roots of a number of plants (ZIMMERMAN and HITCHCOCK, 1933). Root hair formation is also stimulated by the presence of this gas (CHADWICK and BURG, 1967). Leaf expansion, on the other hand, is inhibited by ethylene and this has been attributed to its inhibition of cell division (BURG, APPELBAUM, EISINGER and KANG, 1971).

Ethylene is also produced as a result of the trauma caused by chemicals (hormones and phytotoxins), insect infestations, temperature extremes, drought, disease, mechanical wounding,

water-logging and irradiation (ABELES, 1973; LIBERMAN, 1979). This endogenously produced stress ethylene is thought to accelerate the abscission of those organs damaged by the factors mentioned, although it may also be a part of the disease resistance mechanism of plant tissues (STAHMANN, CLARE and WOODBURY, 1966).

Ethylene, both knowingly and unknowingly, has long been used to promote fruit ripening and with few exceptions most fruits are affected provided the tissues are in a receptive state. Furthermore, many fruits produce ethylene as they ripen and the function of this is probably to accelerate the fruits' own maturation and senescence. Applied ethylene is therefore thought to induce autocatalytic ethylene production (ABELES, 1973). For these reasons ethylene is often referred to as the 'ripening' hormone.

Flower and leaf senescence may also be accelerated by ethylene. By specifically blocking ethylene biosynthesis, flower senescence of a number of different species can be retarded. Thus ethylene is actually produced by many of the flowers themselves and a rise in ethylene production often marks the end of a flower's functional life (LIEBERMAN, 1979). This indicates that ethylene is intimately involved in the natural regulation of flower senescence. The same may also apply to leaf senescence. By having this effect, ethylene sometimes causes serious damage to greenhouse crops as it is usually a component of the exhaust fumes of heating devices. Inadequate ven-

tillation may thus result in a build-up to physiologically effective concentrations (ABELES, 1973).

The action of ethylene in most of the phenomena described (and others not mentioned) has now been associated with its interaction with the other phytohormones. Ethylene itself is regarded as a hormone since it is effective in minute quantities and is neither a substrate nor a co-factor in reactions associated with the major developmental plant processes (LIEBERMAN, 1979). In addition, the dose response curves for a variety of ethylene effects show a remarkable similarity. The threshold concentration for an ethylene response is usually in the region of 0,01 microlitres per litre and half maximal responses are frequently obtained at 0,1 microlitres per litre. Dose saturation usually occurs at 10 microlitres per litre at which point the dose response curve reaches an asymptote. There are seldom any reversals of effects or secondary effects at high ethylene concentrations (ABELES, 1973) although witchweed (*S. asiatica*) seed radicles were found to twist and curl in the presence of only 1,0 microlitre per litre of this gas (EGLEY and DALE, 1970).

A number of compounds exist which may elicit ethylene-like responses in plant tissues. These ethylene analogues include carbon monoxide, acetylene, propene vinyl olefins, vinyl sulphides and some cyanide compounds. BURG and BURG (1967) have proposed that in order for a molecule to have an ethylene effect, it must possess the following charac-

teristics;

(a) the molecule must be unsaturated since single bond compounds are inactive,

(b) the molecule chain length must be short since there is an inverse relationship between chain length and ethylene-like activity,

(c) the molecule must have limited substitutions since these cause electron delocalization resulting in reduced biological activity,

(d) the unsaturated position of the molecule must be adjacent to a terminal carbon atom, and

(e) the terminal carbon atom of the molecule must not be positively charged.

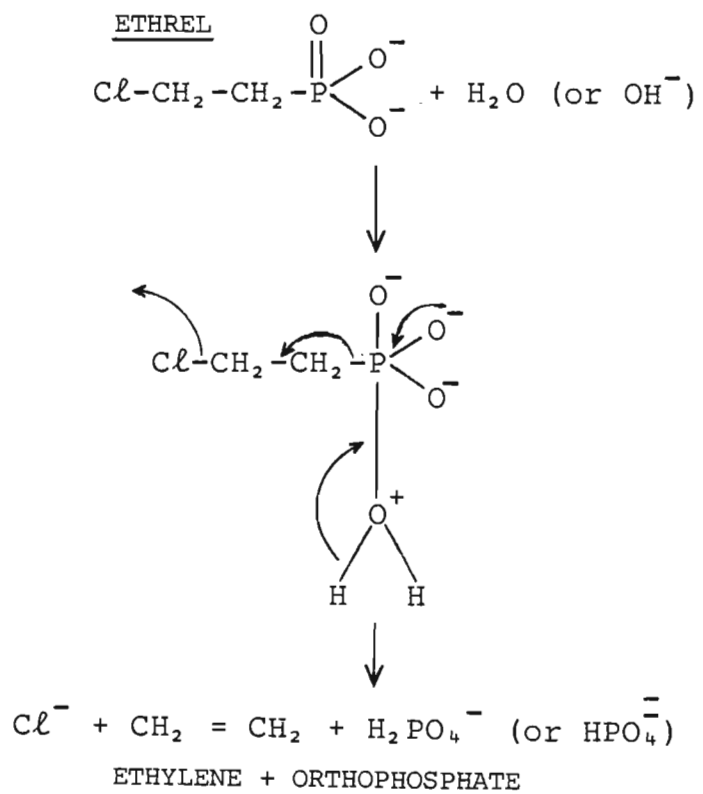
In addition, recent work has shown that all compounds which elicit ethylene-like responses are π acceptors (COTTON and WILKINSON, 1972) and that all such compounds exhibit a strong *trans* effect (SISLER, 1977). However, of all these substances,none has been shown to be as active, concentration-wise, as ethylene.

Any discussion on ethylene would not be complete without some consideration being given to its biosynthesis and site(s) of attachment. Ethylene biosynthesis takes place on the plasma membrane surface (LIEBERMAN, 1979) and the actual synthetic pathway has now been fairly well established. In higher plants the amino acid L-methionine is the precursor of ethylene production and the first step

in the reaction is the conversion of this into S-adenosyl-methionine (SAM). SAM is then converted to 1-aminocyclo-propane-1-carboxylic acid (ACC) in the presence of ACC synthase, this being the overall rate limiting step in the pathway. The well known inhibitor of ethylene biosynthesis, aminoethoxyvinylglycine (AVG) (an enol ether amino acid) has been shown to have its effect at this point in the reaction by inhibiting ACC synthase activity (LIEBERMAN, 1979; SUTTLE and KENDE, 1980; ADAMS and YANG, 1981). Furthermore, auxin, which has long been known to stimulate ethylene production, as well as a number of environmental stress conditions such as water-logging and mechanical wounding, also influences this step by inducing ACC synthase activity (BRADFORD and YANG, 1980; BOLLER and KENDE, 1980; ADAMS and YANG, 1981). The final step in this pathway is the conversion of ACC to ethylene and although the enzyme system which catalyzes this has never been isolated, it does appear to be present in most plant tissues with the exception of preclimacteric fruits (ADAMS and YANG, 1981). By indirect means it has been shown that this enzyme is associated with membranes and requires the presence of oxygen in order to be effective. Furthermore, this enzyme appears to be inhibited by cobalt ions (Co^{2+}), light, temperatures greater than 35°C and uncouplers such as dinitrophenol (ADAMS and YANG, 1981).

The generation of ethylene from such compounds as ethrel or ethephon (2 chloroethyl phosphoric acid) does not involve the biochemical pathway just described but such com-

pounds are important in the manipulation of plant growth for agricultural purposes. It appears that ethrel, like most weak aliphatic acids,is taken up by plants and subsequently breaks down intracellularly at cytoplasmic pH's to form equivalent amounts of ethylene and orthophosphates (WARNER and LEOPOLD, 1969). The minimum pH at which this will occur is 3,5 and therefore ethylene evolution increases linearly with increasing pH. In addition,at any given pH, the increase is also linear over time and the maximum potential conversion is 98 per cent (WARNER and LEOPOLD, 1969; YANG, 1969). The suggested reaction is as follows;



The generation of ethylene from ethrel appears to take place selectively since GIULIVO, RAMINA, MASIA and COSTA (1981), working on peach trees, found that ethrel was translocated in a sugar conjugated form, from the sites of application to

the fruits where the conversion took place. No transport occurred in the opposite direction.

The ethylene synthesized from methionine or generated by the conversion of ethrel is thought to exert its influence by attaching itself to ethylene receptor sites which appear to be the same for most physiological processes. ABELES (1973) estimated that a cell with a volume of 10^4 μm^3 would contain approximately 500 ethylene receptor sites and that this would represent only 0,000001 per cent of the total biomass. This illustrates that information regarding the composition, localization and subsequent action of these receptor sites could only be obtained by more or less indirect means. Recently, however, SISLER (1980) partially purified the ethylene binding component and concluded that it was a lipophilic protein and hence closely associated with membranes. ABELES (1973) suggested that bonding at the ethylene receptor complex was most likely the result of weak van der Waal's forces and by all accounts it seems that plant tissues generally have a low affinity for ethylene.

It is not clear whether or not the metabolism of ethylene is involved in its action on plant growth. At present two hypotheses have been suggested to explain ethylene's interaction with its receptor. The first proposes a dissociable ethylene-receptor complex. In the presence of ethylene the receptor undergoes a conformational change which may then activate a series of key reactions (LIEBERMAN, 1979). This proposal implies that ethylene is neither incorporated

nor metabolized by the tissue concerned and may be released from the receptor intact. Support for this lies in the fact that many ethylene effects cease soon after ethylene removal and that hypobaric atmospheres may delay or even prevent its action. The second hypothesis envisages an actual reaction at the receptor site resulting in the production of physiologically active reactants (LIEBERMAN, 1979). Two pathways for ethylene metabolism have been suggested, the first of which involves the oxidation of ethylene to carbon dioxide. In the second pathway, ethylene is incorporated into a number of soluble components, one of which may be ethylene oxide. This is supported by the fact that oxygen was essential for ethylene metabolism to occur (JERIE and HALL, 1978). However, reports indicate that only very small quantities of exogenous ethylene are actually incorporated into plant tissues and the metabolism of ethylene to carbon dioxide appears to be greater than its incorporation into tissue components (LIEBERMAN, 1979). Thus the metabolism of ethylene to carbon dioxide may represent the initial steps associated with the mode of action of this hormone. On the other hand, it may also represent a disposal pathway for excess ethylene and may thus be totally unrelated to the action of this gas (LIEBERMAN, 1979).

It has never actually been demonstrated that the site of ethylene action is the same as its site of attachment. Furthermore, ethylene may affect specific tissues and cells differently and many of the diverse morphological changes associated with ethylene action may be related to the effects

of this gas on such processes as cell division, cell expansion and auxin transport. Much of the present information regarding these processes and others such as ethylene biosynthesis, was obtained from work carried out on tissues other than seeds and the relevance of this information to the process of seed dormancy has only recently been examined critically (KETRING, 1977). Thus a unified concept on how ethylene promotes programmed death on the one hand and growth (by way of germination) on the other, is at present lacking.

The first reported effect of ethylene on seed germination was that of NORD and WEICHERZ (1929) who demonstrated that this gas increased the rate of germination and growth of barley. The first record of the effects of this gas on seeds which were dormant was that of VACHA and HARVEY (1927). They discovered that the germination of dormant common buckthorn, high bush cranberry, snowberry and tartian honeysuckle seeds could be stimulated with applied ethylene. However, it was not until the 1960's that most of the significant work on the role of ethylene in seed dormancy and germination commenced. To date, the seeds of a considerable number of species have been found to respond to ethylene including; peanut, *Arachis hypogaea* L. (TOOLE, BAILEY and TOOLE, 1964); witchweed, *Striga asiatica* {L.} Knutze {= *S. lutea* L.} (EGLEY, 1972); cocklebur, *Xanthium pensylvanicum* (KATOH and ESASHI, 1975a); *Spergula arvensis* L. (OLATOYE and HALL, 1972); apple, *Malus domestica* (KEPCZYNSKI and RUDNICKI, 1975); tumble pigweed (*Amaranthus*

albus L.), spiny amaranth (*Amaranthus spinosus* L.), redroot pigweed, (*Amaranthus retroflexus* L.), common ragweed (*Ambrosia artemisiifolia* L.), common lambsquarters (*Chenopodium album* L.), common purslane (*Portulaca oleracea* L.), red sorrel (*Rumex acetosella* L.), curly dock (*Rumex crispus* L.) and broadleaf dock (*Rumex obtusifolius* L.) (TAYLORSON, 1979); rape, *Brassica napus* L. (TAKAYANAGI and HARRINGTON, 1971) and subterranean clover, *Trifolium subterraneum* L. (ESASHI and LEOPOLD, 1969). In most cases applied ethylene usually stimulates germination if it has any effect at all. However, the germination of rough cinquefoil (*Potentilla norvegica* L.) and horsenettle (*Solanum carolinense* L.) seeds is inhibited by the presence of this gas (TAYLORSON, 1979). As with other dormancy mechanisms, these examples demonstrate that no phylogenetic relationship exists between ethylene-sensitive seeds. Furthermore, none of these species was found to have an absolute ethylene requirement for germination and there are usually other means to break the dormancy of ethylene-responsive seeds (KETRING, 1977).

From the literature it appears that the dose-response-relationships for seeds and ethylene are typical of those of most other ethylene responses in plants. The threshold ethylene values may be as low as 0,01 microlitres per litre in some species such as *Trifolium subterraneum* (ESASHI and LEOPOLD, 1969) and dose saturation usually occurs at between 1 and 10 microlitres per litre (KETRING and MORGAN, 1970; TAYLORSON, 1979). It is interesting to note that

under certain environmental conditions the sensitivity of seeds to ethylene can change (SCHONBECK and EGLEY, 1981a).

Generally, it appears that for ethylene to be effective, it must be present for at least eight hours with optimum results usually being obtained after 24 hours. However, KETRING and MORGAN (1970) demonstrated that for the inherently more dormant basal seeds of the Virginia type peanut, the concentration of ethylene was more critical than its duration of exposure. Nevertheless, there is considerable evidence to suggest that ethylene is most effective for only a limited period of time during the overall phase of imbibition. *Amaranthus retroflexus* seeds, for example, were originally found to be sensitive to ethylene only during the first 24 hours of imbibition at 30°C and SCHONBECK and EGLEY (1981c) subsequently narrowed this down to a four hour period occurring eight hours after the commencement of imbibition. This corresponded to the lag phase occurring after the initial passive imbibition of water. However, although pre-incubation does enhance ethylene sensitivity in some species (e.g. *X. pensylvanicum*, EGLEY, 1980), this does not appear to be a general phenomenon and the timing of this sensitive period varies between species. In addition more detailed experiments need to be undertaken to determine how much water must be imbibed before an ethylene response will occur.

There is some indication that sensitivity to ethylene may vary under field conditions. In *Amaranthus retroflexus*

seeds it was found that responsiveness to ethylene disappeared after 24 hours but reappeared after eight days and was further enhanced after 32 days pre-incubation (SCHONBECK and EGLEY, 1981a). The reasons for this are not clear but it may serve to prevent premature germination.

The data presented in the discussion so far, indicates that a number of species are well adapted to respond to ethylene and that naturally occurring ethylene may play an important role in releasing dormancy and stimulating germination. SMITH (1976) reported that ethylene is in fact a natural component of soil atmospheres, the source of which appears to be microbial activity. The concentration of this soil ethylene may vary from trace amounts to values in excess of 30 microlitres per litre. Many seeds increase the likelihood of elevated ethylene concentrations in their immediate vicinity by virtue of the fact that they leak considerable quantities of organic nutrients (SMITH, 1976). Furthermore, SCHONBECK and EGLEY (1981b) demonstrated that buried redroot pigweed and cocklebur seeds could respond to ethylene injected into the soil. This, together with the fact that the threshold ethylene concentration for most species lies well within the range occurring in soils, suggests that this source of ethylene may play an important role in the natural decline of dormancy in some species.

A number of interactions between key environmental parameters and applied ethylene have been observed. This provides further evidence that exogenous ethylene may be important in

the field. For convenience these will be considered individually.

In its interaction with temperature, applied ethylene appears to be most effective at between 25 and 35°C. No response has been observed at temperatures below 15°C (ESASHI and LEOPOLD, 1969; KATOH and ESASHI, 1975a), although higher germination percentages were obtained from apple seeds which had been after-ripened at low temperatures in the presence of ethylene (KETRING, 1977). The temperature at which redroot pigweed seeds were pre-incubated prior to ethylene treatment was also found to be important. SCHONBECK and EGLEY (1981a) found that,in the absence of ethylene, normal germination took place at 35°C when these seeds were pre-incubated at 15 or 20°C. However, pre-incubation at 25 or 30°C inhibited germination in the absence of ethylene but increased the sensitivity of the seeds to the gas. In another experiment in this series of investigations, SCHONBECK and EGLEY (1981b) also found that seeds buried in the soil for periods longer than 30 days would not germinate in the absence of ethylene at 35°C but at the same time had become supersensitive to the gas. This served to illustrate that the sensitivity of seeds to ethylene could also be modified by temperature. Conversely, these data also showed that exogenous ethylene could modify the response of redroot pigweed seeds to temperature changes.

Light and ethylene also show some form of interaction but responsiveness to light is not a prerequisite for an ethylene

response. Peanut and witchweed seeds, both of which respond to ethylene, are indifferent to light during germination (TOOLE *et al.*, 1964; EGLEY and DALE, 1970) and the numerous weed species examined by TAYLORSON (1979) exhibited ethylene-light interactions ranging from negligible (*Ambrosia artemisiifolia*) to synergistic (*Rumex crispus*). However, non-pre-imbibed *Spergula arvensis* seeds germinated better in the light than in the dark and both ethylene and carbon dioxide,either individually or collectively,yielded higher germination percentages in the light (JONES and HALL, 1979). These workers were led to the conclusion that,in this species,ethylene and light acted at different sites but whether this is true for all ethylene and light sensitive seeds is not clear. If ethylene and light do interact directly,the most likely component to be affected would be phytochrome. In recent studies on redroot pigweed seeds it was found that the response to ethylene occurred equally as well in both light and dark conditions. However, ethylene and light did exhibit some positive co-action as did ethylene and low temperature pre-incubation. This probably indicates that ethylene and light act at different sites since the positive effect of low temperature pre-incubation was thought to result from reduced phytochrome reversion whereas pre-incubation at higher temperatures enhanced this (SCHONBECK and EGLEY, 1981a). Definite evidence for a phytochrome-ethylene interaction was found in seeds of *Potentilla norvegia*. In this species ethylene inhibited the P_{FR} effect but appeared to do so by acting at a site where phytochrome must associate to act (SUZUKI and

TAYLORSON, 1981) implying that ethylene was not causing the reversion of P_{FR} to P_R. Thus where ethylene and light show positive co-action it appears that ethylene does not have any direct effect on phytochrome levels and may act by removing some block to the full expression of phytochrome as was suggested for *Spergula arvensis* seeds (OLATOYE and HALL, 1972). Similarly light does not stimulate germination by promoting ethylene production since in thermodormant Grand Rapids *Lactuca sativa* seeds, germination was promoted by a combination of red light and ethylene but not by ethylene alone (BURDETT and VIDAVER, 1971).

Ethylene and carbon dioxide have also been shown to interact to some extent in relation to seed germination. Carbon dioxide may either promote or inhibit germination depending on the species involved. The germination of dormant subterranean clover, cocklebur and *Spergula arvensis* was stimulated by this gas (ESASHI and LEOPOLD, 1969; KATOH and ESASHI, 1975a; JONES and HALL, 1979) and other reports suggest that carbon dioxide is in fact a prerequisite for germination (JONES and HALL, 1979). Generally, however, some germination does still occur in the absence of carbon dioxide and ethylene alone can quite often partially satisfy the carbon dioxide requirement. Nevertheless, germination was always greatest when carbon dioxide and ethylene were applied together. It is not clear whether this represents a synergistic interaction or whether the two gases interacted more directly to promote germination. If the interaction is synergistic then ethylene and carbon dioxide

would probably have different sites of action as proposed for subterranean clover (ESASHI and LEOPOLD, 1969). In this regard the proposal by HART and BERRIE (1968) that the incorporation of carbon dioxide into malate is essential for germination, may be significant. They found that an inverse relationship existed between malic acid concentration and dormancy in wild oat seeds (*Avena fatua* L.) and suggested that malate stimulated the tricarboxylic acid cycle which in turn would result in greater activity of the electron transport chain. However, JONES and HALL (1979), although supporting the theory that carbon dioxide is incorporated into the TCA cycle, suggested that in the case of *S. arvensis* seeds, the malate stimulated the glyoxalate cycle rather than having a direct effect on the electron transport chain. Where carbon dioxide and ethylene are thought to interact more directly, it may be via carbon dioxide stimulated ethylene production as was found to be the case in cocklebur and subterranean clover seeds (KATOH and ESASHI, 1975a).

The period of sensitivity to carbon dioxide usually occurs during the early stages of imbibition and precedes the ethylene-sensitive phase. This may indicate that stimulation of electron transport or the glyoxalate cycle is necessary before ethylene can have its optimum effect. Alternatively, endogenous ethylene production may be stimulated during this carbon dioxide-sensitive period. Further research is required to resolve this.

It seems anomalous that carbon dioxide competitively inhibits the effects of ethylene in most ethylene mediated plant responses and yet may promote seed germination in many species. However, in seeds of witchweed (*Striga asiatica*), this more frequently observed interaction between ethylene and carbon dioxide does occur. Here it was found that carbon dioxide reduced both the ethylene induced response and the germination of control seeds. Other species such as *Amaranthus retroflexus* are indifferent to carbon dioxide (SCHONBECK and EGLEY, 1980b).

Another environmentally related phenomenon on which ethylene sometimes has an effect is secondary dormancy. For example, water-stressed *Amaranthus retroflexus* seeds do not germinate. This was attributed to the fact that water stressing affects the active growth phase of germination by reducing the water potential gradient between the seed and the environment, a gradient which usually provides the force for cell expansion. Some germination resulted from ethylene applied during the first day and thus the ultimate effect of water stress was thought to be the inhibition of endogenous ethylene synthesis (SCHONBECK and EGLEY, 1980a). Another form of secondary dormancy is that induced by high temperatures(as in lettuce seeds). BURDETT (1972) found that not only did imbibition at 30°C induce this thermodormancy in lettuce seeds, but also curtailed the synthesis of endogenous ethylene. As with water stressing, applied ethylene relieved this condition. Thermodormant cocklebur seeds (ESASHI *et al.*, 1979) behaved in a similar manner.

Based on their findings, SCHONBECK and EGLEY (1981c) suggested that ethylene may have a different site of action in thermodormant seeds. In view of the findings of other workers, this site may be the ethylene synthesizing apparatus.

Some practical benefits have been derived from the knowledge that many weed species respond to ethylene. In certain regions of the United States ethylene is injected directly into the soil to control witchweed (SMITH, 1976). Similar treatments have been suggested for other species such as cocklebur and redroot pigweed (EGLEY, 1980). The discovery that ethylene sensitivity of redroot pigweed seeds may be modified under certain environmental conditions, adds an additional dimension to the possible control of this species.

As mentioned previously, ethylene in many instances is not the only means of removing the dormancy of ethylene responsive seeds. However, a factor which all these additional treatments have in common was that they stimulated the production of endogenous ethylene. This suggested that it was the relative ability of these seeds to produce ethylene which regulated their dormancy. The first indication of this came from studies on peanut seeds. Here it was demonstrated that the non-dormant Spanish type seeds produced ethylene during germination as did those of the more dormant Virginia variety once they had been stimulated. Unstimulated Virginia type seeds were found to produce 40 times less ethylene than their stimulated counter-

parts (KETRING and MORGAN, 1969). Furthermore, it was also found that the ethylene producing ability of this dormant variety increased as natural dormancy declined and a quantitative relationship was found to exist between the amount of ethylene produced and the degree of dormancy. The threshold ethylene production rate above which peanut seed dormancy was broken was estimated to be in the region of two to three milli-microlitres of ethylene per gramme fresh weight per hour (KETRING and MORGAN, 1970). These authors (1971) also demonstrated that other dormancy breaking treatments such as heating to 40 - 45°C for 15 days, 5 per cent carbon dioxide or applied cytokinins all stimulated the production of endogenous ethylene. It was thus concluded that the controlling process in dormant peanut seed germination is an enhancement of the natural low capacity to produce ethylene. This hypothesis may also apply to other ethylene-responsive seeds. Ethylene production was enhanced in germinating subterranean clover seeds prior to radicle emergence (ESASHI and LEOPOLD, 1969) and the effect of carbon dioxide on the ethylene production of cocklebur seeds prior to germination has already been mentioned. As with peanut seeds, the natural decline in dormancy of cocklebur seeds as a result of after-ripening is characterized by an increase in ethylene production and the impotency observed in some of the after-ripened small seeds of this species may be the result of lower endogenous ethylene production (KATOH and ESASHI, 1975a). Endogenous ethylene production is also thought to play a role in the breaking of apple seed dormancy. This was based on the

finding that low temperature after-ripening of intact apple seeds in the presence of 0,1 to 5,0 per cent ethylene for 70 days promoted their germination. Furthermore, this after-ripening process could be delayed if the seeds were kept under reduced pressures (KEPCZYNSKI and RUDNICKI, 1975). Most important, however, was the observation that ethylene production by excised apple embryos paralleled their germination capacity during after-ripening (KEPCZYNSKI, RUDNICKI and KHAN, 1977). WAN (1980) contests this hypothesis that endogenous ethylene participates in the natural regulation of apple seed dormancy on the grounds that although inhibitors of ethylene synthesis or action did in fact reduce the amount of ethylene evolved by the after-ripening apple seeds, this did not affect subsequent germination. Furthermore, no consistent relationship was obtained between the amount of ethylene evolved from apple seeds and their germination. Thus, there is some doubt surrounding the role of ethylene in this particular species. Studies on the site of endogenous ethylene production in apple, peanut and cocklebur seeds revealed that it was the embryonic axis which was mainly responsible for this (KEPCZYNSKI *et al.*, 1977; KETRING and MORGAN, 1969; ESASHI and KATOH, 1975c).

Ethylene production was also found to occur during the germination of a number of non-dormant seeds such as those of oats (*Avena fatua*), pea (*Pisum sativum* L.) and castor bean (*Ricinus communis* L.) (MEHERIUK and SPENCER, 1964; SPENCER and OLSON, 1965). This suggested that ethylene may simply

be a by-product of the catabolic reactions occurring in germinating seeds. KETRING and MORGAN (1971) argue against this and proposed that ethylene does play a direct role in the dormancy release of peanut seeds on the following grounds;

(a) there is a threshold ethylene production rate above which dormancy is released (as previously mentioned),

(b) substances which enhance production rates to in excess of these threshold levels within 24 to 48 hours of incubation effectively stimulated germination,

(c) ethylene production of inherently more dormant basal seeds equalled that of the less dormant apical seeds when stimulated to germinate in a manner which approached that of the less dormant seeds, and

(d) applied ethylene always stimulated germination.

Recent work on non-dormant *Phaseolus vulgaris* L. seeds showed that embryo development was closely related to the time of ethylene production and this could not be attributed to mechanical stress imposed by the seed coat since similar results were obtained for intact and de-coated seeds (DE GREEF and DE PROFT, 1977). This may support the view that ethylene is simply a by-product of germinating tissue or it may indicate that ethylene is more intimately involved in angiosperm seed germination than previously thought. The

reduction of endogenous ethylene synthesis may represent another dormancy mechanism which has evolved to suit particular conditions. When ethylene is applied to such seeds the initial event would probably be a biochemical or biophysical one which would initiate the autocatalytic production of ethylene. This in turn would raise the ethylene level in the seed tissues to a critical concentration that would initiate the removal of dormancy. The natural release of dormancy would thus depend on the ability of the seeds to increase their capacity for ethylene production.

Ricinodendron rautanenii seeds appear to be unique in that ethylene was the only naturally occurring stimulus that would relieve their dormant condition. A study was thus undertaken to establish;

(1) the dose-response relationship,
(2) the effect of ethylene under various environmental conditions,
(3) the time period during which seeds were sensitive to ethylene, and
(4) the degree of imbibition required before a response would be obtained.

The results obtained from studies such as this frequently provide useful clues as to what physiological processes are blocked in the initially dormant seeds (SHONBECK and EGLEY, 1981c).

MATERIALS AND METHODS

1.0 General

The experiments detailed in this Chapter were carried out for two main reasons. The first was to establish the sensitivity of *Ricinodendron rautanenii* seeds to applied ethylene and to determine how this sensitivity would be affected by various physical and environmental factors. The second was to establish a reliable means of treating these seeds with ethylene for use in later experiments where an attempt would be made to determine which metabolic processes are affected by this gas. It will be noticed that in this Chapter no mention is made of the experiments carried out to determine whether ethrel or ethylene treatments stimulated the production of endogenous ethylene. The reason for this is that such a response would constitute a mode of action and would therefore be more appropriately included in Chapter 3.

Three replicates of 20 seeds were used in all the experiments which follow and each experiment was repeated at least once. In those instances where pre-imbibition of 'nuts' and seeds was necessary, this was achieved by soaking in distilled water for 48 hours. This was based on the previously reported observation that most 'nuts' and seeds were fully imbibed after this period. Germination was recorded on a daily basis and was considered complete using the same standard criteria mentioned in Chapter 1.

The substrate used in these experiments was autoclaved vermiculite. All incubators were accurate to within 0,5°C and the standard germination temperature used was 30°C except where the effect of different temperatures was being examined. All chemicals were reagent grade and gaseous ethylene was obtained from BDH Chemicals, England and was certified as 99,8 per cent pure.

2.0 Influence of the endocarp on the response to ethylene

A preliminary experiment was carried out to establish whether the presence of the endocarp in any way influenced the response to ethylene. This was necessary in view of the finding that intact 'nuts' did not respond to the dormancy breaking gibberellin and ethrel treatments. For this, pre-imbibed intact and scarified 'nuts' were placed on moist vermiculite in a large gasing chamber. The regions of the 'nut' which were scarified included;

(1) the radicle end,
(2) the end opposite the radicle, and
(3) the side region midway between (1) and (2).

In the latter treatment two groups were prepared and these were placed either with or without the exposed area in contact with the moist substrate. The gas chamber was flushed with pure ethylene for one minute after which the vessel was sealed. To maintain the saturated ethylene atmosphere, the flushing procedure was repeated at three hourly intervals.

After 12 hours the ethylene was replaced with air and the vessel was then left unsealed and incubated at 30°C. Germination was recorded daily.

On the basis of the results obtained from this preliminary experiment, all subsequent treatments were applied to seeds (endocarp removed) only. An explanation for this is given in the Results and Discussion.

3.0 Effect of different ethylene concentrations on germination and subsequent seedling growth

An experiment was conducted to determine the ethylene dose-response relationship for *R. rautanenii* seeds. Pre-imbibed seeds were placed in plastic containers filled with moist vermiculite. These containers were in turn placed in large glass jars of known volume. Two hundred millilitres of water was poured into each jar to maintain a high relative humidity throughout the experimental period. A sheet of glass having a single opening, 25 millimetres in diameter, was placed over the opening of each jar. The jars were sealed by inserting a tightly fitting rubber 'suba-seal' into each opening. Ethylene was then injected into each jar after having first removed the required volume of air. The concentrations used were 10, 1,0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} microlitres per litre. In determining the amount of ethylene required to obtain these concentrations, allowance was made for the volume occupied by the water, plastic containers, vermiculite and seeds in each jar. A saturated

ethylene atmosphere was obtained by flushing the jars with ethylene for 30 seconds prior to sealing. All jars were incubated in the dark at 25°C and germination was recorded daily. The experiment was terminated after seven days and the effect of the various ethylene concentrations on seedling growth was recorded.

4.0 Effect of 2-Chloroethyl phosphonic acid (Ethrel)

The sensitivity of manketti seeds to ethrel (also known as ethephon or CEPA) and the effect of this compound on subsequent seedling growth, was also investigated. Dose response curves were obtained by incubating seeds at 30°C on vermiculite moistened with different ethrel concentrations. The concentrations used were those which corresponded to an effective ethylene concentration range of 10^4, 10^3, 10^2, 10^1, 1,0, 10^{-1}, 10^{-2} and 10^{-3} microlitres per litre. These were obtained by making a serial dilution series from a 21 millilitre per litre stock solution of ethrel. According to the manufacturers, such a solution would have an effective ethylene concentration of 10 000 microlitres per litre.

In addition to this, seeds were also exposed to the different ethrel concentrations for varying lengths of time. For this, seeds were removed from the different ethrel concentrations after 24, 48, 72, 96 and 120 hours and transferred to ethrel-free conditions. After eight days incubation at 30°C, the resultant seedlings were separated into their embryo and endosperm components. The fresh mass

of these tissues was then recorded. Dry mass determinations were obtained by drying this material in an oven set at 100°C for 24 hours.

5.0 The effect on germination of incubating seeds in an ethylene enriched air-stream

Under natural conditions it is unlikely that seeds would be subjected to a closed system such as that used in some experiments. Thus, the effect of an ethylene enriched stream of air on germination was investigated. This was achieved using a modified version of the gas dispensing apparatus described by SALTVEIT (1978), illustrated in Figure 2.1. This system works on the principle that ethylene placed in the large reservoir (ER) diffuses through the Tygon tubing (Tt) at a fixed rate. The resulting concentration of ethylene in the air stream is directly proportional to the tubing length and inversely proportional to the flow rate of the air stream. An ethylene concentration of 10 microlitres per litre was used in this experiment and was obtained by having a flow rate of 200 millilitres per minute and a tubing length of 136 millimetres (SALTVEIT, 1978). The ethylene enriched air stream was then passed continuously over pre-imbibed seeds incubated on moist vermiculite in a one litre ehrlenmeyer flask. The entire apparatus was placed in an incubator set at 30°C and germination was recorded daily.

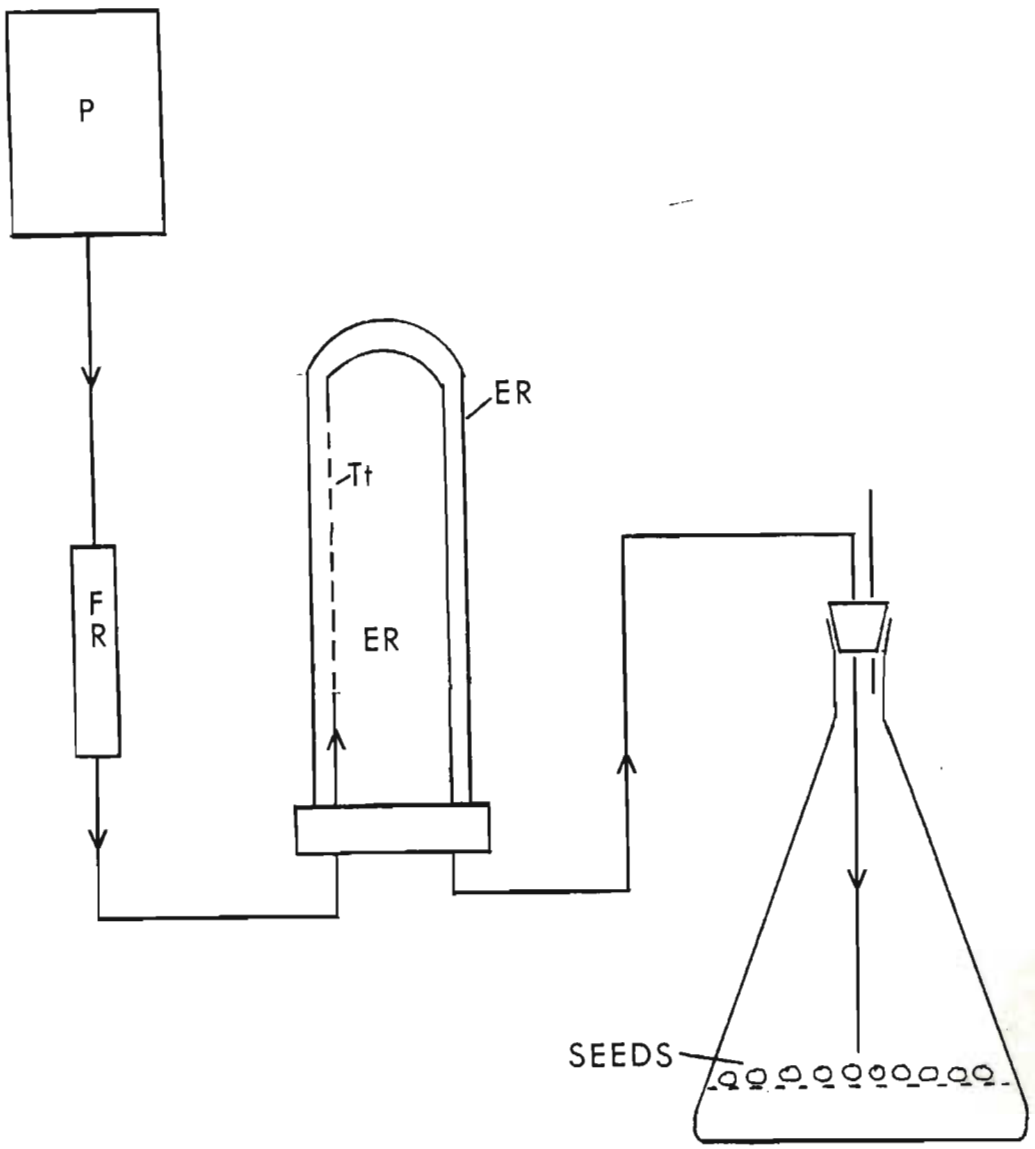


Figure 2.1 The apparatus (adapted from that of SALTVEIT, 1978) used to dispense known concentrations of ethylene into a continuously flowing stream of air. An air stream was generated by a small diaphram pump (P) and the rate of this was regulated by a flow regulator (FR). The air then entered an ethylene reservoir (ER) and ethylene diffused into the air stream via a section of tygon tubing (Tt). The ethylene concentration obtained is a function of flow rate and length of the tubing. The ethylene enriched air was then passed over seeds placed in an

6.0 Effect of temperature on the response to ethylene

The effect of ethylene on seeds incubated at different temperatures was investigated. This was done in order to determine whether or not this could be related to temperatures which occur in the field. For this, suitable replicates of pre-imbibed seeds were placed in 500 millilitre ehrlenmeyer flasks containing moist vermiculite. The atmosphere in each flask was then saturated with ethylene as previously described. The flasks were subsequently incubated at either 5, 10, 15, 20, 25, 30 or 35°C. After 12 hours air was flushed through the flasks to remove the ethylene. Germination was then recorded daily and any seeds which had not germinated after 21 days incubation were transferred to the standard germination temperature of 30°C. This was done in order to determine whether the seeds had responded to ethylene and were merely prevented from germinating by some other factor.

7.0 The effect on germination of different periods of exposure to ethylene

An experiment was conducted to determine the minimum period of exposure to ethylene required by seeds to germinate. To achieve this, pre-imbibed seeds were incubated at 30°C on moist vermiculite in an ethylene saturated atmosphere. Samples were removed after 0,5; 1; 2; 3; 6; 9 and 12 hours. Each sample was transferred to a separate incubator, also set at 30°C, and maintained under ethylene-free conditions

for the remainder of the experimental period. Germination was recorded daily.

8.0 The effect of ethylene on seeds imbibed for varying lengths of time.

A number of experiments were carried out to determine at what point during imbibition manketti seeds exhibited their maximum sensitivity to ethylene.

8.1 The effect of ethylene on dry seeds

In the first of these, an attempt was made to determine whether ethylene could break the dormancy of unimbibed seeds. For this, a number of dry seeds were incubated for 12 hours at 30°C in an ethylene saturated atmosphere. The seeds were then transferred to ethylene-free conditions and kept at the same temperature. It was possible, however, that any germination obtained from this treatment could, in fact, be attributed to ethylene remaining in the testa and being carried into the tissues during imbibition. Thus, two additional treatments were included in this experiment. In the first, the testae of the dry seeds were removed prior to the ethylene treatment. In the second, ethylene was physically removed from intact seeds by placing them in a mild vacuum for three minutes. This was done directly after the 12 hour ethylene treatment and just prior to moist incubation at 30°C.

8.2 The effect of different periods of imbibition on the ethylene response

In the second experiment, a method similar to that described by EGLEY (1980) was used to determine, as accurately as possible, the minimum imbibition period required before an ethylene response was obtained. For this, dry manketti seeds were incubated on moist vermiculite at 30°C and samples were removed after 3, 6, 9, 12, 18, 24, 36 and 48 hours of imbibition. Each sample was then placed in a 500 millilitre ehrlenmeyer flask containing vermiculite. These flasks were flushed with pure ethylene, sealed and then incubated at 30°C. After 12 hours the ethylene in the flasks was replaced with air and any subsequent germination was recorded daily.

8.3 The effect on germination of simultaneous gasing and imbibition

In most experiments carried out on seeds of other ethylene sensitive species, the gas treatment was usually applied at the commencement of imbibition. Thus, for comparative purposes, the effect of a similar treatment on manketti seeds was also investigated. This was achieved by incubating dry seeds on moist vermiculite at 30°C in an ethylene saturated atmosphere for 12 hours. After this period the seeds were transferred to ethylene-free conditions and incubated at the same temperature as before.

9.0 Persistence of the ethylene response

Under natural circumstances it is possible that favourable environmental conditions and optimum ambient ethylene concentrations may not prevail at the same time. Thus, an experiment was conducted to determine whether ethylene treated manketti seeds retained their ability to germinate even after a period of desiccation. For this, pre-imbibed and dry seeds were incubated in an ethylene saturated atmosphere for 12 hours at 30°C. During this phase only the pre-imbibed seeds were provided with moisture. After treatment with ethylene, the seeds were removed from the flasks and placed in an open vessel in the laboratory. (The mean temperature experienced by the seeds during this period was approximately 20°C). Pre-imbibed seeds were allowed to desiccate and samples were taken from both groups after one, four and eight days respectively. These samples were then incubated at 30°C on moist vermiculite and germination was recorded daily.

10.0 The role of CO_2 in the ethylene response

As previously mentioned, carbon dioxide appears to be a prerequisite for the germination of some ethylene-sensitive species (JONES and HALL, 1979). To determine whether such a system existed in *R. rautanenii* seeds, carbon dioxide was removed from the atmosphere surrounding the seeds either during imbibition or during the period of ethylene treatment. The actual removal of carbon dioxide was achieved

by placing a small vial containing five millilitres of a 20 per cent potassium hydroxide solution and a fluted filter paper wick inside the respective flasks. Two controls were included in this experiment. The first control consisted of a group of seeds which was incubated in the absence of carbon dioxide during both imbibition and the gasing period. In the second, the seeds were imbibed and gased normally. Incubation was at 30°C and germination was recorded daily. In addition to this a representative sample of pre-imbibed seeds was subjected to a 5 per cent carbon dioxide treatment (TOOLE *et al.*, 1964) using the same apparatus and method as that described in section 3.0.

RESULTS AND DISCUSSION

The tough, woody endocarp which surrounds *Ricinodendron rautanenii* seeds appears to play an important role in determining the response of this species to ethylene. Figure 2.2 shows that germination only occurred when a portion of this structure was removed from the radicle end of the seed (irrespective of whether the scarified region was in contact with the substrate or not). In the remaining treatments, swelling of the endosperm was frequently observed, although no radicle protrusion was noted. These findings indicated (once again) that water penetration was not being retarded and it was concluded that the endocarp inhibited germination by physically restricting embryo expansion. After eight days incubation, the endocarp was removed from all seeds which had not germinated. These excised seeds were re-incubated on moist vermiculite at 30°C. No further germination was recorded, indicating that the effect of the dormancy breaking treatment had been lost. This demonstrated that the manketti has a double dormancy mechanism (*viz.* coat imposed and physiological) and that satisfaction of the physiological requirements does not necessarily result in germination. Thus, in order for manketti seeds to respond to ethylene in the field, some decay of the endocarp would be necessary. On the basis of this assumption, all subsequent experiments were carried out on seeds only.

The germination response of manketti seeds to different

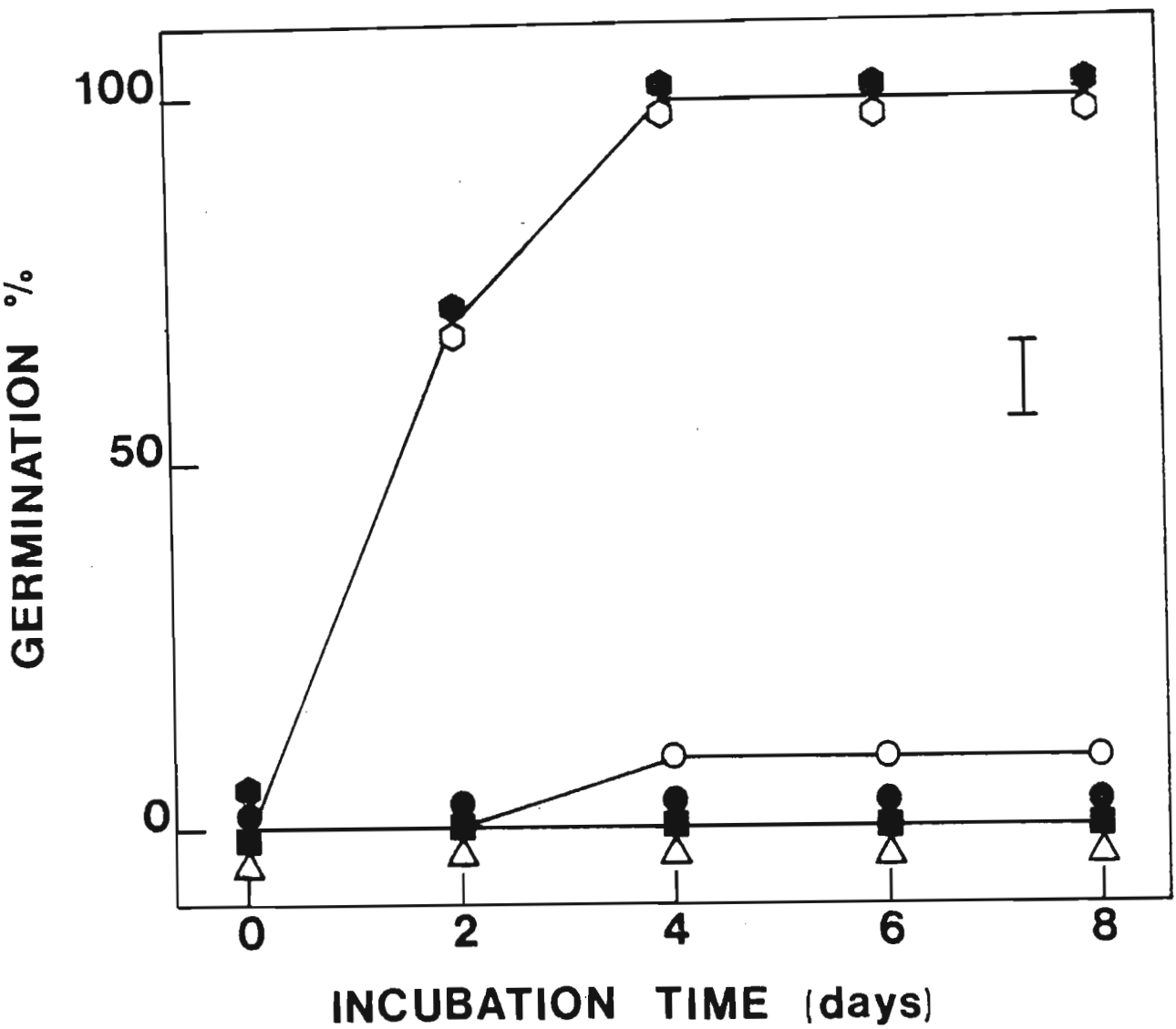


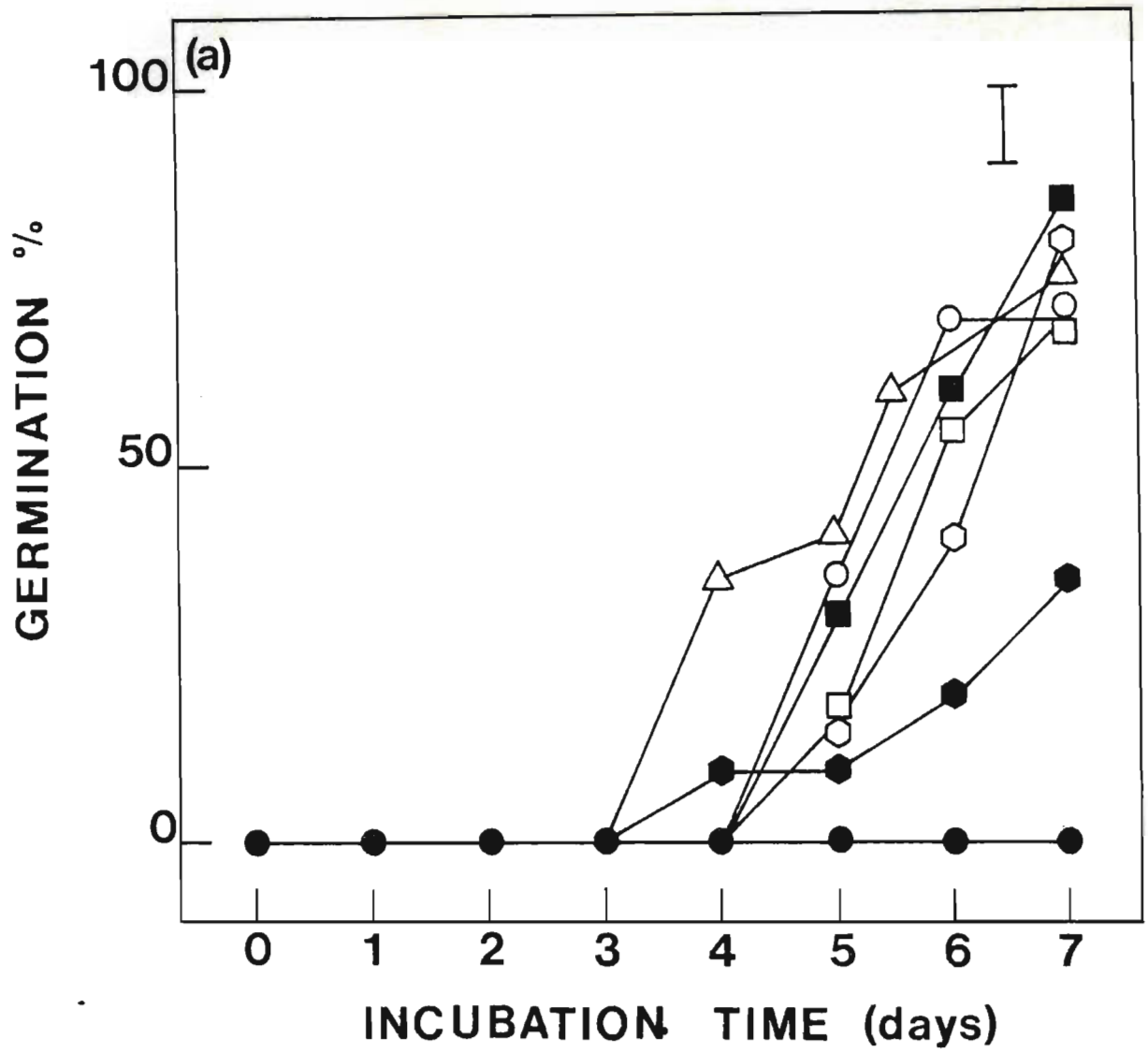
Figure 2.2 Germination curves for ethylene treated 'nuts' scarified at the radicle end (⬢ , ⬡), the end opposite the radicle (■ , □) or at a point between the two ends (● , ○). Control 'nuts' (▲ , △) were left intact. Open symbols represent those 'nuts' which were placed with the scarified portion in contact with the substrate. The bar represents the maximum 95 per cent confidence limit.

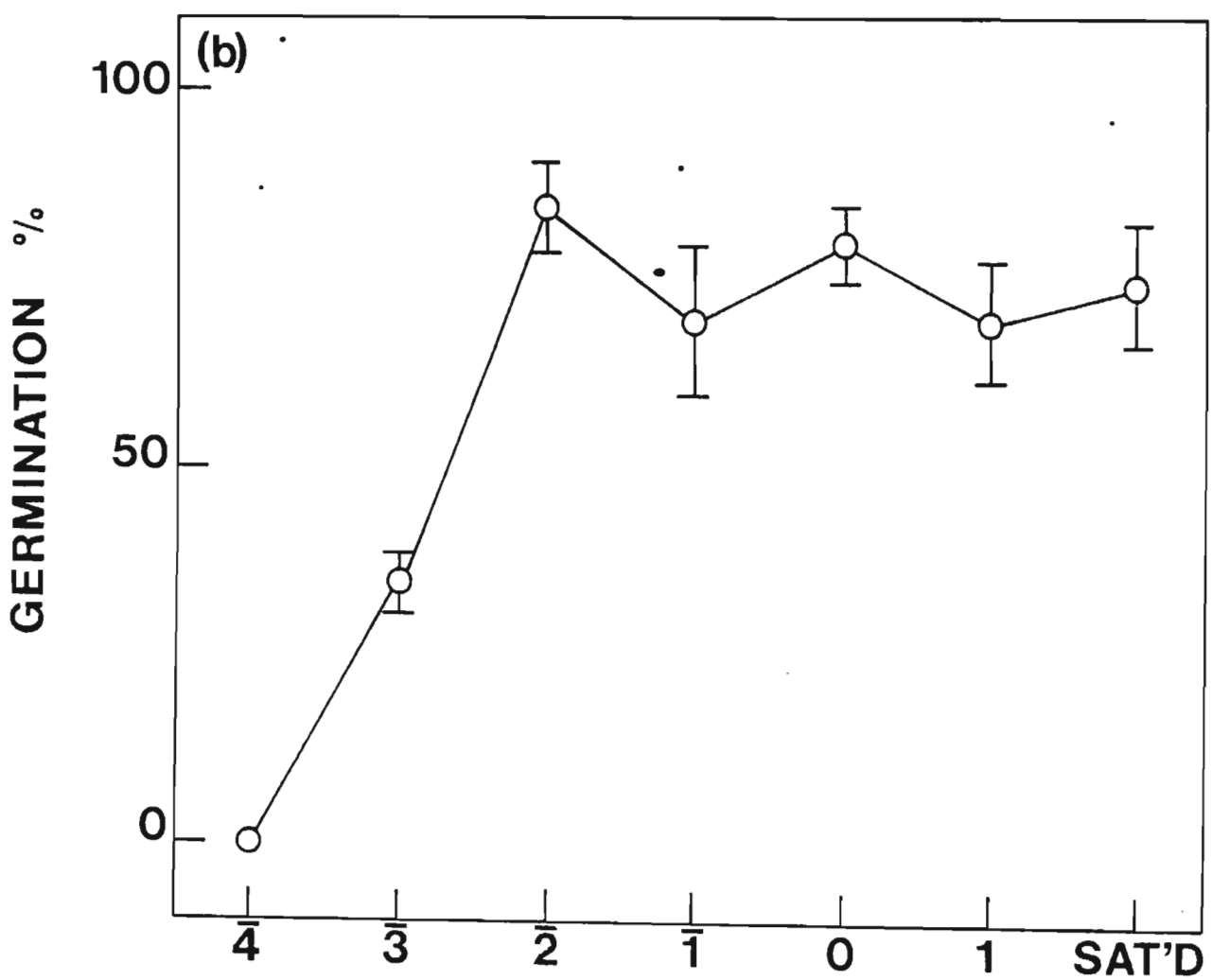
concentrations of ethylene is presented in Figure 2.3a. In this experiment, the lowest effective ethylene concentration was found to be 10^{-3} microlitres per litre. This treatment resulted in only 35 per cent of the seeds germinating and represented the threshold ethylene concentration for *R. rautanenii*. In the seeds of other ethylene sensitive species, the most frequently recorded threshold concentration was in the region of 10^{-1} microlitres per litre (EGLEY and DALE, 1970; KETRING and MORGAN, 1970) although ESASHI and LEOPOLD (1969), working on *Trifolium subterraneum* seeds, obtained some response at 10^{-2} microlitres per litre. Thus, by comparison, it appeared that manketti seeds were considerably more sensitive to this gas. In addition, most seed lots germinated to their full potential at concentrations above 10^{-3} microlitres per litre and the fact that these values did not differ significantly from each other indicated that dose saturation had occurred. This was made even more obvious when the data was expressed as a log dose-response relationship as in Figure 2.3b. The response obtained is clearly unlike the linear dose-response observed in seeds of other species such as *Amaranthus retroflexus*, *Xanthium pensylvanicum* (EGLEY, 1980) and *Striga asiatica* (EGLEY and DALE, 1970). In these instances, dose saturation usually occurred at higher ethylene concentrations of between 1 and 10 microlitres per litre.

With regard to the rates of germination, no differences were observed at concentrations above 10^{-3} microlitres per litre (Figure 2.3a). However, one exception to this was

Figure 2.3a The effects of different ethylene concentrations on the germination of intact *Ricinodendron rautanenii* seeds incubated in the dark at 25°C. Concentrations used were: 10^{-4} (●), 10^{-3} (⬢), 10^{-2} (■), 10^{-1} (○), 1,0 (□), and 10 (△) $\mu\ell\ \ell^{-1}$. One group of seeds was incubated in a saturated (SAT'D) ethylene atmosphere (⬡). The bar represents the maximum 95 per cent confidence limit.

Figure 2.3b The ethylene dose-response relationship for manketti seeds. Results are expressed as the final germination percentages obtained after seven days against the log of the ethylene concentrations. The bars represent the 95 per cent confidence limits.


(a)
100
50
0
GERMINATION %
0
1
2
3
4
5
6
7
INCUBATION TIME (days)


(b)
100
50
0
GERMINATION %
4̄
3̄
2̄
1̄
0
1
SAT'D

the seeds incubated in a saturated ethylene atmosphere and this probably resulted from binding site saturation. These results further illustrated the remarkable sensitivity of *R. rautanenii* seeds to ethylene.

The effects of the different ethylene concentrations on seedling growth were also examined and the results are presented in Table 2.1. The data shows that no trend existed with regard to root length; although those seeds incubated in an ethylene concentration of 10^{-1} microlitres per litre were significantly longer than the rest (Table 2.1a). It is thus possible that, at this concentration, root growth in this species is promoted but more data would be required to support this. Fresh and dry mass determinations of the root, shoot and endosperm tissues also showed no significant differences (Table 2.1b), suggesting that these seedlings grow equally well in both high and low ethylene concentrations.

The results obtained in these experiments support the view that ethylene plays a regulatory role in the natural germination of this species. The concentration range over which ethylene was effective was found to lie well below the mean values recorded in soils (SMITH, 1976) and this species also showed exceptional tolerance to a gas which, in many cases, promotes senescence. However, it is likely that subsequent growth phenomena would have been affected had the seedlings been maintained in ethylene for prolonged periods of time.

Table 2.1a

Applied Ethylene Concentration ($\mu\ell\ \ell^{-1}$)	Root Length (Millimetres)
Control (dH_2O)	0
Saturated *	6,2 ($\pm$ 1,6)
Saturated **	5,6 ($\pm$ 0,7)
10	4,4 ($\pm$ 1,0)
1,0	3,6 ($\pm$ 1,45
10^{-1}	8,91 ($\pm$ 1,21)
10^{-2}	6,18 ($\pm$ 1,4)
10^{-3}	4,71 ($\pm$ 2,6)

* unsealed ** sealed

Table 2.1b

Applied Ethylene Concentration ($\mu\ell\ \ell^{-1}$)	Moisture Content (%)		
	R	S	E
Control (dH_2O)	0,03($\pm$0,005)		0,78($\pm$0,05)
Saturated *	89,7 ($\pm$ 4,1)	69,1 ($\pm$ 6,6)	55,9 ($\pm$ 5,1)
Saturated **	90,0 ($\pm$ 5,0)	68,6 ($\pm$ 5,2)	54,7 ($\pm$ 5,5)
10	90,7 ($\pm$ 4,6)	67,2 ($\pm$ 6,7)	53,4 ($\pm$ 5,5)
1,0	90,0 ($\pm$ 6,0)	69,7 ($\pm$ 4,5)	59,2 ($\pm$ 5,7)
10^{-1}	88,2 ($\pm$ 5,2)	65,2 ($\pm$ 4,3)	53,2 ($\pm$ 5,9)
10^{-2}	92,0 ($\pm$ 4,1)	70,6 ($\pm$ 3,9)	55,6 ($\pm$ 6,0)
10^{-3}	88,5 ($\pm$ 5,6)	72,7 ($\pm$ 5,3)	60,3 ($\pm$ 5,4)
	Dry Mass Grammes $Seed^{-1}$		
	R	S	E
Control (dH_2O)	0,028)$\pm$0,004)		0,74($\pm$0,07)
Saturated *	0,02($\pm$ 0,001)	0,1 ($\pm$0,002)	0,81 ($\pm$ 0,19)
Saturated **	0,02($\pm$ 0,001)	0,08($\pm$0,001)	0,75 ($\pm$ 0,28)
10	0,02($\pm$ 0,001)	0,08($\pm$0,002)	0,83 ($\pm$ 0,17)
1,0	0,02($\pm$ 0,001)	0,1 ($\pm$0,003)	0,80 ($\pm$ 0,3)
10^{-1}	0,02($\pm$ 0,001)	0,08($\pm$0,002)	0,80 ($\pm$ 0,3)
10^{-2}	0,02($\pm$ 0,001)	0,1 ($\pm$0,001)	0,82 ($\pm$ 0.3)

Further support for a 'natural' role for ethylene was obtained from the experiments carried out using a gas dispensing device (SALTVEIT, 1978). Eighty per cent of the seeds incubated in the ethylene-enriched stream of air germinated within four days. This showed that under natural conditions ethylene produced during the breakdown or organic material (e.g. fruit tissues) and carried to seeds in wind currents, could still have its effect. Unfortunately the apparatus proved unreliable on occasions and was therefore not used as a standard means of breaking manketti seed dormancy. (The treatment which was in fact selected for standard use in the remaining experiments was the incubation of seeds in a saturated ethylene atmosphere). For future studies, however, the apparatus described by SALTVEIT (1978) would represent an inexpensive method of treating material with this phytohormone, particularly in view of the prohibitive cost of pure ethylene gas.

The germination curves of seeds incubated with ethylene at different temperatures are presented in Figure 2.4. In accordance with the findings of other workers (ESASHI and LEOPOLD, 1969; KATOH and ESASHI, 1975a; and SCHONBECK and EGLEY, 1981a) these results show that ethylene has little or no effect at temperatures below 20°C. Furthermore, ethylene treated seeds incubated at these temperatures (i.e. 5, 10, 15 and 20°C) for 21 days failed to germinate when transferred to 30°C. This suggested that either ethylene binding cannot occur at low temperatures, or that the metabolic processes necessary for dormancy breaking in this

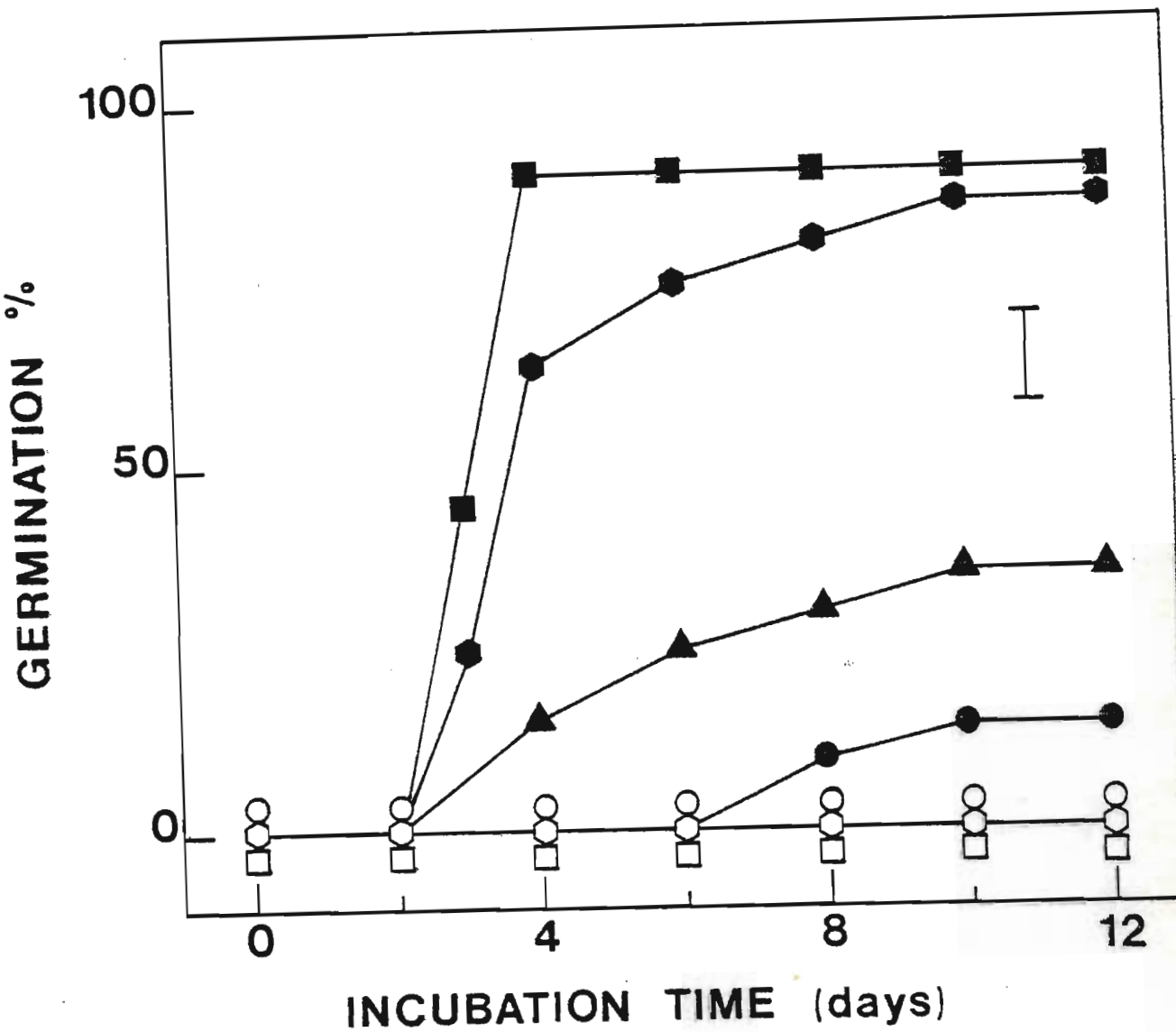
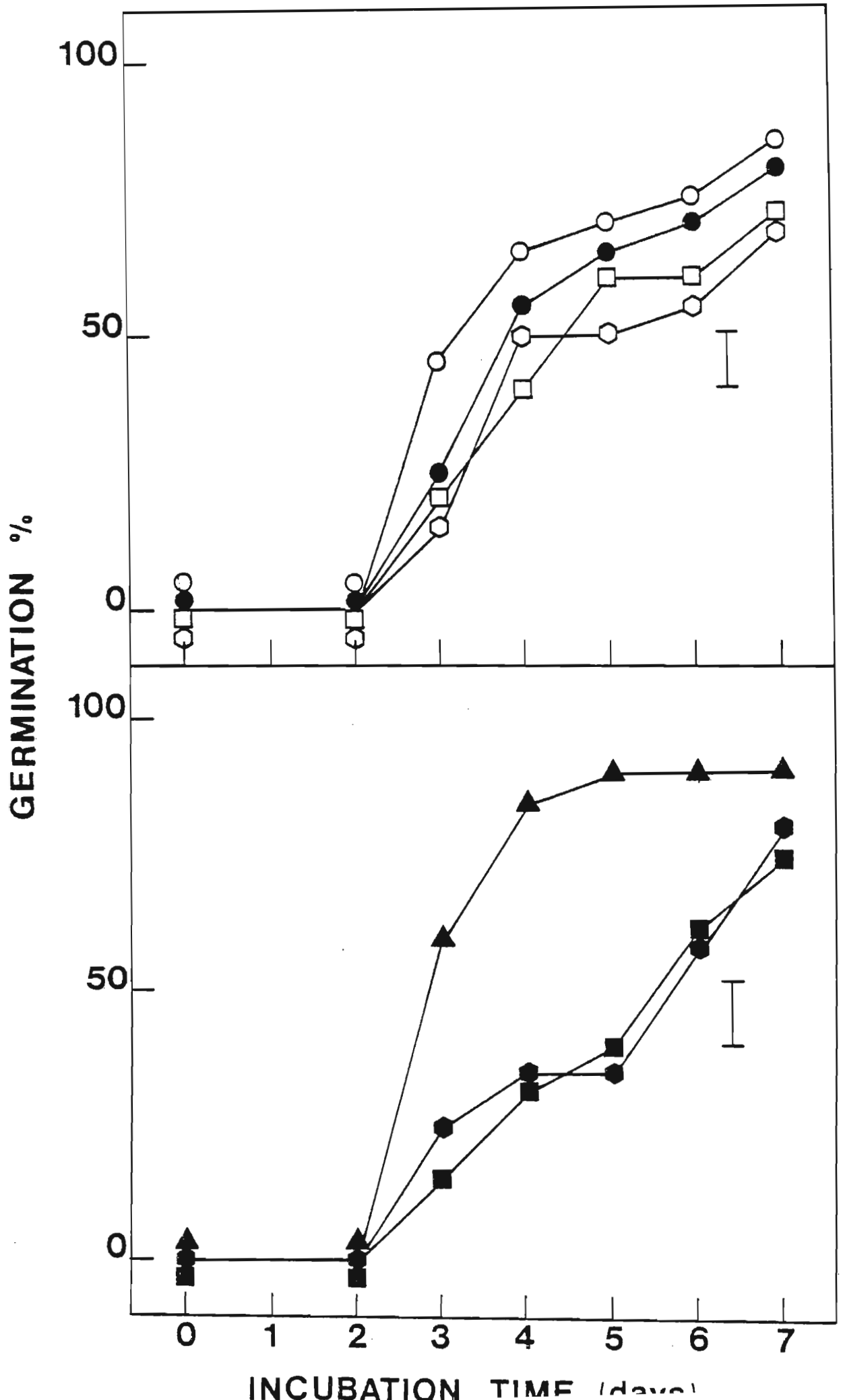


Figure 2.4 The germination responses of ethylene treated manketti seeds incubated at 5 (○), 10 (⬡), 15 (□), 20 (●), 25 (⬢), 30 (■) and 35°C (▲). The dormancy breaking treatment consisted of incubating the seeds for 12 hours in an ethylene saturated atmos-

species require higher temperatures. At 20°C, only 20 per cent germination was recorded after 14 days, indicating that even this temperature was still too low to support optimum germination. At the higher temperatures of 25 and 30°C, 85 and 90 per cent germination were obtained respectively. However, at 35°C the response was again reduced and only 35 per cent germination was recorded. These results agree with those obtained for other ethylene sensitive species where it was found that, in general, optimum germination occurred at between 25 and 35°C. These specific temperature requirements may be related to the activity of various enzyme systems (e.g. in cocklebur seeds ESASHI and LEOPOLD (1975a) suggested that the effect of these temperatures was to stimulate endogenous ethylene production). The reduced germination response of manketti seeds at 35°C was probably not due to any physical property of ethylene (e.g. increased volatility)since, at this temperature, ethylene was shown to have its optimum effect on other species such as *Xanthium pensylvanicum* (EGLEY, 1980). In *R. rautanenii* seeds this depressed response may represent an adaptive mechanism which prevents germination at excessively high temperatures (particularly in view of the plants' habitat).

Manketti seeds exposed to ethylene for varying lengths of time responded very differently from other species kept under similar conditions. The results presented in Figure 2.5 show that germination was as high after a 30 minute exposure period as it was after exposure for 12 hours. In addition, the rates of germination were similar for all ex-


100
50
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GERMINATION %
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6
7
INCUBATION TIME (days)

posure periods except the 12 hour treatment which was probably higher due to binding site saturation. In other ethylene sensitive species such as *Amaranthus retroflexus* (EGLEY, 1980) and *Arachis hypogaea* (TOOLE *et al.*, 1964) it was found that in order to appreciably stimulate germination, ethylene was required for approximately 16 and 8 hours respectively. In addition, a very different response was reported for *Spergula arvensis* seeds where germination was found to increase linearly with increasing exposure times (JONES and HALL, 1979). Thus, in comparison, it seems that *R. rautanenii* seeds require only brief periods of exposure to ethylene. It could be argued, however, that these data are unrepresentative since the seeds are unlikely to experience a saturated ethylene atmosphere in the field. In considering this criticism, it should be borne in mind that in previous experiments it was demonstrated that only the rates of germination were different between seeds exposed to high and low concentrations of ethylene. Thus, extrapolations to events which may occur in the field are not without substance. The ability of *Ricinodendron rautanenii* seeds to respond to such low ethylene concentrations and after such brief exposures to the gas makes this species one of the most sensitive to ethylene yet recorded, and demonstrates their unique receptivity to this gas.

Another parameter which may affect germination in the field is the amount of water imbibed by the seeds prior to receiving the ethylene stimulus. Obviously, the first consideration in this series of experiments was whether or not

dormancy could be broken in dry seeds. The results presented in Figure 2.6 indicate that dry seeds, when treated with ethylene and subsequently incubated on a moist substrate, could in fact germinate. Dry excised seeds (testae removed) also responded favourably, suggesting that the germination observed in intact seeds may not necessarily be due to residual ethylene present in the testae. However, previous experiments had shown that removal of the testa alone could promote germination in this species (Chapter 1). In addition, the observation that ethylene treated intact seeds failed to germinate after being subjected to a mild vacuum, conflicts with the view that dormancy can be broken in the absence of moisture. Nevertheless, it should be borne in mind that if the bond between ethylene and its receptor site is weak, then this interaction would easily be disrupted by hypobaric conditions. Thus, it is not possible to discount the possibility that unimbibed seeds are able to perceive the ethylene stimulus.

The germination rates of seeds imbibed on moist vermiculite for varying lengths of time prior to ethylene treatments were not significantly different (Figure 2.7). Furthermore, the final germination percentages obtained were also similar. On the basis of this it appeared that the amount of water imbibed prior to the ethylene treatment was not crucial to the breaking of dormancy. This was also supported by the finding that seeds which were simultaneously gased and imbibed (for 12 hours) germinated to their full potential (Figure 2.8). These results are in accordance with those

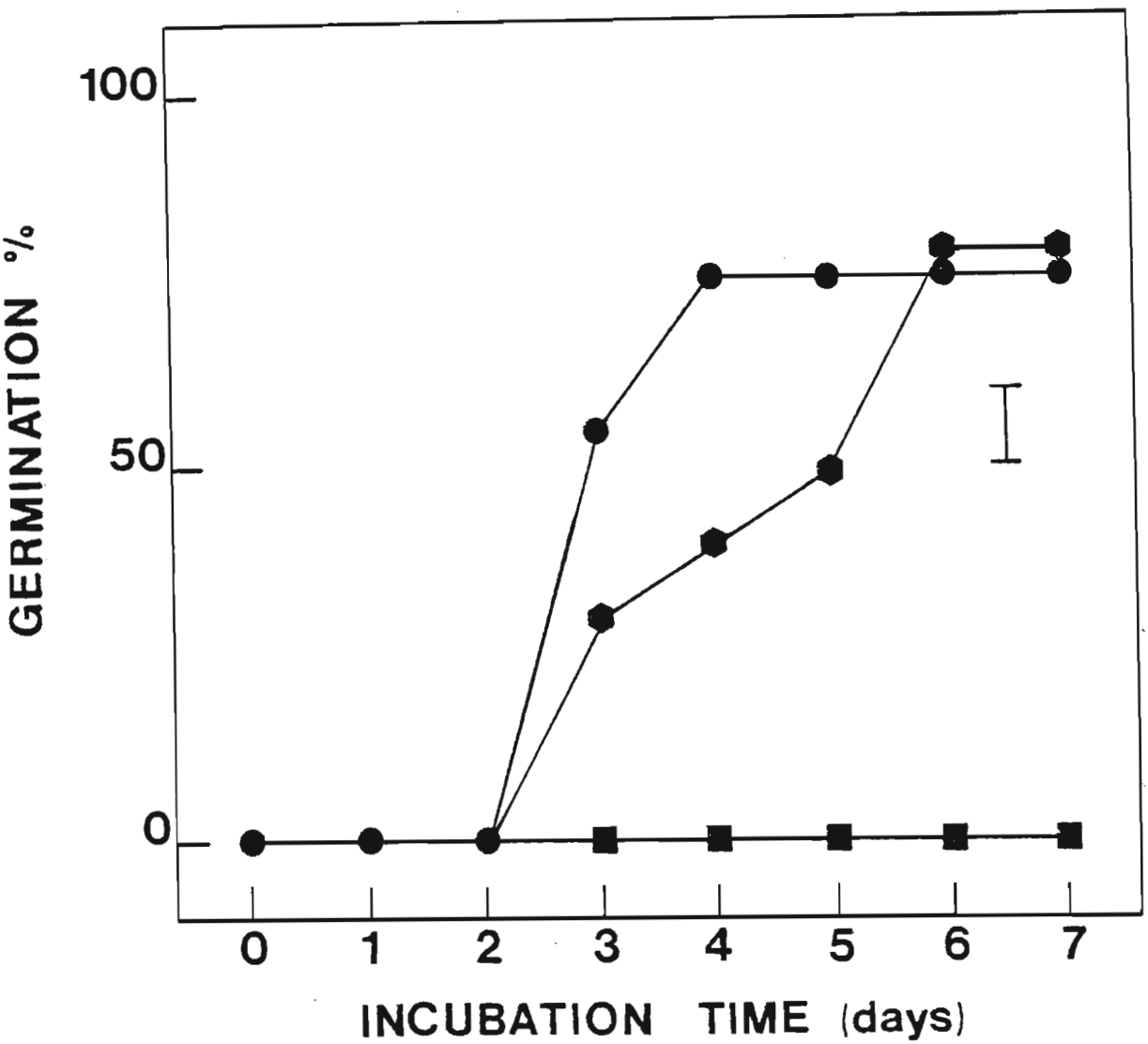
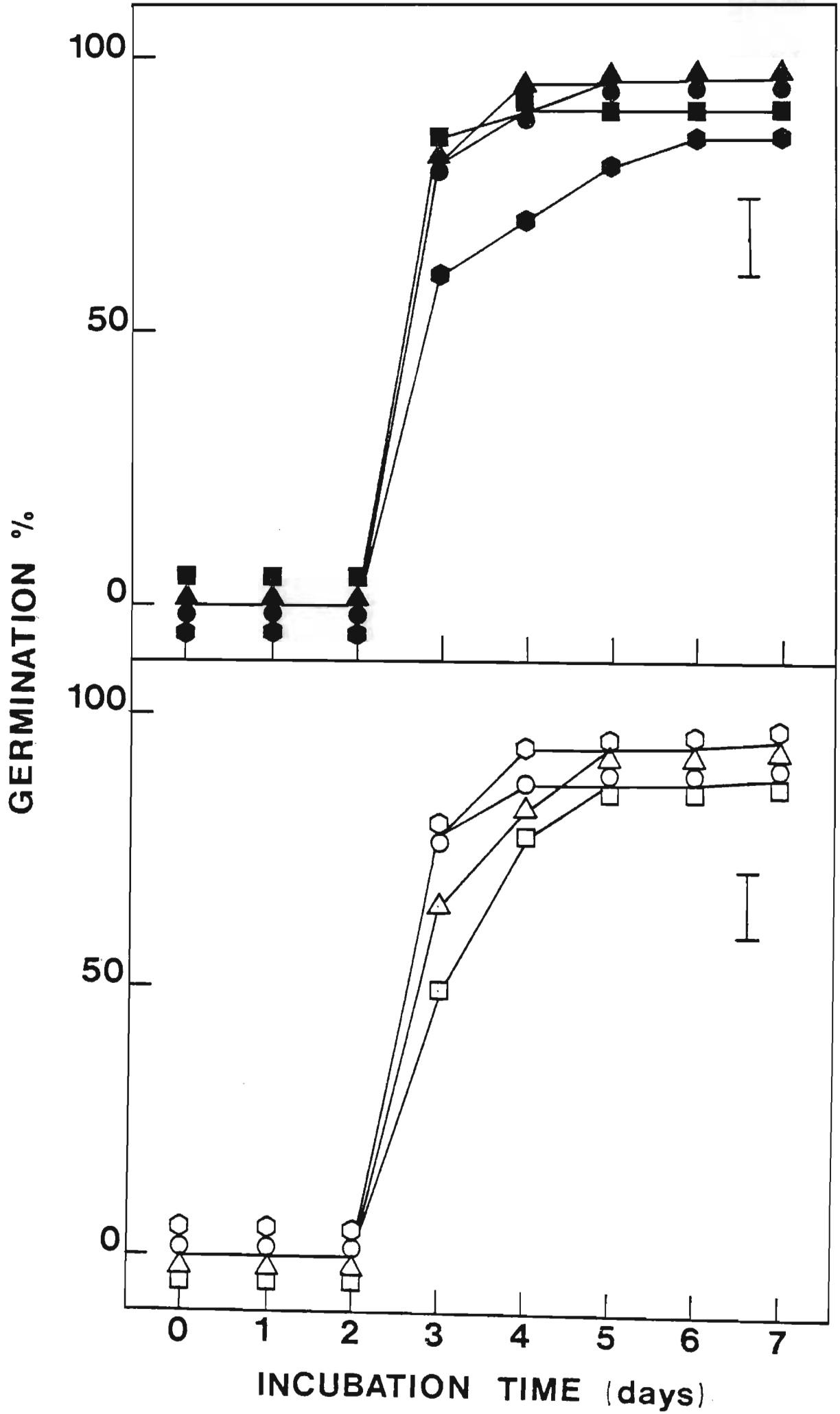


Figure 2.6 The germination response of dry, intact (⬢ , ■) and excised (●) seeds treated with ethylene for 12 hours prior to moist incubation at 30°C. Intact seeds represented by ■ were subjected to a mild vacuum prior to moist incubation. The bar represents the


100
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GERMINATION %
100
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0
1
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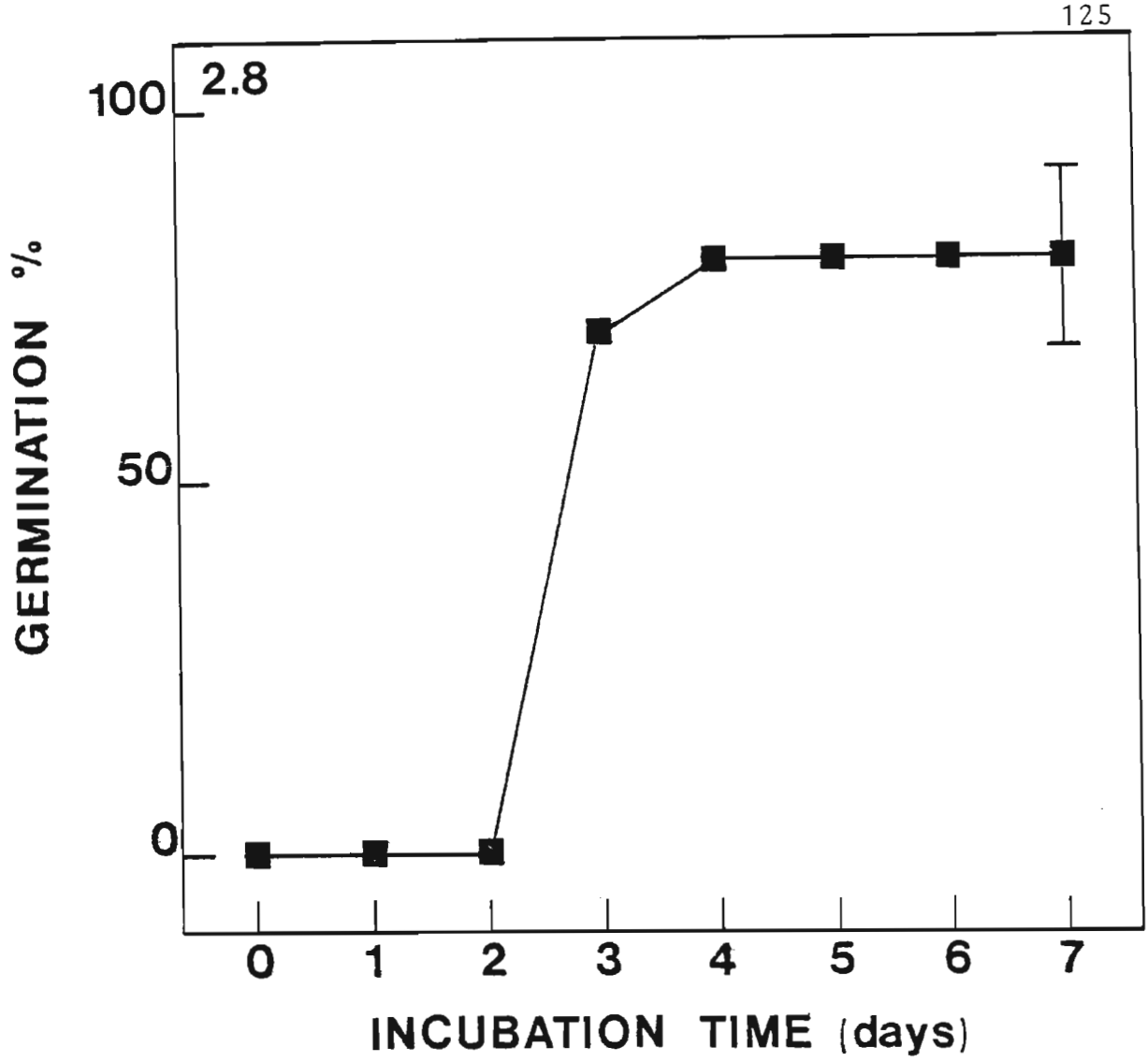
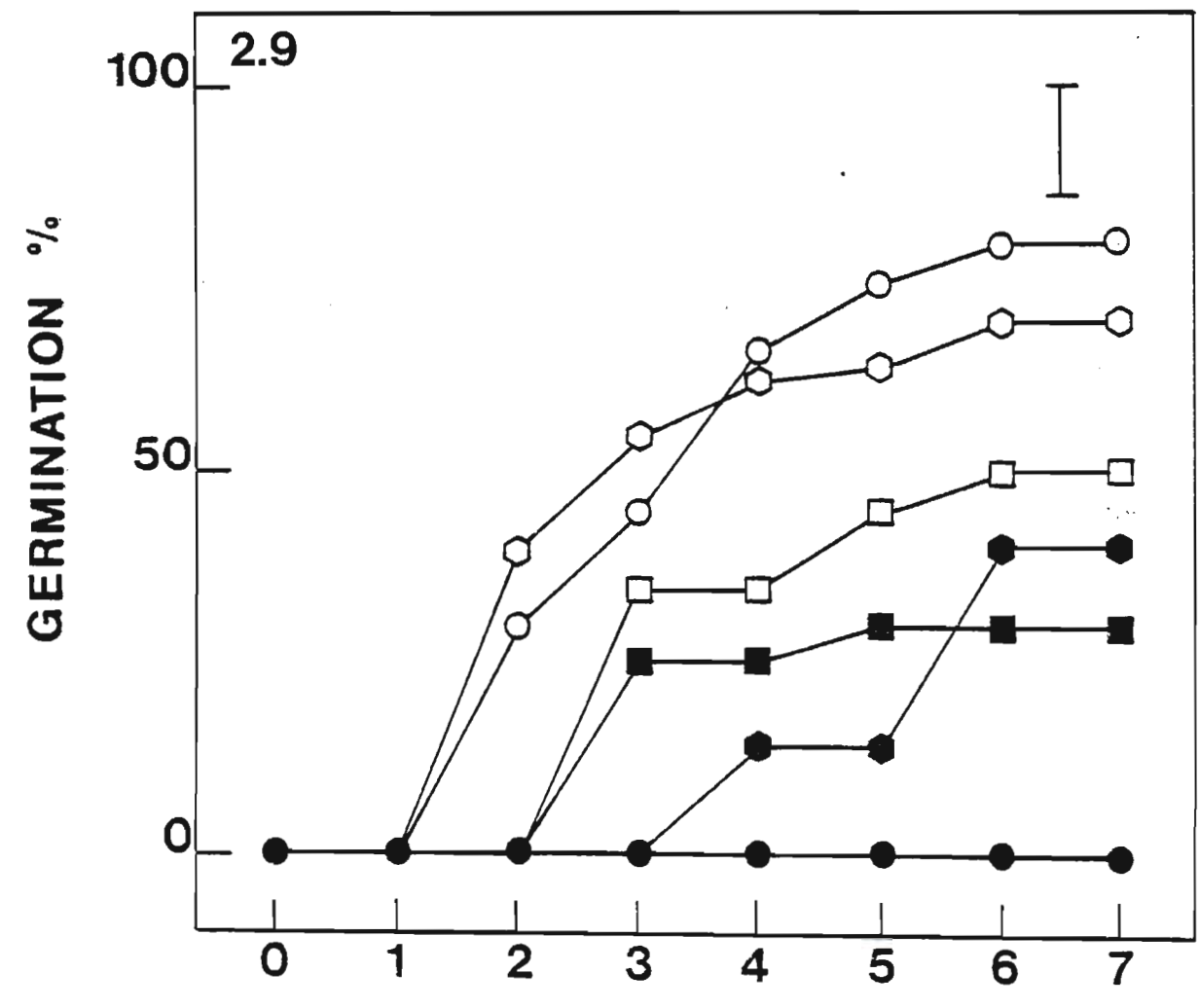
6
7
INCUBATION TIME (days)

Figure 2.8 The germination response of manketti seeds which were imbibed and gased with ethylene simultaneously for 12 hours. Subsequent incubation on moist vermiculite was at 30°C under ethylene-free conditions. The bar represents the confidence limit where p = 0,05.

Figure 2.9 The germinated responses of dry (shaded symbols) and pre-imbibed (open symbols) manketti seeds incubated with ethylene for 12 hours at 30°C and subsequently left in an open vessel at room temperature for 1 (● , ○); 4 (⬢ , ⬡); and 8 (■ , □) days. After the respective periods of dessication, the seeds were returned to moist conditions and re-incubated at 30°C. The bar represents the maximum 95 per cent confidence limit.


2.8
100
50
0
GERMINATION %
0 1 2 3 4 5 6 7
INCUBATION TIME (days)


2.9
100
50
0
GERMINATION %
0 1 2 3 4 5 6 7

obtained by JONES and HALL (1979) for *Spergula arvensis* seeds. In this instance it was found that no differences existed in the germination potential of seeds which had been imbibed for two days prior to gasing and those which were simultaneously gased and imbibed. Thus, the importance of water in the actual perception of the dormancy breaking stimulus is not clear, although there are some indications that, initially, it may not be required at all.

Some clarification on this issue was obtained from the experiments conducted to determine whether the effects of ethylene were retained in dry and imbibed seeds after a period of desiccation. The results presented in Figure 2.9 show that dry treated seeds, left for 24 hours before incubation on moist vermiculite, did not germinate. However, when left for four and eight days prior to moist incubation, 40 and 25 per cent respectively of these non-pre-imbibed seeds germinated. From this result it seemed reasonable to assume that ethylene had, in some way, entered the dry seed tissues since most of the gas would have diffused into the atmosphere during the delay between gasing and imbibition. Much greater germination responses were obtained with pre-imbibed seeds. When desiccated for one day after the ethylene treatment, 80 per cent of these pre-imbibed seeds germinated when re-incubated under normal conditions. During the period of desiccation, the moisture content declined by 40 per cent. After four and eight days, on the other hand, the moisture content fell by a further 75 and 96 per cent respectively. In spite of this, re-

incubation under normal conditions resulted in 90 per cent of the four day group and 45 per cent of the eight day group germinating within seven days. In all the pre-imbibed seeds, germination was found to commence one day sooner than usual suggesting that some of the major metabolic adjustments required for germination had already taken place. However, the rates of germination were lower than those of seeds left to germinate directly after the ethylene treatment. This may be due to the physiological recovery of the seeds to water stressing.

These data supported, to some extent, the earlier findings that dormancy could be broken in dry seeds. Furthermore, they also demonstrated that the effects of ethylene could be retained in these seeds even when they had experienced considerable dehydration. The high germination percentages obtained for pre-imbibed seeds treated with ethylene and then desiccated for one, four and eight days respectively, has important ecological implications. Under natural conditions it is likely that seeds buried in the soil or lying on the soil surface would undergo periods of wetting and drying. Most seeds appear to tolerate this process provided that re-dehydration does not occur too late in the overall germination process. There are even reports that some species actually benefit from this treatment (HEYDECKER, 1977). Manketti seeds, not only appear to tolerate this re-dehydration, but also retain the dormancy breaking effects of ethylene during this process. This implies then, that it is not necessary for optimum ethylene and environmental

factors to prevail simultaneously. In this regard, it is unfortunate that comparisons with other ethylene sensitive species are not possible, since no similar studies have been carried out. Future studies on manketti seeds (and those of other ethylene sensitive species) should attempt to determine precisely how long the effects of ethylene are retained and, whether or not, these are reversible.

As mentioned previously, carbon dioxide and ethylene may act synergistically in stimulating the germination of dormant *Arachis hypogaea*, *Trifolium subterraneium*, *Xanthium pensylvanicum* and *Spergula arvensis* seeds (KETRING, 1977; JONES and HALL, 1979). The results presented in Figure 2.10 show that *R. rautanenii* seeds germinated to their full potential irrespective of whether or not carbon dioxide was present during the periods of imbibition or gas treatment. The onset of germination, however, was clearly delayed in those seeds which did not receive carbon dioxide during the ethylene treatment phase. Furthermore, it is possible that, had these seeds remained in carbon dioxide-free conditions for greater lengths of time, germination would have been retarded altogether. This has been reported in other species such as *Arachis hypogaea* (KETRING and MORGAN, 1969) and *Trifolium subterraneum* (ESASHI and LEOPOLD, 1969). A criticism which can be leveled at such experiments is that prolonged absence of carbon dioxide may affect processes (such as carboxylation (KATOH and ESASHI, 1975b)) which are essentially unrelated to the effects of ethylene. Nevertheless, even if this argument is taken into consideration,

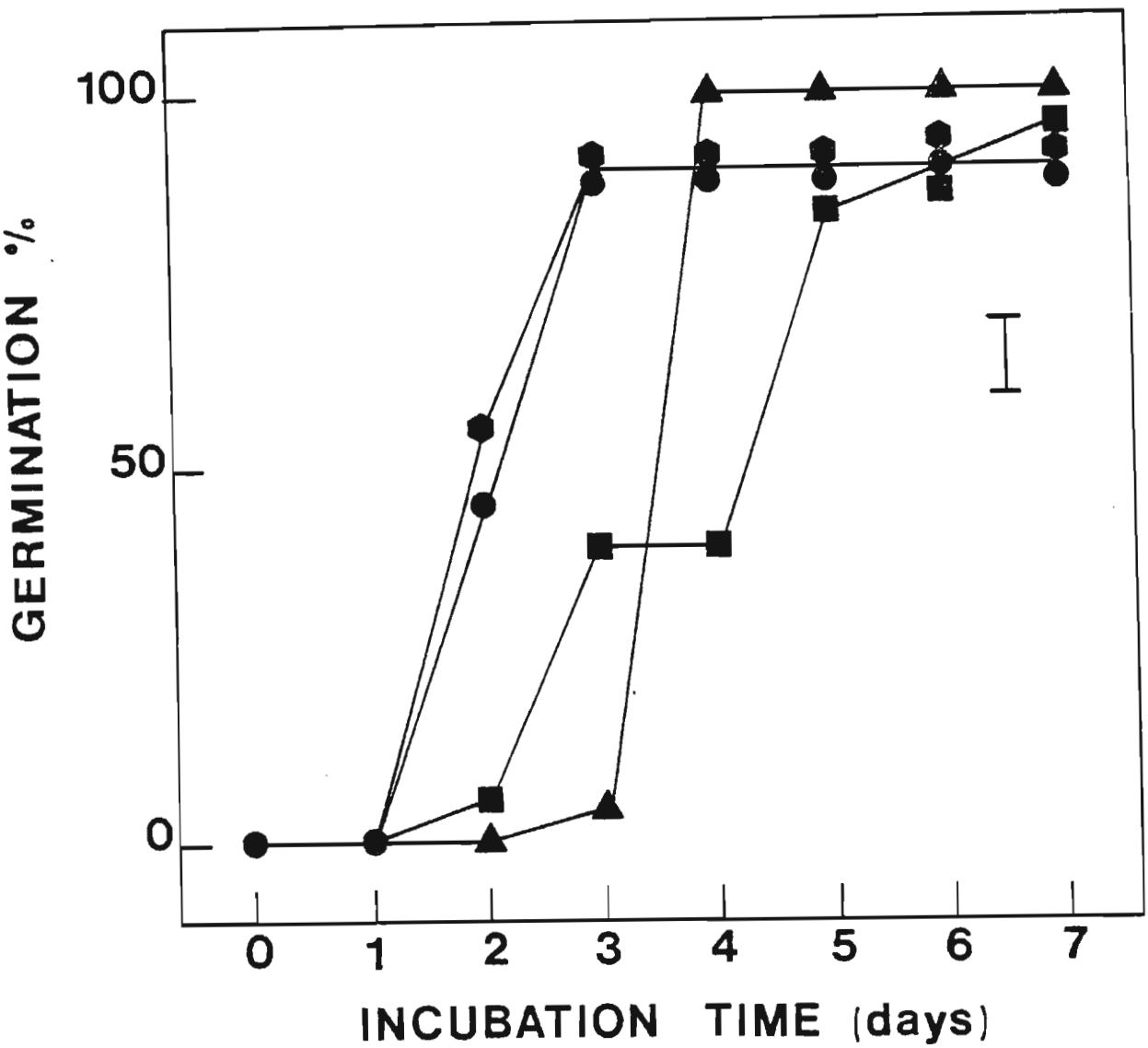
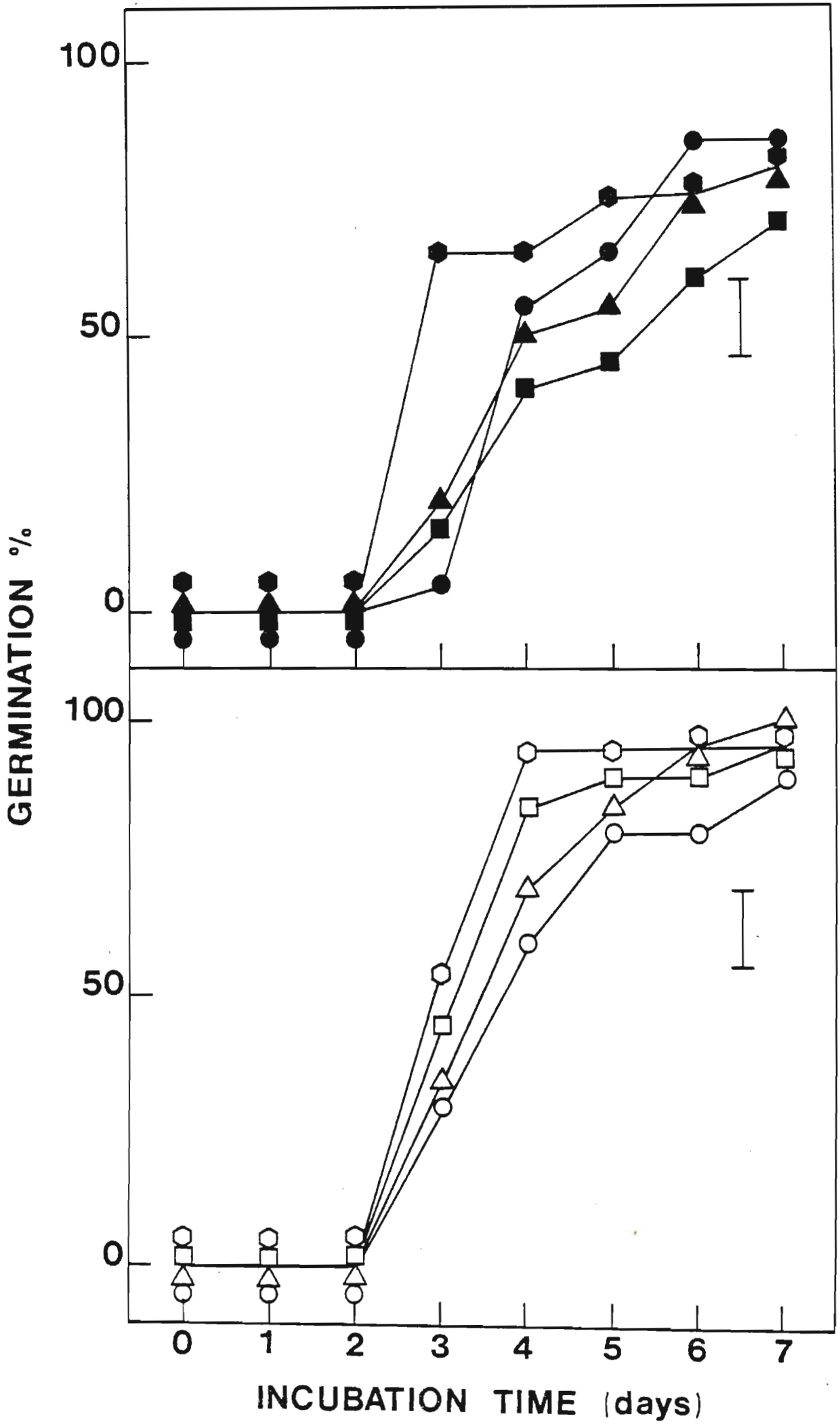


Figure 2.10 The germination of *Ricinodendron rautanenii* seeds incubated either in the presence (▲) or absence (■) of carbon dioxide throughout the experimental period. Seeds were also deprived of CO_2 during imbibition (⬢) and gasing (●) only. Incubation was at 30°C. The bar represents the maximum 95 per cent

it still seems that *R. rautanenii* seeds are unlike those of *Xanthium pensylvanicum* (KATOH and ESASHI, 1975b) and *Spergula arvensis* (JONES and HALL, 1979) in that no carbon dioxide sensitive period preceeds the period of ethylene sensitivity.

In dormant cocklebur (*X. pensylvanicum*) seeds it was also found that a 10 per cent carbon dioxide treatment stimulated germination (albeit to a lesser extent than ethylene). A similar treatment had no effect on manketti seeds. The results obtained in these experiments suggested that carbon dioxide plays little or no role in the breaking of manketti seed dormancy although it may be required in the normal metabolism of the seeds.

In recent years, the ethylene releasing compound ethrel has been shown to produce the full spectrum of ethylene effects. With regard to seeds, it was found to release dormancy in a number of species including *Striga asiatica* (EGLEY and MORGAN, 1970), *Xanthium pensylvanicum* and Indian rye grass (*Lolium* sp.) (TAO, McDONALD and KHAN, 1974). Results of the experiments carried out with this compound on *R. rautanenii* seeds are presented in Figure 2.11 and Tables 2.2a and b. These data show that no threshold concentration was evident and that a response was obtained at all the concentrations used. Of these, those concentrations which corresponded to 100, 1 000 and 10 000 microlitres per litre ethylene were clearly more effective in terms of both the final percentages obtained and the rates of germination. The reason for this could be that during passive imbibition very little moisture


100
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GERMINATION %
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INCUBATION TIME (days)

Table 2.2a Moisture content (%) of the embryo and endosperm tissues from seeds incubated at 30°C on different concentrations of ethrel for varying lengths of time. Seedlings were harvested eight days after the experiment commenced. Figures in parentheses represent the confidence limits where p = 0,05.

Effective Ethylene Concentration	MOISTURE CONTENT (%)				
	Period Incubated on Ethrel				
	24	48	72	96	120
	Embryo Component (Root and Shoot)				
10^{-3}	0	82,2 (± 3)	75,4 (± 7)	74,1 (± 8)	69,6 (± 7)
10^{-2}	79,5 (± 7)	78,8 (± 7)	81,8 (± 5)	81,7 (± 4)	81,3 (± 8)
10^{-1}	82,4 (± 9)	81,8 (± 5)	83,1 (± 9)	81,6 (± 5)	81,9 (± 4)
1,0	77,8 (± 2)	77,4 (± 3)	81,7 (± 7)	67,0 (± 5)	70,6 (± 6)
10^{1}	83,5 (± 5)	82,5 (± 5)	84,1 (± 5)	73,0 (± 4)	81,8 (± 3)
10^{2}	83,0 (± 7)	79,0 (± 4)	81,8 (± 6)	83,6 (± 7)	80,0 (± 6)
10^{3}	89,1 (± 6)	79,0 (± 7)	81,0 (± 6)	77,4 (± 6)	83,2 (± 5)
10^{4}	80,5 (± 7)	76,8 (± 6)	78,0 (± 4)	65,2 (± 6)	67,8 (± 6)
	24	48	72	96	120
	Endosperm Component				
10^{-3}	0	60,3 (± 7)	52,1 (± 2)	37,2 (± 4)	35,8 (± 6)
10^{-2}	52,5 (± 6)	59,0 (± 4)	55,0 (± 8)	56,9 (± 5)	60,3 (± 5)
10^{-1}	35,6 (± 6)	54,2 (± 6)	36,2 (± 8)	47,1 (± 3)	63,5 (± 6)
1,0	37,1 (± 5)	49,1 (± 5)	59,4 (± 7)	40,3 (± 3)	50,1 (± 6)
10^{1}	58,6 (± 7)	56,5 (± 4)	67,4 (± 4)	44,2 (± 7)	55,6 (± 3)
10^{2}	58,8 (± 5)	50,0 (± 5)	58,7 (± 5)	67,5 (± 4)	54,6 (± 6)
10^{3}	68,7 (± 6)	58,8 (± 3)	66,2 (± 7)	40,5 (± 6)	53,5 (± 7)
10^{4}	58,4 (± 5)	51,2 (± 39	65,0 (± 9)	52,9 (± 4)	48,4 (± 4)

Table 2.2b Dry mass values for the embryo and endosperm components of seeds incubated at 30°C on different concentrations of ethrel for varying lengths of time. Seedlings were harvested eight days after the experiment commenced. Figures in parentheses represent the confidence limits where p = 0,05.

Effective Ethylene Concentration	DRY MASS (PER SEED)				
	Period Incubated on Ethrel				
	24	48	72	96	120
	Embryo Component				
10^{-3}	0	0,16 (± 0,15)	0,14 (± 0,06)	0,07 (± 0)	0,07 (± 0)
10^{-2}	0,105 (± 0,03)	0,17 (± 0,06)	0,155 (± 0,09)	0,13 (± 0)	0,245 (± 0,15)
10^{-1}	0,17 (± 0,06)	0,155 (± 0,10)	0,37 (± 0,09)	0,18 (± 0,1)	0,145 (± 0,09)
1,0	0,10 (± 0,04)	0,115 (± 0,06)	0,19 (± 0,07)	0,10 (± 0)	0,10 (± 0)
10^{1}	0,13 (± 0,05)	0,145 (± 0,03)	0,235 (± 0,1)	0,10 (± 0)	0,16 (± 0,4)
10^{2}	0,16 (± 0,06)	0,11 (± 0,06	0,215 (± 0,1)	0,37 (± 0,09)	0,20 (± 0,04)
10^{3}	0,10 (± 0,03)	0,09 (± 0,06)	0,215 (± 0,09)	0,125 (± 0,03)	0,11 (± 0,04)
10^{4}	0,14 (± 0,06)	0,094 (± 0,01)	0,110 (± 0,06)	0,07 (± 0,003)	0,06 (± 0,02)
	24	48	72	96	120
	Endosperm Component				
10^{-3}	0	0,69 (± 0,2)	0,795 (± 0,03)	0,735 (± 0,1)	0,815 (± 0,15)
10^{-2}	0,705 (± 0,4)	0,77 (± 0,24)	0,715 (± 0,03)	0,56 (± 0,36)	0,567 (± 0,23)
10^{-1}	0,745 (± 0,4)	0,925 (± 0,5)	1,9 (± 0,6)	0,725 (± 0,4)	0,52 (± 0,2)
1,0	0,956 (± 0,3)	0,865 (± 0,2)	0,69 (± 0,2)	0,68 (± 0,2)	0,69 (± 0,1)
10^{1}	0,765 (± 0,2)	0,735 (± 0,2)	0,57 (± 0,2)	0,725 (± 0,03)	0,71 (± 0,2)
10^{2}	0,725 (± 0,2)	0,76 (± 0,06)	0,595 (± 0,09)	0,54 (± 0,12)	0,725 (± 0,3)
10^{3}	0,705 (± 0,15)	0,725 (± 0,15)	0,625 (± 0,15)	0,68 (± 0,2)	0,77 (± 0,06)
10^{4}	0,75 (± 0,3)	0,815 (± 0,03)	0,805 (± 0,03)	0,875 (± 0,03)	0,871 (± 0,02)

is actually imbibed by the seeds (see Figure 1.6, Chapter 1) and hence only small quantities of ethrel would be incorporated into the tissues. Thus, because ethrel is converted to ethylene intracellularly (ESASHI and LEOPOLD, 1969), the greater the number of ethrel molecules present, the higher the internal ethylene concentration is likely to be.

The effects on subsequent seedling growth of incubating manketti seeds on different ethrel concentrations for varying lengths of time are presented in Tables 2.2a and b. From this it was obvious that the period of time seeds were incubated with this compound was not critical in terms of the final germination percentages obtained. Furthermore, the fresh and dry mass values for the endosperm and embryo tissues of the resultant seedlings also remained unaffected by the concentration and period of exposure to, ethrel. This was supported by the results of an analysis of variance which demonstrated that within-treatment variance and between-treatment variance were similar. It was therefore concluded that, like the gas, high concentrations of ethrel had no adverse effects on the germination and initial seedling growth of manketti seeds. In addition, the duration of exposure to these high concentrations was equally non-inhibitory. Thus, for commercial purposes, ethrel would represent an inexpensive means of rapidly propagating this species. However, ethrel was not suitable for analytical work since, because seeds vary in the amount of water they imbibe, the number of ethrel molecules responsible for a

given response would not always be the same. It would therefore be impossible to estimate the effective ethylene concentration.

The results obtained in this series of experiments demonstrated that *R. rautanenii* seeds had the lowest threshold ethylene concentration yet recorded for any species (prior to any pre-treatments). Thus, provided the restrictive influence of the endocarp has been removed, manketti seeds are uniquely sensitive to this gas. The data also showed that unusually high concentrations of ethylene (and ethrel) did not adversely affect the germination or early growth of these seeds. This suggested that the metabolic processes associated with the initial stages of germination are extremely tolerant of a substance which in many other instances promotes senescence (ABELES, 1973).

The temperature range over which ethylene was found to be most effective was narrow but agreed with that established for many other species. Furthermore, this range did not conflict with temperatures which probably occur in the natural habitat of these plants. This seemingly well defined range of optimum temperatures may be related to the requirements of certain enzyme reactions which are necessary to either perceive the ethylene stimulus or bring about the release from dormancy. Also with regard to metabolism, manketti seeds were notably different from many other ethylene sensitive species in that they lacked a carbon dioxide requirement. This may indicate that the trigger

mechanism in this species is somewhat different and that they do not require prior stimulation of the electron transport chain or glyoxalate cycle in order to germinate.

Support for the view that ethylene is the natural germination stimulus came mostly from the experiments carried out on dry seeds and on the retention of the effects of the dormancy breaking treatment under adverse environmental conditions. One of the most striking findings to emerge, was the apparent ability of unimbibed seeds to respond to ethylene. This suggested that additional water was not necessary in order for the seeds to perceive the dormancy breaking stimulus. However, this is difficult to explain in view of the fact that ethylene would normally enter the tissue in solution (unless the seeds already contain sufficient moisture). Manketti seeds also demonstrated the ability to retain the effects of exogenous ethylene even after a period of virtual complete re-dehydration. This indicated that the effects of ethylene were rapidly mediated in the dormant tissues and that these were of a permanent nature. The ecological value of this would be, that once the stimulus has been perceived, the potential to germinate would be retained even after the seeds had undergone a period of desiccation. In addition, unlike *Lactuca sativa* seeds which entered a period of secondary dormancy as a result of water-stressing (BURDETT, 1972), manketti seeds germinated normally after being returned to a moist substrate.

The lack of any autecological studies on *R. rautanenii* seeds

makes it difficult to relate the results obtained with the situation which may exist under natural conditions. Thus, to speculate on the natural course of events, it seems that some form of endocarp scarification must occur before the seeds are able to respond to any dormancy breaking stimuli. This could take the form of degradation by micro-organisms although the reputed partiality that elephants have for these fruits may also play a significant role in this regard. The corrosive acids present in the digestive tracts of these animals may cause considerable breakdown of the endocarp. However, irrespective of how this coat is weakened, the ultimate result would be the increased ability of these seeds to respond to ambient ethylene. The substrates necessary to support the micro-organisms which generate this gas may be present in the form of the abundant mesocarp tissues. Thus, under warm, humid conditions it is possible that considerable quantities of ethylene would be produced, and since these conditions would probably also favour germination, propagation of the species would continue. In addition, should environmental conditions deteriorate immediately after the appearance of the dormancy breaking stimulus all would not be lost, since the seeds are able to retain the effect of the gas even after almost complete dehydration.

In spite of this speculation, the results showed that ethylene is most probably the primary means of overcoming dormancy in manketti seeds. This led to the question as to which aspects of metabolism are affected by the gas and the experiments carried out in this respect are detailed in Chapter 3.

CHAPTER 3

THE EFFECT OF ETHYLENE ON SOME ASPECTS OF THE PHYSIOLOGY AND BIOCHEMISTRY OF DORMANT *RICINODENDRON RAUTANENII* SEEDS

INTRODUCTION

In recent years much has been written on the topic of seed dormancy and germination, both in the form of journal articles and advanced texts. Notable reference works in this field include those compiled or edited by KOZLOWSKI (1972), MAYER and SHAIN (1974), MAYER and POLJAKOFF-MAYBER (1975), KHAN (1977) and BEWLEY and BLACK (1978). The discussion which follows concentrates mainly on those aspects of metabolism which are thought to be in some way related to the actual control of germination.

As mentioned previously, dormancy may be defined as "the failure of otherwise viable seeds to recommence development immediately when supplied with water and oxygen at temperatures recognized as normally favourable for plant growth" (VILLIERS, 1972). Factors which relieve the dormant condition are thought to act on specific sites since germination is probably not controlled by a general depression or activation of the seeds' entire metabolic apparatus (MAYER and SHAIN, 1974). However, the precise nature of these sites and their intracellular location is still something of an enigma.

In this respect, few attempts have been made to determine

whether there are any visible ultrastructural differences between the dry tissues of dormant and quiescent seeds of the same species. Work which has been done in this field has revealed that most dry seed tissues appeared to share a number of common ultrastructural features (VIGEL, 1970; ABDUL-BAKI and BAKER, 1973; BAIRD, LEOPOLD, BRAMLAGE and WEBSTER, 1979). The plasma membrane of such tissues was usually disrupted and, in those areas where it remained intact, numerous inclusions were found to occur between it and the cell wall. Dictyosomes were seldom observed, whereas plastids and mitochondria were commonly visible. A striking feature of these mitochondria was that their internal membrane structures were usually poorly developed, and they often lacked the essential enzymes necessary for phosphorylation (WILSON and BONNER, 1971). Food reserve organelles, such as protein bodies, amyloplasts or spherosomes, were always present and occurred in different ratios depending on the species involved. Preformed ribosomes were also reported in most species examined, and these were usually distributed throughout the scant cytosol present. The nuclei of the dry cells were generally found to be irregular in outline and bounded by a distinct membrane. In addition, large agglomerations of chromatin were frequently found scattered throughout the nucleoplasm. It should be noted, however, that the seeds examined by these authors were not necessarily dormant. In a recent study on *Ilex opaca* Ait. seeds HU, ROGALSKI and WARD (1979) reported that the ultrastructure of the quiescent, rudimentary embryos closely resembled that of the quiescent mature embryos of

many other species. Thus, it appeared that the embryos of seeds experiencing morpho-physiological dormancy (underdeveloped embryos) were not ultrastructurally different from those of non-dormant seeds. More detailed studies on this aspect of dormancy are required, and clearly much would be gained from knowing whether the degree of embryo maturity at the time of maternal abscission is reflected in the elaboration of the organelles.

Another feature of dry seed materials, which was common to most species examined, is their ability to respire, albeit at extremely low levels (BEWLEY and BLACK, 1978). Present reports indicate that this aspect of metabolism is not lacking in dormant species thus indicating that dormancy probably does not result from an inability to produce energy rich compounds. These findings, together with the ultrastructural information, suggest that there are no ultrastructural or metabolic features by which a dry seed could be characterized as dormant.

Under moist conditions, most dry seeds, with the exception of those experiencing hardseededness, imbibe water. This process usually results in the resumption of metabolic activity, culminating in the germination of non-dormant species. Three distinctive phases have been recognized during this period of renewed metabolism (Figure 3.1) and the duration of these varies, depending on the species and on the prevailing environmental conditions. Phase one is simply a rapid but passive uptake of water and is common to all species

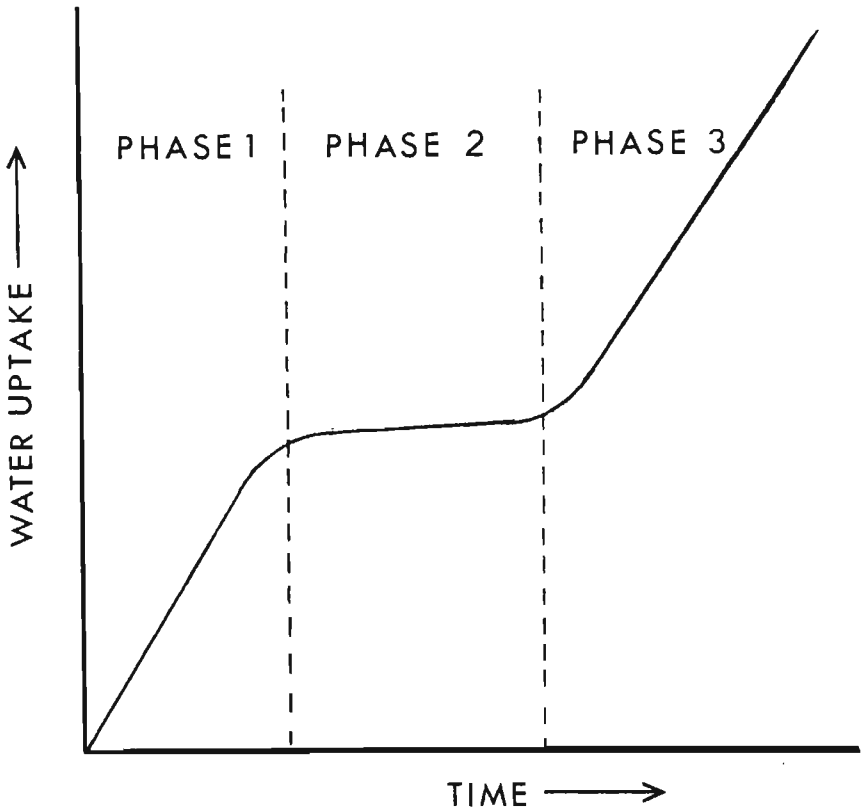


Fig. 3.1 A generalised triphasic water uptake curve typical of many germinating seeds. The duration of each 'Phase' and the amount of water taken up varies between different species.

with permeable seed coats. The rates of this initial water uptake are a function of the type of food reserves present in the storage organs and embryonic axes of the respective seeds. In this regard, it has been found that proteinaceous and fatty seeds appear to imbibe water more rapidly than those seeds which contain starch as their principal food reserve (ALLERUP, 1958). This water uptake results in the rehydration of bicolloids (CHING, 1972) and it is during this period that the "switched-off" genome is reactivated. Another notable feature of phase one is the marked upsurge in respiratory activity, the substrates for which are derived from the hydrolysis of certain food reserves (BEWLEY

and BLACK, 1978).

After a period of time, this initial water uptake and respiratory activity reaches an asymptote in many species and it is this 'lag period' which represents the second phase. Two possibilities have been proposed to account for this phenomenon. The first explanation attributes the lag in respiratory activity to a restriction in the supply of oxygen to the embryo by the testa or surrounding, rehydrated tissues. This was based on the observation that, during this period respiratory quotient values frequently exceeded one, which is usually indicative of anaerobic respiration (SPRAGG and YEMM, 1959). The second explanation, and probably the more likely of the two, is that this lag phase represents a period during which a more efficient respiratory system develops to replace the less efficient system operative during phase one. This would account for the anomoly which is apparent in the first explanation, where the testa is considered freely permeable to oxygen during phase one, whereas this same structure is said to prevent its uptake during phase two (BEWLEY and BLACK, 1978). Not all species exhibit this lag phase. In seeds of *Avena fatua* and *Ricinus communis*, water uptake and respiratory activity were found to increase linearly over time, although the reasons for this were not clear. It has been suggested that in these species, the development of efficient mitochondrial respiratory systems is extremely rapid (BEWLEY and BLACK, 1978).

Dormant seeds do not appear to proceed beyond phase two. This suggests that it is those processes which are responsible for initiating the onset of phase three that are lacking in such seeds. One of the major features of phase three is the protrusion of the radicle through the testa, a process which results most frequently from cell elongation (BEWLEY and BLACK, 1978). In contrast to the relative inactivity of phase two, this phase is marked by a sharp resumption of water uptake and respiratory activity. CHING (1972) suggested that the increased osmotic potential of the radicle noted during this period, resulted from the increased number of small molecules created by the breakdown of the major food reserves. The increased respiratory activity, on the other hand, and its return to an aerobic nature, is probably due to an increase in the availability of oxygen via the disrupted testa. In this respect, other contributing factors may be the appearance of new mitochondria and the increased efficiency of existing ones (BEWLEY and BLACK, 1978).

The events described are those which are most characteristic of the three phases. In addition to these, there are also a large number of other metabolic processes which occur during this period, each of which is a potential control point. Thus, in the discussion which follows, the important metabolic events of the three phases will be considered in more detail. However, before discussing these, it is necessary to first consider the numerous ultrastructural changes which occur during the three phases, in order to gain a visual perspective of these events.

Current literature shows that the ultrastructure of germinating dicotyledonous seeds is well documented. In lipid rich seeds, distinctive features of this process include;

(1) a rehydration of the cytosol and reorganization of the membrane systems;
(2) a reduction in the number of protein bodies and spherosomes and a corresponding increase in the number of vacuoles and vesicles;
(3) the formation of polysomes and the appearance of rough endoplasmic reticulum and dictyosomes;
(4) the repair or synthesis of organelles such as plastids and mitochondria;
(5) the extensive mobilization of reserves in an organized sequence, commencing with protein hydrolysis;
(6) the formation of glyoxysomes from endoplasmic reticulum, an event which marks the time at which all the enzymes of the glyoxalate pathway are operative; and
(7) the rehydration of the nucleus with an associated increase in the size of the nucleolus (VIGEL, 1970; ABDUL-BAKI and BAKER, 1973; BAIRD *et al.*, 1979).

However, as with dry seed material, these changes were observed in non-dormant seeds and few studies have been conducted on rehydrated, dormant seed tissues. In one such study, VILLIERS (1971) demonstrated a clear ultrastructural

distinction between the processes of maturation and dormancy release in *Fraxinus excelsior* L. embryos. The dormancy breaking chilling treatments were found to initiate changes in the nucleii of cells from the embryo. A similar study was carried out on dormant and non-dormant *Setaria lutescens* Hubbard caryopses (ROST, 1972). In this study, it was found that the reserve mobilization and organelle formation observed in the germinating seeds were absent from the re-hydrated, dormant caryopses. Thus, there do not appear to be any fundamental ultrastructural features of imbibed seeds which would characterize them as dormant. Furthermore, there is no evidence to suggest that ultrastructural changes themselves actually control the early stages of germination. On the contrary, it seems more likely that such changes are closely related to (and probably dependent on) changes in the basic metabolism of the seeds. Thus, the suggestion by MAYER and SHAIN (1974) that changes in membranes are one of the control mechanisms in germination, probably refers to the actual metabolism of membrane components and changes in their permeability. There is now sufficient evidence available to suggest that the synthesis of new membranes and changes in the permeability of existing ones are important early events in germination. However, the present data does not permit the conclusion that membrane transformations play a primary role in dormancy release. In this regard, it is interesting to note that the effects of ethylene have, in some cases, been attributed to its effect on secretory phenomena and it appears that this gas can influence the transport of certain enzymes through membranes

(ABELES, 1973).

In earlier studies, serious consideration was given to the possibility that dormancy was imposed by some or other interference with conventional respiration. This view was supported by the fact that the dormancy of many species could be overcome by an increase in the partial pressure of oxygen. However, ROBERTS (1964) reported that common inhibitors of cytochrome oxidase, such as potassium cyanide, sodium azide, carbon monoxide, hydrogen sulphide and hydroxylamine actually promoted germination in some instances, rather than inhibit it. It thus seemed paradoxical that both increased oxygen partial pressures and respiratory inhibitors promoted germination. It has now been postulated that an 'alternative' form of respiration takes place via the pentose phosphate pathway. The current hypothesis is based on that of ROBERTS (1969), which briefly states that within tissues there are at least two respiratory pathways which compete for oxygen. The first is conventional respiration which involves glycolysis, the Krebs cycle and the electron transport chain culminating in cytochrome oxidase. The other pathway involved is the pentose phosphate pathway, the terminal oxidase of which is unknown but which is thought to have a much lower affinity for oxygen than cytochrome oxidase. Thus, any oxygen entering the seed tissue will preferentially be incorporated by cytochrome oxidase and any restriction on oxygen permeability would, therefore, virtually exclude the pentose phosphate pathway from operating. Inhibitors of cytochrome oxidase (such as cyanide, etc.), on

the other hand, would favour the activity of the P.P. pathway as would an increase in the availability of oxygen. However, an alternative explanation for the effects of cyanide and nitrates has been proposed by TAYLORSON and HENDRICKS (1977). These authors proposed that such compounds inhibit the ability of catalase enzymes to decompose hydrogen peroxide (H_2O_2). In dormant seeds, it is thought that this catalase enzyme removes hydrogen peroxide from a pathway in which oxidizing power is passed through quinones to the oxidizing-reducing co-enzyme nicotinamide adenine dinucleotide phosphate (NADP). Thus, inhibition of catalase activity would result in the availability of H_2O_2 for the reduction of NADP. The final result of this would be the stimulation of the pentose phosphate pathway.

The actual role of the pentose phosphate pathway is somewhat enigmatic, but generally it appears to have two main functions in plant tissues. Firstly, it it thought to provide NADPH for various reductive synthetic reactions. (It is interesting to note that a similar function is attributed to nitrates, nitrites and methylene blue, all of which have been found to promote germination) (ROBERTS and SMITH, 1977). The other function of this pathway may be to produce various four and five carbon intermediates which can then be used as structural components in various synthetic processes (ROBERTS and SMITH, 1977). The synthesis of pentose sugars by this pathway could be important in terms of nucleic acid synthesis (and thus, cell division) since this aspect of metabolism relies on a constant supply

of these sugars.

It was recently suggested that the pentose phosphate pathway is very likely one of the capabilities depressed or missing in dormant seeds, and that its operation is essential for the loss of dormancy (JANN and AMEN, 1977). This is supported by the fact that in *Avena fatua* seeds the levels of two pentose phosphate enzymes, glucose-6-dehydrogenase and 6-phosphogluconate dehydrogenase, increased in imbibed quiescent seeds but not in the imbibed dormant ones (KAVACO and SIMPSON, 1976). In addition, it now seems that the dormancy status of *Arachis hypogaea* seeds can be determined simply by measuring the relative activity of these two enzymes (SWAMY, UMPATHI and REDDY, 1980). With regard to the present study, it is interesting to note that there is some indirect evidence to suggest that ethylene may influence this pathway. This was obtained from work carried out on *Xanthium pensylvanicum* seeds in which it was found that ethylene stimulated respiratory activity prior to radicle emergence. This was further supported by the fact that inhibitors of cytochrome oxidase and alternative respiration also promoted the germination of this species (ESASHI, WAKABAYASHI, TSUKADA and SATOH, 1979). However, not all ethylene-sensitive species exhibit this response, and a recent study on *Spergula arvensis* seeds showed that ethylene did not initiate a switch from conventional to alternative respiration (JONES and HALL, 1981).

The proposed control of the pentose phosphate pathway would

provide an ideal unifying concept for dormancy. However, BEWLEY (1979) has stated that this model for dormancy cannot have universal application at this stage for the following reasons;

(1) it has never actually been demonstrated that the enzyme systems reputed to be involved in this model are linked in the manner described;

(2) no shifts in the C_6/C_1 ratios were observed when lettuce seed dormancy was broken by light, gibberellins or mercaptoethanol (where the C_6/C_1 ratio is taken to be indicative of which pathway is operative, and which is obtained by feeding tissues either glucose-1-^{14}C or glucose-6-^{14}C and then measuring the relative amounts of $^{14}CO_2$ released);

(3) it is only assumed that NADPH oxidation is essential for dormancy breaking;

(4) the source of hydrogen peroxide for endogenous activity prior to visible germination has not yet been confirmed and;

(5) more work is required to determine the timing of catalase inhibition (an apparent prerequisite for the commencement of the pentose phosphate pathway) since much of the data currently available reflects measurements taken after the seeds have already germinated.

In spite of these drawbacks, however, this model still holds

considerable potential for providing a unified concept of seed dormancy in the future.

The concept that interference with normal respiration is a possible dormancy mechanism, has not been ignored entirely. CHING and CHING (1972), suggested that germination may be controlled by the availability of adenosine phosphate levels. In this regard, it is worth noting that ethylene has been shown to increase the hydrolysis of ATP in certain instances (OLSON and SPENCER, 1968). It was thus suggested that, although ethylene may not affect normal respiration *per se*, it may indirectly affect this process by stimulating ATPase activity (KETRING, 1977).

Another indirect means of influencing respiratory activity, and hence germination, would be via the control of reserve hydrolysis. Reserve materials within seeds can usually be divided into two major categories; those which are present in the seed and are available for immediate use; and those which are stored in an insoluble form in the endosperm (and axis tissues), and which require hydrolysis and transport (MACLEOD, 1969). Respiration during the early stages of germination would thus be maintained by the rapid utilization of small amounts of mono-, di- and trisaccharides and any control over the hydrolysis of these compounds would effect germination (MAYER, 1977). An example of this may be found in *Avena fatua* where non-dormant seeds readily convert maltose to sucrose, whereas this does not occur in the dormant ones (CHEN and WARNER, 1969). Furthermore,

reports that the dormant, excised embryos of some species such as *Calliopsis basalis* Dietr. (SHARMA, 1979) will germinate on nutrient culture media, suggests the presence of deficient metabolic pathways. In such instances, the embryos may be unable to produce vital intermediates *in vivo*, thereby preventing germination. Other possible substrates for initial respiration are the stored amino acids and hexoses derived from storage proteins. As yet, however, there are no reports of any control being exerted over these particular pathways.

The role of hormones in controlling these early catabolic reactions is fairly well understood in certain species. Work carried out on barley (*Hordeum vulgare* L.) seeds demonstrated that the embryo controlled germination by producing gibberellic acid (RADLEY, 1969). This hormone, it was found, is transported via the scutellum to the aleurone layer surrounding the endosperm where it stimulates the production of hydrolytic enzymes. These enzymes, such as α-amylase, are then responsible for the digestion of the starch reserves (BRIGGS, 1972). Ethylene has also been shown to influence the production of α-amylase in barley seeds (NORD and WEICHHERZ, 1929), although this could not be repeated in a more recent study by SCOTT and LEOPOLD (1967). This subject is much favoured by researchers and further evidence showing the intimate involvement of phytohormones in the mobilization of food reserves is accumulating rapidly.

Most of the insoluble food reserves are usually only

mobilized once the 'lag phase' has been reached. One such reserve is lipid and its hydrolysis commences with the activity of lipase enzymes (CHING, 1972). These enzymes are present in the dry seed tissues and,once rehydrated, split the lipid into its fatty acid and glycerol components (BEWLEY and BLACK, 1978). These fatty acids are then acted upon by enzymes of the glyoxalic acid cycle located in the mitochondria and glyoxysomes (CHING, 1972). Any control over germination by this pathway would probably involve the integration of the component reactions and the intracellular communication of reaction products between the organelles involved. Studies on *Corylus avellana* cotyledons have demonstrated that during after-ripening, sucrose levels increased as a result of lipid hydrolysis (BRADBEER and COLEMAN, 1967). This suggested that one effect of the dormancy breaking treatment may have been a stimulation of the glyoxalate pathway. In addition, the activity of isocitrate lyase, a key enzyme in the glyoxalate pathway, was found to increase rapidly in after-ripening *Pinus ponderosa* Laws seeds prior to germination (CHING, 1970). However, lipid breakdown may also occur in the absence of any dormancy breaking treatments. VILLIERS (1971) reported that during the maturation of *Fraxinus excelsior* seeds, the amount of extractable lipid declined from 20 to 4 per cent. In spite of this, the seeds remained dormant. Thus, there is still insufficient evidence to invoke the control of lipid mobilization as a means of controlling dormancy. With regard to the lipid-rich manketti seeds, it is worth noting the suggestion by ABELES (1973), that ethylene may influence

lipase activity.

A food reserve which is frequently found in association with lipids are the stored proteins. During germination, these compounds are hydrolyzed by the action of proteinase enzymes into their constituent amino acid components (CHING, 1972). These amino acids are then used for the synthesis of new proteins or provide energy by oxidation of the carbon skeleton. The proteinase enzymes responsible for this are present in the dry seeds and are reactivated during imbibition (BEWLEY and BLACK, 1978). The only reported species in which protein degradation is in some way controlled is in lettuce (*Lactuca sativa*) seeds. In this species, one of the proteolytic enzymes was found to interact with an endogenous trypsin inhibitor (SHAIN and MAYER, 1965). These authors suggested that this then controlled the onset of activity by the autocatalysis of a trypsin-like enzyme. This view, however, deserves re-evaluation since, in a more recent study, the protease inhibitor in rice seeds did not change during the early stages of germination (HORIGUCHI and KITAGISHI, 1971). Nevertheless, in general terms, proteolytic enzymes may be important in the release or activation of bound, particulate or masked enzymes during germination (MAYER and SHAIN, 1974). Furthermore, there are also a number of reports where the onset of proteolytic enzyme activity is under phytohormone control (WILEY and ASHTON, 1967; JACOBSEN and VARNER, 1967).

A frequent inclusion in protein bodies, are the globoids

which constitute the major storage form of phosphates and other macronutrients in seeds (MAYER and SHAIN, 1974). Dormancy breaking appears to be closely linked to phosphate metabolism in that an increased supply of available energy to the growing centres of the embryo is essential. To support this, OLNEY and POLLOCK (1960) found that inorganic phosphates accumulated in dormant cherry seeds, whereas in the chilled embryos of this species, phosphate (phytin) accumulation was in the form of sugar phosphates and nucleotides. From this study, it was tentatively concluded that the dormant condition was closely associated with a block in phosphate metabolism. Thus, it is possible that in some instances, dormancy may result from an interference with the metabolism of phosphate-rich compounds, although it should be borne in mind that phytin activity is not an early event in the germination sequence and in some cases may actually lag behind germination itself (GUARDIOLA and SUTCLIFFE, 1971).

Numerous studies have now shown that dormant, imbibed seeds are metabolically active and BEWLEY (1979) is of the opinion that, in general terms, the breaking of dormancy is probably not mediated via the promotion of reserve hydrolysis. Dormancy mechanisms are, therefore, more likely to operate at the level of enzyme activation, protein and nucleic acid synthesis, growth regulators and gene activation. This does not imply, however, that there is no value in examining the levels of various metabolic substrates at different times during dormancy release, since such studies often

provide valuable clues as to the site of the metabolic 'block'.

The emergence of the radicle at the onset of the third phase is, in many cases, the result of cell elongation and it appears that cell division plays only a limited role in this process (BEWLEY and BLACK, 1978). Nevertheless, it is now widely accepted that protein synthesis is an essential pre-requisite for this radicle emergence. Thus, a convenient control of dormancy would be the repression or de-repression of DNA or an activation of the RNA, associated with the process of protein synthesis. In many species, all the necessary equipment for protein synthesis is present in the dry seeds. However, synthesis itself only commences once the cells are sufficiently hydrated to allow preformed cytoplasmic ribosomes to associate with the long-lived messenger (m)RNA (MAYER and SHAIN, 1974; BEWLEY and BLACK, 1978). One of the most disputed issues concerning protein synthesis in newly imbibed seeds, is whether the mRNA conserved in dry embryos is sufficient to code for all the proteins synthesized *de novo* prior to germination, or whether new mRNA must first be synthesized. The fact that some seeds will germinate in the presence of an inhibitor of RNA synthesis is often used to substantiate the claim that mRNA synthesis is not required for germination.

Protein synthesis has also been reported to occur in dormant seeds. REGER, EGLEY and SWANSON (1975) noted the presence of polysomes in the imbibed but dormant seeds of purslane

(*Portulaca oleracea*). This, in their opinion, suggested the initiation of the protein synthesizing complex. Actual measurements of protein synthesis in dormant seeds of *Avena fatua* (CHEN and VARNER, 1970) and *Agrostemma githago* L. (DE KLERK and LINSKENS, 1979) revealed that considerable activity took place prior to dormancy release. In a similar study on the dormant embryonic axes of *Xanthium pensylvanicum* seeds, SATOH and ESASHI (1979) found that these embryos initially synthesized more protein than the non-dormant, after-ripened ones. These data suggest, then, that the preformed mRNA fractions present in the dry embryos serve primarily to allow a rapid resumption of growth upon re-hydration. However, they do not function to regulate early development (BROOKER, CHEUNG and MARCUS, 1977). It therefore seems more likely that dormancy results from some interference with the synthesis of new RNA and the related proteins.

In this regard, KHAN (1966) suggested that protein synthesis itself was not involved in the release of *Xanthium* seed dormancy and instead attributed it to a reversal of hormone induced inhibition of mRNA synthesis. However, a somewhat different mechanism was thought to exist in dormant pear embryos. In this species, it was found that the capacity to synthesize nucleic acids increased during cold treatments. At the same time, the concentration of a certain hybrid RNA-DNA molecule was also found to increase. On the basis of this, KHAN, HEIT and LIPPOLD (1968) suggested that although the general increase was probably of some significance,

it was the increase in the hybrid molecule which may be the important factor in dormancy release. Yet another mechanism has been observed by EPHRON, EVENARI and DE GROST (1971). Their work on dormant and stimulated lettuce seeds revealed the existence of differing mRNA synthesizing abilities. They found that dormancy breaking light treatments resulted in the increased incorporation of (^{14}C)-phe-tRNA into polypeptides. Thus, no single underlying mechanism has been located and numerous studies in this field have failed to provide any evidence to suggest that germination is controlled by a lack in the availability of ribosomes, tRNA, amino acids or synthetase enzymes (BEWLEY and BLACK, 1978).

OSBORNE (1981) summarized the major early and late synthetic activities occurring during the imbibition of isolated dormant and non-dormant embryos. This summary is reproduced in Table 3.1. It is apparent from this table that both dormant and non-dormant embryos achieve the early stages of germination equally well. In addition, biochemical studies on the changes in lipid and protein levels during the early stages of renewed growth, as well as the synthesis of various types of RNA, have so far failed to reveal any major differences between seeds in the dormant and non-dormant condition. This ultimately leads to the question of the importance of DNA in the overall dormancy breaking process.

An increase in the DNA template availability was suggested

Table 3.1 Early and late synthetic activities of isolated non-dormant (ND) and dormant (D) embryos on imbibition of water.

	ND	D
(a) Early syntheses		
Proteins	+	+
Phospholipids	+	+
RNA classes		
25 S	+	+
18 S	+	+
5 S	+	+
4 S	+	+
Poly A-rich	+	+
DNA repair	+	+ (?)
(b) Late syntheses		
DNA replication	+	-

as the cause of the rise in RNA synthesis in *Corylus avellana* embryos during dormancy breaking (JARVIS, FRANKLAND and CHERRY, 1968). More recently, DNA levels themselves were found to increase markedly during the emergence of *Agrostemma githago* radicles, whereas only low levels of DNA synthesis were recorded in the dormant embryos (HECKER and HUTH, 1975). However, it seems that the involvement of DNA may vary between species and in lettuce seeds, germination still took place in spite of the DNA complement being disrupted by ionizing radiation (HABER and LUIPPOLD, 1960). Furthermore, the observation that radicle protrusion in many species is the result of cell expansion only, would

tend to exclude the involvement of DNA replication. Thus, more research is required in order to establish the precise role of DNA in the dormancy breaking process.

It is now becoming increasingly evident that plant growth regulators play a predominant role in dormancy mechanisms. These compounds are thought to modulate specific environmental cues into biochemical messages. In this way, they may act as switching agents, whereby one physiological state is switched to another by either differential transcription (i.e. gene repression or depression), activation of translation or even by somehow altering membrane permeability. Dormant embryos will not, therefore, transcribe any new genetic information until the hormonal germination agents are supplied either exogenously or endogenously in response to some or other environmental trigger (JANN and AMEN, 1977).

Abscisic acid (ABA) is one of the phytohormones considered important in the phenomenon of dormancy. This is based on the fact that not only is it a potent inhibitor of seed germination when applied exogenously, but it is also a notable endogenous component of many dormant seeds. In developing seeds, it has been suggested that this compound may be imported from the parent tissues, and its purpose would be to prevent premature germination (WALTON, 1981). In mature seeds, the hormone may be located in the covering structures, as in the case of *Corylus avellana* seeds, and this may impose dormancy by its continual movement into the embryo (JARVIS, 1975). Reductions in the levels of this

inhibitor as a result of dormancy breaking treatments have been recorded in some species. In *Acer saccharum* seeds, for example, it was found that after-ripening treatments caused a 98 per cent reduction in ABA levels, whereas only a 37 per cent decline was noted in the untreated control seeds (WEBB, VAN STADEN and WAREING, 1974). However, this is not a universal phenomenon and in *Lactuca sativa* seeds no correlation existed between dormancy and ABA levels (BRAUN and KHAN, 1975).

The observation that many of the effects of abscisic acid are reversible has led to the suggestion that these are simply transient effects, and that the compound is rapidly inactivated. Such inactivation could occur by sequestration into cellular compartments which would mean that in order to obtain a given physiological response, ABA synthesis would take place continuously (WALTON, 1977). Another means of inactivation would be via the metabolism of this compound into phasic acid and it seems that in some species, it is unlikely that dormancy could be attributed to a block in this pathway. For example, it has been shown that both dormant and after-ripened ash seeds were equally adept at performing this conversion (SONDHEIMER, GALSON, TINELLI and WALTON, 1974). The additional possibility that dormant and non-dormant seeds have differing abilities to form less active glucose esters of ABA has received only scant attention and deserves further research.

The level at which this hormone acts is not clear, but it

has been shown to inhibit a variety of gibberellic acid mediated responses. Such inhibition can frequently be reversed by the reapplication of gibberellins suggesting that an inverse relationship may exist between abscisic acid and gibberellins during dormancy breaking. Gibberellins, in fact, are now thought to play an almost universal role in seed germination, since applied gibberellins stimulate the germination of many different species. The results presented in Chapter 1 clearly indicate that *Ricinodendron rautanenii* is no exception in this regard. However, gibberellins may have a different importance in quiescent seeds from that in dormant ones. In both types, gibberellins are probably necessary for the later stages of germination, whereas it is quite possible that they are only critical in the earlier phases of dormant seed germination if the embryos lack key enzymes or adequate nutrients (JANN and AMEN, 1977). One of the most remarkable features of this hormone is the fact that it is effective on such a wide range of dormancy mechanisms including incomplete embryo growth, mechanically resistant seed coats, high inhibitor levels and physiologically incompetent embryos (JONES and STODDART, 1977).

One of the best known effects of gibberellic acid is its ability to substitute for the chilling requirements of some seeds. In *Corylus avellana* seeds, for example, dormancy is induced by dry after-ripening and can be relieved by low temperature treatments or with exogenous gibberellin applications (BRADBEER and PINFIELD, 1967). Dry storage

appears to have its effect on the tissues' ability to synthesize gibberellins since the levels of extractable gibberellin actually declined during this treatment (ROSS and BRADBEER, 1971). The most interesting finding, however, was that dormancy breaking chilling treatments did not raise the endogenous gibberellin levels. It was only when the seeds were transferred from 5° to 30°C that dramatic changes in the levels of GA_1 and GA_9 were observed. This suggested that the low temperature treatments only served to activate the gibberellin producing mechanism and that actual synthesis only occurred at the elevated temperatures (WILLIAMS, BRADBEER, GASKIN and MACMILLAN, 1974). However, this is not the only mechanism which exists and in certain apple (*Pyrus malus* L.) cultivars, spectacular changes occurred in the gibberellin levels during the actual cold after-ripening treatment (BIANCO and BULARD, 1980). A similar increase in gibberellin levels was observed during the cold after-ripening of *Tillia platyphyllos* Scop. seeds and this was attributed to a release from the less active bound forms rather than the synthesis of new ones (NAGY, 1980).

The dry after-ripening requirement of some species can also be replaced by exogenous gibberellins and this phenomenon is remarkably similar to the cold after-ripening requirement just described. For example, it has been shown that dry after-ripened *Avena fatua* seeds have an increased ability to synthesize gibberellin-like substances when placed under optimum germination conditions (SIMPSON, 1965). In

addition, this group of phytohormones has also been shown to replace the red light requirements of several species exhibiting photodormancy. The germination of both positively photoblastic (e.g. *Lactuca sativa*) and negatively photoblastic (e.g. *Phacelia tanacitifolia* Benth.) seeds has been found to be stimulated by gibberellins under conditions not normally conducive to germination (JONES and STODDART, 1977). This apparent close relationship between phytochrome and gibberellins at one time led to the suggestion that red light functioned by increasing the endogenous levels of gibberellins. However, P_{FR} may be active within five minutes of its formation which is too short a period of time for *de novo* gibberellin synthesis (BEWLEY, NEGBY and BLACK, 1968). In addition, no seeds appear to be solely dependent on exogenous gibberellins to induce germination, whereas many seeds have very strict P_{FR} requirements. Hence, P_{FR} appears to be the more primitive element of control and may act as a gibberellin-releasing factor from microbodies (TAYLORSON and HENDRICKS, 1977).

In view of the effectiveness of gibberellins on so many diverse dormancy mechanisms, it seems reasonable to suspect that a single underlying mode of action exists for this hormone. Stimulation of the pentose phosphate pathway could, in fact, represent such a universal mechanism. SIMMONDS and SIMPSON (1971) proposed that gibberellins stimulated the germination of dormant *Avena fatua* seeds by causing a shift in glucose metabolism. The result of this was the increased participation of the pentose phosphate

pathway. Earlier in this discussion it was mentioned that the pentose phosphate pathway could also be stimulated by nitrates, nitrites and hydroxylamine since they made H_2O_2 available for NADPH oxidation. A likely source of peroxide would be the glyoxalate cycle and gibberellins have been shown to stimulate the activity of certain key enzymes of this pathway in dormant almond seeds (HAWKER and BUNGEY, 1976). Thus, by controlling the glyoxalate cycle, gibberellins may indirectly control the pentose phosphate pathway.

Gibberellins have also been shown to be lipophylic compounds and therefore have an affinity for membranes. Glucosidic gibberellin derivatives, on the other hand, are more hydrophilic and have reduced biological activity (JONES and STODDART, 1977). It is therefore possible that gibberellins exert their effect on germination by altering membrane permeability. This aspect deserves more serious consideration in future, since by bringing about changes in the plasmalemma, this hormone could control the import and export of the materials required for growth.

It is not clear what role RNA plays in mediating the effects of gibberellin acid. In after-ripened *Corylus avellana* seeds, newly synthesized gibberellins, together with another unidentified factor appeared to act on one or more sites of the DNA equivalent of an operator gene. Repression of this DNA equivalent was thereby overcome, thus enabling the transcription of a number of 'structural' genes. The re-

sult of this would probably be the derepression of an operator gene and hence the onset of mRNA synthesis (JARVIS, FRANKLAND and CHERRY, 1968). By regulating the synthesis of new mRNA in this manner, gibberellins could determine the quality and quantity of proteins produced.

It was mentioned earlier that gibberellin mediated responses could frequently be inhibited by abscisic acid. However, the converse of this is not always true. Cytokinins are in many cases essential for the completion of gibberellin induced germinative or enzymatic processes, especially in those instances where these have been blocked by inhibitors. It has in fact been found that even excessive quantities of gibberellin could not substitute for this cytokinin requirement (KHAN, 1971; THOMAS, 1977). Results from studies on the endogenous levels of cytokinins and abscisic acid during dormancy release have highlighted the importance of cytokinins in this phenomenon, and provide evidence to support the idea that these two compounds interact. Abscisic acid levels were found to decline during the afterripening of *Acer saccharum* seeds, whereas the cytokinin levels increased during the same period and reached a peak after 20 days (WEBB, VAN STADEN and WAREING, 1973). Furthermore, studies on other aspects of biochemistry have revealed that these two hormones invariably have opposite effects, although both appear to act at the level of translation and transcription (THOMAS, 1977).

It is also possible that some effect of cytokinin other than

its antagonism of ABA may be influencing the germination of numerous species including *Acer saccharum*. THOMAS (1977) suggested that the seemingly unrelated effects of cytokinins on growth may be due to their effects on the function of proteins. Alternatively, cytokinins may also effect membrane permeability (MILLER, 1956). THOMAS, PALEVITCH, BIDDINGTON and AUSTIN (1975) reported that compounds which were known to effect membrane permeability also simulated the effects of a cytokinin-gibberellin mixture on seed germination. Thus, the cytokinin-inhibitor interaction described earlier could be a mechanism to control membrane permeability. In this manner, cytokinins could, in fact, regulate the actual movement of gibberellins (THOMAS, 1977). The mechanism envisaged is that the gibberellins essential for germination are either locked within or out of certain compartments occurring at specific locations in the seed tissues. By their effect on membrane permeability, cytokinins could then control the movement of these gibberellins between the various compartments.

The finding that cytokinins could frequently reverse the inhibition of germination induced by natural inhibitors, provided a useful conceptual tool from which a working model for the hormonal control of dormancy and germination was developed (KHAN, 1971). A summary of this model is presented in Figure 3.2, and in it, gibberellins, inhibitors and cytokinins have been assigned primary, preventative and permissive roles respectively. This is based on the fact that cytokinins appear to compete with ABA for common

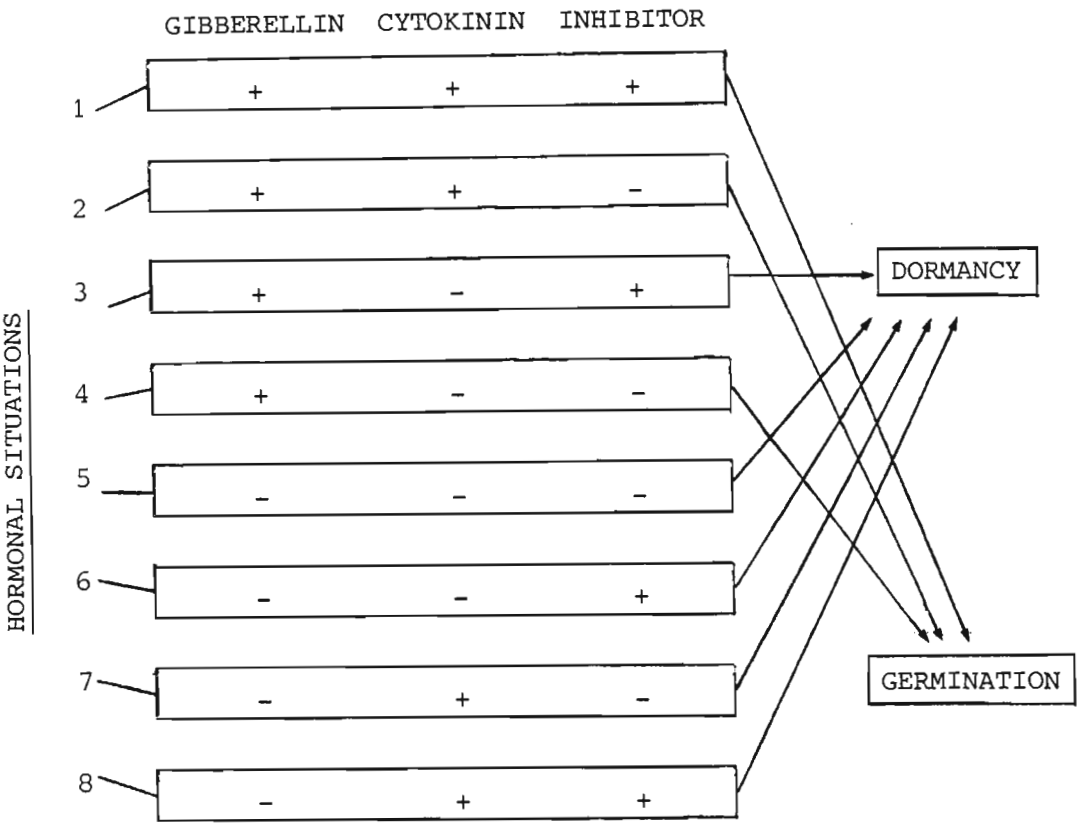


Fig. 3.2 A model of dormancy and germination showing selective functions of hormones. Physiologically effective or ineffective levels, which could be many, are designated here as + or - for convenience. Depending on the varying concentrations of hormones (from seed to seed and even in a single seed) there would be many hormonal situations (eight for convenience) leading to varying depths of dormancy or degrees of germination. The model underscores the selective nature of hormone action (taken from KHAN, 1977).

sites of action, whereas a non-competitive interaction exists between ABA and gibberellins (KHAN, 1971). This model illustrated how it was possible that dormancy and germination could be determined in one of several ways and the arrangement of these hormones into the eight combinations shown was based on their physiologically effective or ineffective levels. The model also explained, for the first time, some of the distinctly anomalous situations such as growth or germination in the presence of inhibitors (situation number 1) or dormancy in the absence of inhibitors (situations 5 and 7).

One of the unique features of this model was that it was derived from the demonstrated abilities of hormones to function selectively in seed processes. As a consequence of this, it initially seemed that the model would not be able to account for those situations where dormancy breaking was associated with phasic changes in hormone levels as in the case of *Acer saccharum* (WEBB, VAN STADEN and WAREING, 1973). However, the view that discreet interactions may occur between these hormones during the phasic changes (KHAN, 1977) makes it possible to accommodate those species exhibiting this phenomenon. By implication then, seeds of the same plant may switch from one hormonal situation to another as the environmental conditions change.

KHAN'S model for germination is appealing, but does not yet take full account of the two other major growth regulators, auxins and ethylene. This situation is no fault of the model itself but results from the current gaps in our understanding of the role of these substances. For example, the role of auxins in the phenomenon of seed dormancy has long been in dispute. Recent studies on *Hyoscyamus muticus* L. seeds showed that auxin (IAA) levels increased under conditions promoting germination (ELKINAWY and HEMBERG, 1974). However, NIKOLAEVA (1975) reported that, in general, IAA levels decreased during the germination of deeply dormant seeds. MAYER and POLJAKOFF-MAYBER (1975) have summarized the available information and stated that auxins will only stimulate germination under very special conditions. Clearly, the involvement

of auxin in seed dormancy and germination needs to be re-assessed.

The involvement of ethylene in seed dormancy has been well documented and this was discussed in considerable depth in Chapter 2. However, this hormone's interaction with other growth regulators and the level at which it operates in seed dormancy was deliberately omitted from that discussion and will be considered here. Auxins have been shown to regulate endogenous ethylene production in various tissues (ABELES, 1973). This phenomenon has, in fact, been used on a number of occasions, to account for various auxin induced growth responses. Such an interaction would appear to present itself as an ideal means for controlling germination, particularly in view of the fact that ethrel was found to increase the endogenous auxin levels in *Prunus salicina* Lindl. seeds (DOMOTO and HEWITT, 1973). However, ethylene sensitive peanut seeds (*Arachis hypogaea*) showed no response to exogenous auxin applications (KETRING and MORGAN, 1970) and there is in fact no evidence to support the existence of such a mechanism.

Cytokinins, on the other hand, do exhibit a positive interaction with ethylene during seed germination. They were found to stimulate both the germination of, and the endogenous ethylene production by, dormant *Arachis hypogaea* seeds (KETRING and MORGAN, 1972). However, cytokinins could not replace the ethylene (or light) requirements of dormant *Spergula arvensis* seeds, although this gas was

found to increase the endogenous cytokinin levels during dormancy breaking (VAN STADEN, OLATOYE and HALL, 1973).

The effects of gibberellins on ethylene sensitive phenomena are variable (ABELES, 1973). This hormone was reported to have no effect on the endogenous production of ethylene in rape seeds (TAKAYANAGI and HARRINGTON, 1971), whereas in peanut seeds (*Arachis hypogaea*) some stimulation, albeit slight, was noted. In contrast, there are no reports of ethylene-stimulated gibberellic acid production during dormancy breaking.

The observation that ethylene may overcome the inhibition of germination imposed by abscisic acid and other inhibitors in both dormant and non-dormant seeds has also led to the suggestion that it is involved in the promotor-inhibitor system (KETRING, 1977). This is supported by the fact that ethylene was found to overcome ABA induced inhibition of RNA and protein synthesis in dormant *Arachis hypogaea* seeds (KETRING, 1975). This also suggests that ethylene may operate at the gene level and it may be significant in this regard that ethylene was also found to stimulate RNA synthesis in various leaf and fruit tissues (ABELES, 1973). On the other hand, the possibility that ethylene may effect DNA metabolism remains a moot point (LESHEM, 1973). Thus, the basic question as to how the effects of ethylene are mediated in dormant seeds remains unresolved.

This general discussion has highlighted the fact that, al-

though much is known about the biochemistry and physiology of germinating seeds, details regarding the differences between dormant and non-dormant seeds are lacking. There also seems to be a general paucity of information regarding the actual level at which dormancy mechanisms operate, although this would not be from a lack of effort on the part of researchers in this field. The improved techniques available for extraction and detection of certain substances will, no doubt, assist in rectifying this situation. In addition, much can still be gained from studying the changes in respirable and structural substrates during dormancy release since these must surely provide valuable information as to the relative activity of the various metabolic pathways. Furthermore, the role of plant growth regulators in the control of seed dormancy is still not clearly understood and there is still much to be learnt regarding the level at which these compounds operate. At present, it appears that the role of ethylene, in particular, is least understood, although this situation is changing rapidly. This final Chapter details the experiments carried out to determine the effects of this gas on the ultrastructure and physiology of *R. rautanenii* seeds. From this it was hoped that some contribution could be made to the current understanding of the role of ethylene in seed dormancy and germination.

MATERIALS AND METHODS

1.0 General

In this series of experiments, an attempt was made to determine the effect of ethylene on the physiology of *Ricinodendron rautanenii* seeds during dormancy release. Treatments were applied to seeds from the 1978 harvest which had been stored at 5°C. In all instances the seeds were incubated at 30°C and each experiment was repeated at least once. As in previous experiments, the incubators used were accurate to within $\pm$ 0,5°C and all chemicals were reagent grade.

For the biochemical and ultrastructural studies, the effects of two separate treatments were examined and the methods used were as follows:

(1) In the first treatment dry, dormant seeds were incubated on vermiculite moistened with an ethrel solution having an effective ethylene concentration of 200 microlitres per litre. From these, samples of 60 seeds (3 x 20 sub-samples) were taken at 24 hourly intervals. Each sample was dissected into its embryonic axis, cotyledonary and endosperm components and their fresh mass determined. A small sub-sample of each component was then dried for 24 hours at 100°C in a ventilated oven for estimation of the dry mass. At the same time a small sample

was also prepared for electron microscopy. The remaining tissues were frozen in liquid air, freeze dried and stored in sealed containers at -20°C until required for analysis.

(2) In the second treatment, gaseous ethylene was used to bring about release from dormancy. Seeds were placed on moist vermiculite and imbibed under ethylene free conditions for 48 hours. After removing a sample of 60 seeds, the remaining seeds were subjected to a 12 hour ethylene treatment. This was achieved by flushing the vessels containing the seeds with 99,8 per cent pure ethylene for one minute. The vessels were then sealed and the flushing procedure was repeated at three hourly intervals to ensure a saturated atmosphere. After the gas treatment, the seeds were transferred to ethylene-free conditions and left to germinate. During this experiment samples were removed after, (a) the 48 hour imbibition period, (b) the 12 hour gasing treatment and, (c) at subsequent intervals of 24 hours. The experiment was terminated four days after the gasing treatment. As in the ethrel experiment, the seeds were separated into their embryonic axis, cotyledonary and endosperm components and their fresh mass determined. Dry mass determination and tissue preparation for electron microscopy and storage were carried out in the same manner as previously described.

In the discussion which follows, these two treatments will

simply be described as either the '*ethrel treatment*' or '*gas treatment*'.

In those experiments where the effects of various compounds on the ethylene response were examined, use was made of the '*gas treatment*' method. In these experiments the compounds were applied either throughout the entire experimental period or during either the imbibition or germination phases. The effects of these compounds on seedling growth was also examined. This was achieved by terminating the experiment seven days after the gasing treatments and then determining the fresh and dry mass values of the respective root, shoot and endosperm tissues.

2.0 Microscopy techniques

At each of the sampling periods mentioned (both treatments), three millimetre squares of endosperm and cotyledonary tissue were removed from the regions shown in Figure 3.3 In addition, the first three millimetres of the embryonic root were also removed for observation. These tissues were fixed at 4°C in 6 per cent glutaraldehyde, buffered at pH 7,2 with 0,05 Molar sodium cacodylate. After 24 hours the glutaraldehyde was removed and the fixed material was given three 30 minute washes in 0,05 Molar sodium cacodylate buffer. The tissues were then post-fixed for two hours in 2 per cent osmium tetroxide also buffered with 0,05 Molar sodium cacodylate. This was followed by a further three 30 minute washes in the buffer alone in order to remove all

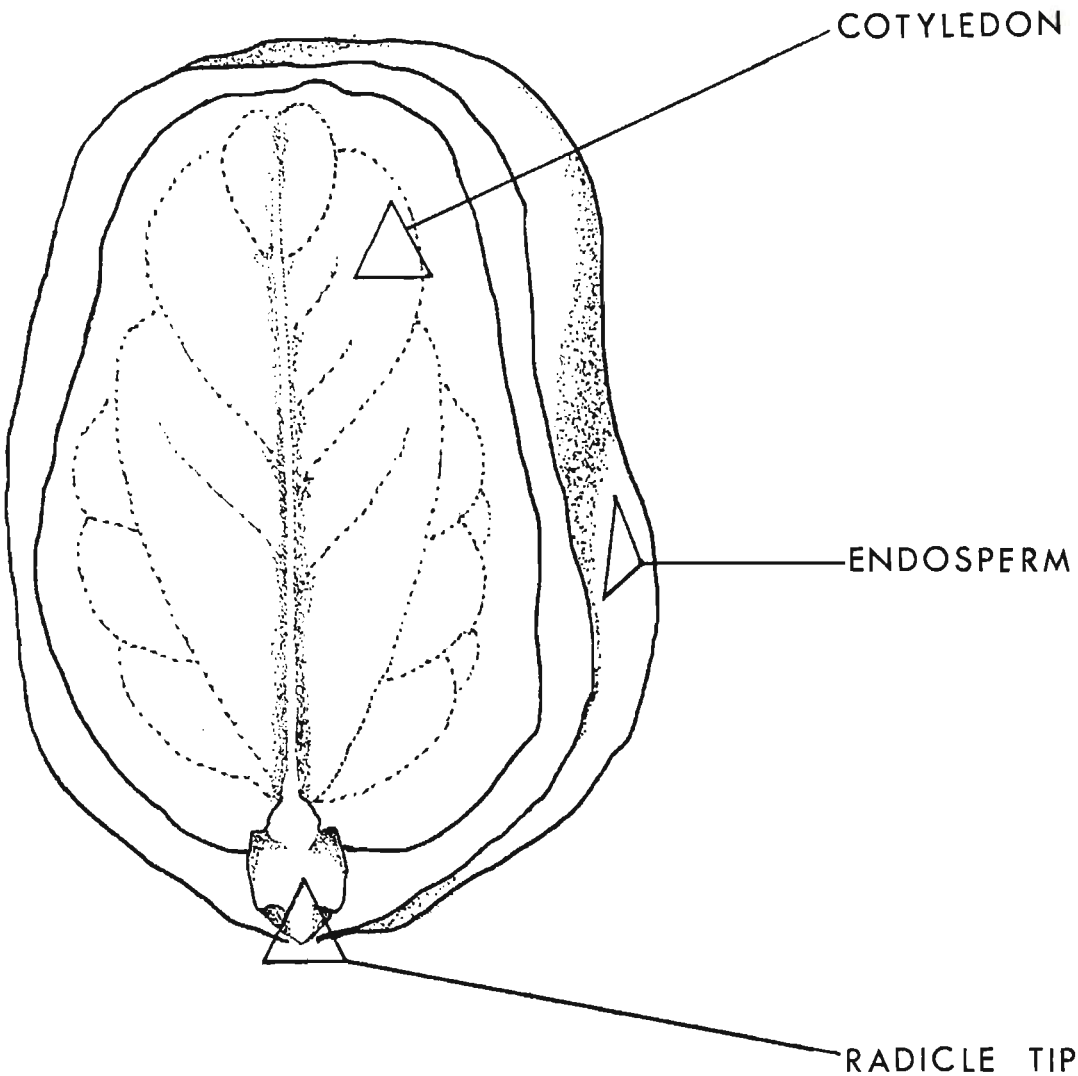


Fig. 3.3 Regions of *Ricinodendron rautanenii* seeds from which samples were removed for electron microscopy.

traces of the osmium tetroxide. The tissues were dehydrated in an alcohol series followed by a double wash in pure propylene oxide. Impregnation of the tissue with Epon-Araldite resin was achieved by adding increasing amounts of the resin to subsequent propylene oxide washes. For final impregnation, the tissues were placed in pure resin and subjected to a mild vacuum (400 torr) for 12 hours. Polymerization lasted 48 hours at 70°C. Sections for both light and electron microscopy were cut with glass and diamond knives respectively. The monitor sections cut for light microscopy were stained using 1 per cent toluidine blue in 1 per cent borax:1 per cent pyronin Y (1:1 v/v). The sections of electron microscopy were stained with uranyl acetate and lead citrate as described by REYNOLDS (1963). These sections were examined with a Jeol 100 CX electron microscope at an accelerating voltage of 80 kV and photographed.

3.0 Biochemical analyses I. Food reserves

Determination of food reserve levels was conducted on the finely ground, freeze-dried samples from both ethrel and gas treated tissues. The sequence followed is depicted in Figure 3.4

3.1 Extraction and identification of lipids

Lipids were extracted from the component seed tissues using a method adapted from MEARA (1955). Ten grammes of freeze-

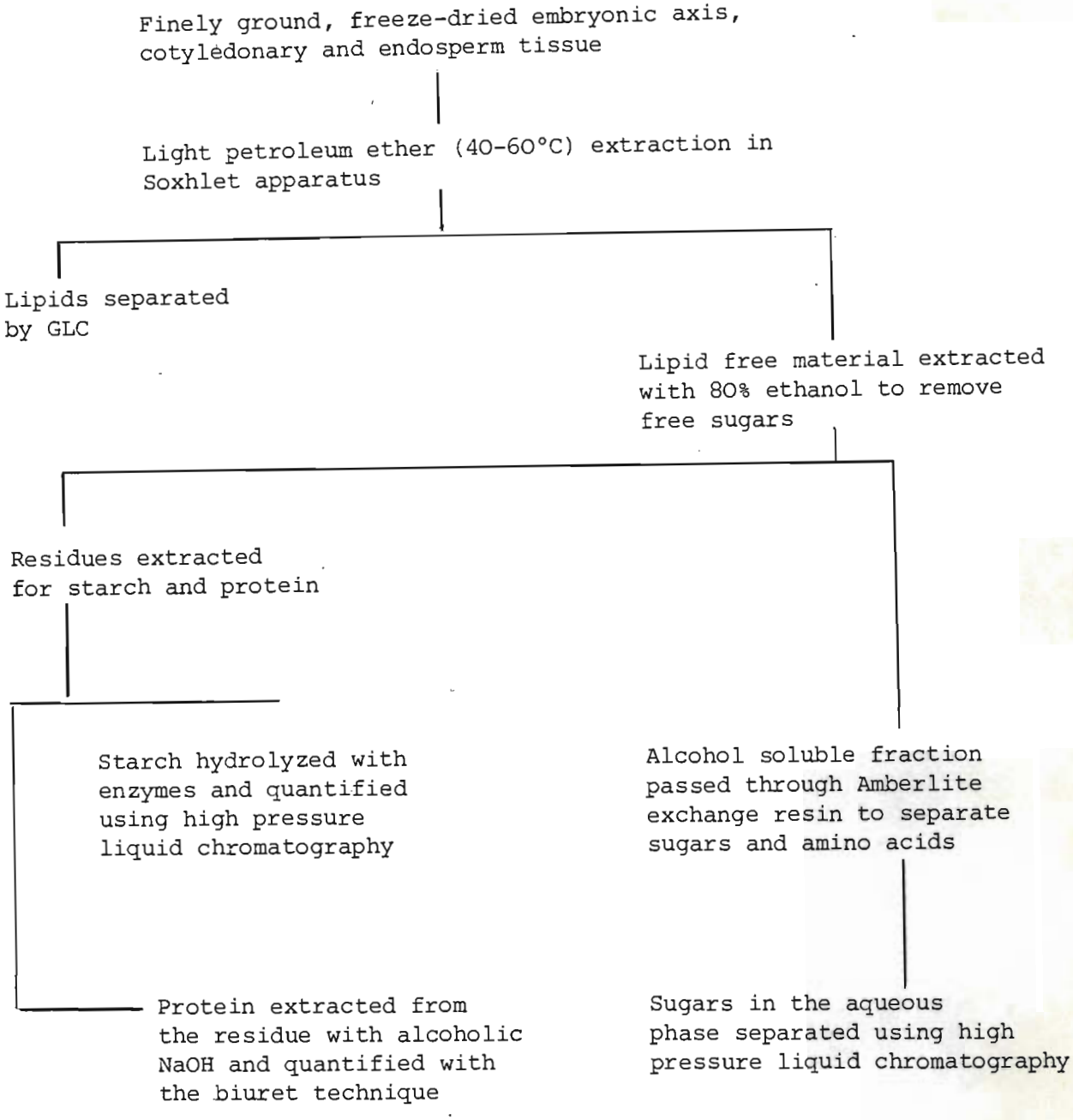


Fig. 3.4 Flow diagram for the extraction of lipids, carbohydrates, protein and starch from the seed components of *Ricinodendron rautanenii*.

dried endosperm and two grammes of both embryonic axis and cotyledonary tissue were massed to an accuracy of one milligramme and placed in previously washed Whatman cellulose extraction thimbles of known mass. The thimbles were them placed in a Soxhlet apparatus and the lipid extracted with light petroleum ether (boiling point 40-60°C) by

percolation for eight hours. Subsequently, the thimbles were removed from the Soxhlet apparatus, air-dried in a desiccator and remassed. The mass lost from each thimble represented the mass of the petroleum ether soluble fraction. Each sub-sample was extracted separately and each estimate represents the mean of the three values obtained. The petroleum ether fractions of embryonic axis material were taken to dryness in a rotary evaporator at 30°C. The lipids were then redissolved in five millilitres of petroleum ether, placed in glass vials and evaporated to dryness under a stream of nitrogen. Once dry, the vials were sealed and sent to the Council for Scientific and Industrial Research in Pretoria for identification of the constituent lipids. The fatty acid composition of the neutral lipids was determined by saponifying approximately 0,3 grammes of the oil with 5 per cent alcoholic potassium hydroxide. The soaps were then immediately converted to methyl esters by boiling with BF_3-methanol (METCALF, SCHMITZ and PELKA, 1966). The methyl esters were dissolved in hexane and analyzed with a Varian 3700 gas chromatograph fitted with a flame ionization detector. Separation was carried out using a glass capillary column coated with XE-60 as the stationary phase. The column was maintained at 210°C and helium was used as the carrier gas. The results are presented as the individual fatty acid composition expressed as a percentage of the total fatty acid content.

3.2 Sugar analyses

3.2.1 *Extraction of soluble sugars*

Sugar analyses were carried out on the defatted tissues. For this 2,5 grammes of endosperm tissue and 1,0 gramme of both cotyledonary and embryonic axis material were placed in 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 pre-massed Whatman No. 42 filter paper into beakers of known mass. 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 mass loss by the residues and the mass of the ethanolic extracts were averaged. This final value was used to estimate the percentage ethanol soluble fraction on a dry mass basis.

The dried extracts were dissolved in 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 slowly washing the resin with 200 millilitres of 5N HCℓ. This was then washed repeatedly with distilled water until all the chloride ions had been removed. The extracts were then passed through the columns at a rate of approximately 20 millilitres per hour. This resulted in the amino acid remaining on the column and the free sugars passing through unaffected. The column was then washed with 100 milli-

litres of distilled water and the combined aqueous phase, containing the free sugars, was reduced to dryness. The dried extracts were then resuspended in 10 per cent *iso*-propanol for storage.

3.2.2 *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 and then filtered to remove the inorganic salts. The pyridine was subsequently evaporated off and the dried samples resuspended in 0,1 millilitres of 10 per cent *iso*-propanol. Aliquots of the extracts and standard sugars were then injected into a Varian 5000 High Pressure Liquid Chromatograph fitted with a µ Bonda-Pak Carbohydrate Analysis Column (Waters Associates, Milford, Massachusetts). The solvent was acetonitrile:water (3:1 v/v) and this was passed through the column at a rate of 4,0 millilitres per minute. Sugars were detected with a Varian Refractive Index Detector and quantified using an in line Varian Computer Data System (Model 111L).

3.3 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). To ensure the complete removal of all residual free sugars, 0,2 gramme sub-samples of each com-

ponent were added to five millilitres of water and 25 millilitres of hot 80 per cent ethanol. These were agitated for 10 minutes. The samples were then centrifuged at 1 700g for a further ten minutes and the supernatant removed. Two subsequent extractions were carried out using 30 millilitres of hot 80 per cent ethanol. The combined supernatant fractions were discarded. The insoluble residue was autoclaved at 120°C for 60 minutes. After this, the gelatinized starch was hydrolyzed by adding five millilitres of 0,1 Molar citrate buffer, pH 5,0, containing five milligrammes of amyloglucosidase and one milligramme α-amylase. The mixture was diluted to 30 millilitres and incubated at 30°C for 4,5 hours. The reaction mixture was then filtered and taken to dryness on a rotary evaporator. The dry extracts were redissolved in 0,1 millilitres of *iso*-propanol and the glucose content was determined with a liquid chromatograph using the same technique described in section 3.2.2. The results are presented as starch equivalents per gramme dry mass and are the means of three separate extractions.

3.4 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 a 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 also added to the flasks. This mixture was then heated on a magnetic stirrer to 70°C in two minutes followed by a further two minutes of rapid stirring without heat. The mixtures were cleared by filtering through a fibre-glass filter and the transmittance of the solutions was measured at 500 nanometres 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 are presented as percentage milligramme equivalents of BSA per gramme dry mass and are the means of three extractions.

4.0 Biochemical analyses II. Respiration

The respiratory behaviour of dormant and ethylene treated *R. rautanenii* seeds was assessed by measuring the quantity of carbon dioxide released by the tissues at various times. Determinations were carried out on both ethrel and ethylene treated seeds and samples were taken every 12 hours. This was achieved by sealing the flasks with rubber 'Suba-Seals' for a period of 15 minutes. After this period, a ten millilitre sample of air was withdrawn from each of the flasks and injected into pre-evacuated test tubes (10 millilitres). The carbon dioxide concentration was estimated using a Perkin Elmer F 11 gas chromatograph. This instrument was fitted with a 6 metre x 3 millimetre Poropak S

column connected to a thermal conductivity detector. The column and detector were both run at 30°C. The results obtained are expressed as microlitres per litre per seed.

5.0 Biochemical analyses IV. Protein synthesis

5.1 Determination of protein synthesis

The protein synthetic ability of gas treated seeds was examined according to the technique employed by SATOH and ESASHI (1979). At each sampling period, the seeds were separated into their embryonic axis, cotyledonary and endosperm components. From this, ten axes (of known mass) and 0,2 grammes of both cotyledonary and endosperm (finely sliced) tissue were selected. These were rinsed in distilled water and transferred to flasks containing one millilitre of eight millimolar potassium phosphate buffer (pH 5,6) containing 0,4 microcuries of L-(U-^{14}C)leucine (50 microcuries per millimole, Amersham Radiochemicals Ltd, England). The flasks were incubated in the dark for two hours at 23°C. After this period, the labelled segments were given three, ten minute washes in ice-cold distilled water to wash away any free ^{14}C-leucine. The material was then blotted dry and subsequently homogenized for three minutes in a mortar containing one millilitre of ice-cold 20 millimolar potassium phosphate buffer (pH 7,0). The homogenates were transferred to centrifuge tubes with a further three millilitres of homogenizing buffer and centrifuged at 1 000g for 10 minutes. The supernatant was recovered and the pelleted material was washed once with four

millilitres of the same buffer and recentrifuged. Two millilitres of 25 per cent (w/w) trichloroacetic acid solution was added to the combined supernatant and allowed to stand overnight at 4°C. The resultant precipitate was separated from the supernatant by centrifugation at 10 000g for 10 minutes. After two further washes with four millilitres of 5 per cent w/w trichloroacetic acid, the precipitate was dissolved in one millilitre of two Molar ammonium hydroxide. The supernatant represented the trichloroacetic acid soluble fraction and included that fraction of the label which was taken up but not incorporated into protein. The trichloroacetic acid insoluble component represented the protein fraction. One millilitre aliquots of both fractions were mixed with 10 millilitres of 'Redy-Solv' EP scintillation fluid. Radioactivity was measured with a Beckman LS 8100 spectrometer and correction for quenching was achieved by the channels ratio method.

5.2 Application of protein synthesis inhibitors

An additional series of experiments was carried out using inhibitors of protein and ribonucleic acid synthesis. In the first experiment, the effects of cycloheximide were examined. This compound is a specific inhibitor of protein synthesis on extra mitochondrial, cytoplasmic ribosomes (SIEGEL and SISLER, 1964) and is active at very low concentrations of 0,5-2,0 microgrammes per millilitre (KROON and DE VRIES, 1969). A 100 milligramme per litre solution of this antibiotic was applied to gas treated seeds either

throughout the entire experimental period or during either the imbibition or germination phases. Germination was recorded daily and the experiment was terminated after seven days. Germinated seedlings were separated into their root, shoot and endosperm components. The fresh and dry mass values of these components was then obtained in the same manner as previously described. In addition to this, the protein synthetic capacity of the embryonic axes was also determined at various times for each of the respective treatments. For this, the same labelling technique as that described above was employed except that where necessary, cycloheximide was included in the incubation medium at a concentration of 100 milligrammes per litre.

In the second of these experiments, the above procedure was repeated except that a 500 milligramme per litre solution of actinomycin D was used instead of cycloheximide. This antibiotic is an antimetabolite which suppresses DNA-dependent RNA synthesis (REICH, FRANKLIN, SHATKIN and TATUM, 1961) by binding to DNA (KIRK, 1960). The ultimate effect of this compound then, would be a reduction in the synthesis of new proteins.

6.0 Biochemical analyses V. Interference with DNA replication

The effect of a DNA replication inhibitor on gas treated seeds was investigated. The compound used was acridine orange and a 100 milligramme per litre solution of this

substance was applied to gas treated seeds either throughout the entire experimental period or during either the imbibition or germination phases. Acridine orange has its effect by intercalating between adjacent stacked base pairs of the DNA double helix. The result of this is that both DNA synthesis and DNA dependent RNA synthesis is inhibited (FRANKLIN and SNOW, 1975). The parameters measured in this experiment were percentage germination and seedling growth.

7.0 Biochemical analyses VI. Activity of the pentose phosphate pathway

An experiment was carried out to determine whether or not compounds which stimulate the activity of the pentose phosphate pathway could replace the ethylene requirement of *R. rautanenii* seeds. To achieve this, seeds were incubated at 30°C on vermiculite moistened with the compounds listed in Table 3.2.

Table 3.2 A list of the compounds applied to *R. rautanenii* seeds which have been shown to stimulate the activity of the pentose phosphate pathway

Compound	Conc. (mM) I	II	III
H_2O (control)			
KNO_3	50	10	1,0
KNO_2	50	10	1,0
Methylene blue	50	10	1,0
$CS(NH_2)_2$ (Thiourea	50	10	1,0
KCN	50	10	1,0
NaN_3	10	1,0	0,1

The concentration of methylene blue used was based on that published by ROBERTS (1973). The concentration of the remaining compounds are the same as those used by ESASHI, OHARA, OKAZAKI and HISHINUMA (1979) on *Xanthium pensylvanicum* seeds. Germination was recorded daily and the experiment was repeated once.

8.0 Hormone studies I. Inhibitors of gibberellin synthesis

The effect of two gibberellin synthesis inhibitors on the germination of gas treated seeds was examined. This was done in order to determine whether or not the effects of ethylene were mediated via increased gibberellin synthesis. The compounds used were Cycocel((2-chloroethyl)trimethyl-ammonium chloride) and phosphon D (2,4-dichlorobenzyl-tributylphosphonium chloride). Cycocel inhibits gibberellin synthesis by preventing the conversion of *trans*-geranyl-geranyl pyrophosphate to copalyl pyrophosphate, whereas phosphon D prevents the conversion of copalyl pyrophosphate to kaurene(-)-kaur-16-ene (LANG, 1970). The concentrations of cycocel and phosphon D used were 2,5 and 0,5 milligrammes per millilitre respectively (BLACK, 1969). Germination was recorded daily and the experiment was terminated after seven days. The fresh and dry mass values for the various seedling components were also determined.

9.0 Hormone studies II. Ethylene

The quantity of ethylene released by seeds subjected to

various treatments was investigated. The treatments applied were; (a) the *ethrel treatment*, (b) the *gas treatment* and (c) a 500 milligramme per litre gibberellin-A_3 solution. In the latter treatment, seeds were incubated at 30°C on vermiculite moistened with the hormone. At six hourly intervals the flasks containing the seeds were sealed and after 15 minutes a 10 millilitre sample of air was removed. These were injected into pre-evacuated test tubes which were then submerged in a flask containing a saturated solution of ammonium sulphate to prevent ethylene loss during storage (NELSON, ISEBRANDS and RIETVELD, 1980). Ethylene determinations were carried out using a Varian 3700 gas chromatograph fitted with a Poropak S column and a flame ionization detector. The column was run at 80°C with the injector port set at 50°C and the detector at 150°C. Ethylene concentrations are expressed as milli-microlitres per litre.

10.0 Hormone studies III. Cytokinins

A preliminary investigation was carried out on the cytokinin levels present in the embryonic axes of dry and imbibed dormant manketti seeds. In addition, changes in these levels which may occur in the embryonic axes as a result of ethylene treatments were examined.

10.1 Extraction of embryonic axes for cytokinins

Cytokinins were extracted from the embryonic axes by

homogenizing appropriate quantities of the material (on a dry mass basis) in 50 millilitres of 80 per cent ethanol. This was agitated at room temperature for 24 hours. The extracts were then filtered through Whatman No. 1 filter paper and reduced to dryness under vacuum at 40°C. The residues were dissolved in five millilitres 80 per cent ethanol and strip-loaded directly onto Whatman No. 1 chromatography paper (DAVEY, 1978).

10.2 Paper chromatography of cytokinins

The extracts were applied as a one centimetre strip to sheets of Whatman No. 1 chromatography paper. The chromatograms were allowed to equilibrate in the chromatography tanks for two hours and then developed with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v) (PAW) in a descending manner. Once the solvent front had progressed approximately 30 centimetres from the origin, the chromatograms were removed and dried 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, each R_f strip was placed into an ehrlenmeyer flask and assayed for cell division promoting activity using the soyabean callus bioassay of MILLER (1963, 1965).

10.3 Soyabean callus bioassay

This particular bioassay was chosen since the range of

concentrations 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 bioassay 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 prepared and combined in the proportions indicated in Table 3.3. Fifteen millilitres of this nutrient medium were placed in 25 millilitre flasks containing 0,15 grammes of agar and the respective R_f strips. The flasks were stoppered with non-absorbent 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 soyabean callus of approximately ten milligrammes each were placed on the basal medium. The flasks were then incubated in a growth room where conditions of constant temperature (26°C $\pm$ 2°C) and continuous low intensity light were maintained. After 21 days, each piece of callus in each flask was massed. Callus growth in each fraction was plotted on a histogram relative to the control value. The significant limit at the 0,01 per cent level was calculated and is indicated in the histograms as a dotted line. Standards of kinetin were included in each bioassay. In order to estimate gross levels of cytokinin activity at a

TABLE 3.3. Basal medium for soybean callus bioassay (adapted from MILLER, 1963 1965)

Stock Solution	Chemical	gl^{-1} stock solution	ml stock solution for litre medium
STOCK 1	KH_2PO_4	3,0	100
	KNO_3	10,0	
	NH_4NO_3	10,0	
	$Ca(NO_3)_2 \cdot 4H_2O$	5,0	
	$MgSO_4 \cdot 7H_2O$	0,715	
	KC1	0,65	
	$MnSO_4 \cdot 4H_2O$	0,14	
STOCK 2	NaFeEDTA	1,32	10
	$ZnSO_4 \cdot 7H_2O$	0,38	
	H_3BO_3	0,16	
	KI	0,08	
	$Cu(NO_3)_2 \cdot 3H_2O$	0,035	
	$(NH_4)\ Mo_7O_{24} \cdot 4H_2O$	0,01	
STOCK 3	myo-inositol	10,0	10
	nicotinic acid	0,2	
	pyridoxine HC1	0,08	
	thiamine	0,08	
STOCK 4	NAA	0,2	10
ADDITIONAL	Sucrose		30 gl^{-1} medium
	Agar		10 gl^{-1} medium

pH adjusted to 5,8 with NaOH

given time, the bioassay results were expressed in kinetin equivalents. The inherent difficulties in making such an estimate were recognized.

RESULTS AND DISCUSSION

In recent years a large volume of literature has been amassed on the ultrastructure and metabolism of germinating seeds. Careful examination of this literature, however, shows that few studies have been carried out on species which exhibit true dormancy. One possible reason for this is the lack of examples in which there is a clear distinction between the hydrated dormant and non-dormant conditions. In addition, most studies have concentrated on species from the Gramineae and Leguminosae because of their obvious economic importance.

With regard to ultrastructure, several authors (e.g. KLEIN and BEN-SHAUL, 1966; OPIK, 1966; PAULSON and SRIVASTAVA, 1968; JONES, 1969; VAN STADEN, GILLILAND and DIMALLA, 1979) have reported on work carried out on seeds which they regarded as dormant. However, closer examination of these species shows that they were mostly quiescent and merely required water to initiate the resumption of embryonic growth. The fine structure of truly dormant seeds has, therefore, only been investigated in very few instances (e.g. VILLIERS (1971) and ROST (1972)). Thus, seeds of *Ricinodendron rautanenii* provided a unique opportunity to examine both the fine structure and biochemistry of the truly dormant condition.

Samples of *R. rautanenii* seeds for the various ultrastructural and biochemical studies were removed at specific

intervals during the periods of imbibition and germination. These are illustrated in Figure 3.5.

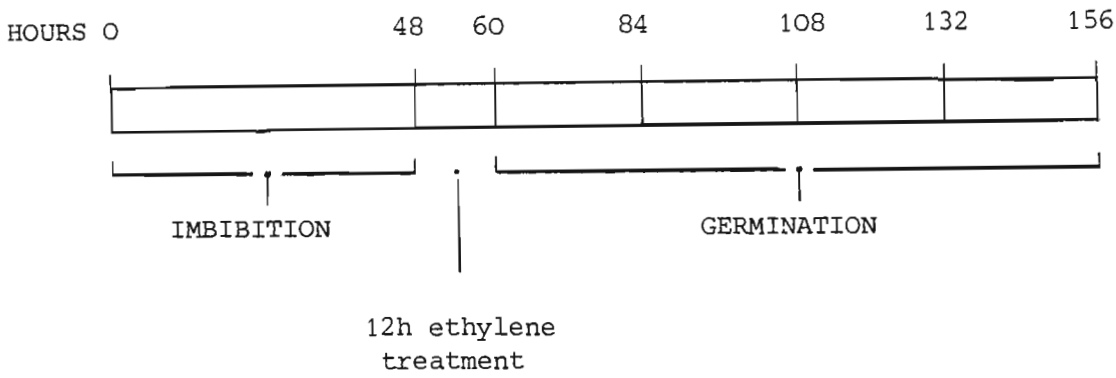


Fig. 3.5 Line diagram showing the intervals at which samples were removed for ultrastructural and biochemical investigations.

Dormant tissues were represented by samples taken from the dry and 48 hour imbibed seeds. Samples taken after the 12 hour ethylene treatment, on the other hand, represented the period of dormancy breaking and germination. The final sample was taken four days after the gasing treatment and by this time most of the seeds had germinated. An additional experiment was carried out in which seeds were incubated on vermiculite moistened with ethrel (having an effective ethylene concentration of 200 microlitres per litre). This was done in order to compare the changes obtained as a result of simultaneous imbibition and dormancy breaking with those obtained in the gasing experiment. In this instance, samples were removed at 24 hourly intervals after the commencement of incubation. All samples were separated into their respective endosperm, cotyledonary and embryonic axis components and analyzed individually.

The fine structure of cells from the radicle tip of dry *R. rautanenii* seeds is illustrated in Plate 3.1. These cells are characterized by massive stores of reserve materials, the most predominant of which are the spherosomes. These organelles are typically smooth and spherical and vary from 0,25 to 2,0 micrometres in diameter. In this respect, these spherosomes closely resemble those recorded in seeds of *Fraxinus excelsior* (VILLIERS, 1971) and *Corya illinoensis* Wang. (VAN STADEN, GILLILAND and DIMALLA, 1979). In earlier studies, workers associated deep dormancy with a distinct peripheral alignment of the lipid bodies (KLEIN and BEN-SHAUL, 1966; PAULSON and SRIVASTAVA, 1968). However, this is clearly not the case in *R. rautanenii* tissue since the spherosomes are randomly dispersed throughout the cell matrix. Nevertheless, as in many other species, the spherosomes can be seen to lie in close contact with protein body membranes. Actual identification of these lipid bodies was achieved using the Sudan IV staining technique and the importance of these organelles was highlighted by the fact that the total lipid contained within them was estimated at 70 per cent of the total dry mass.

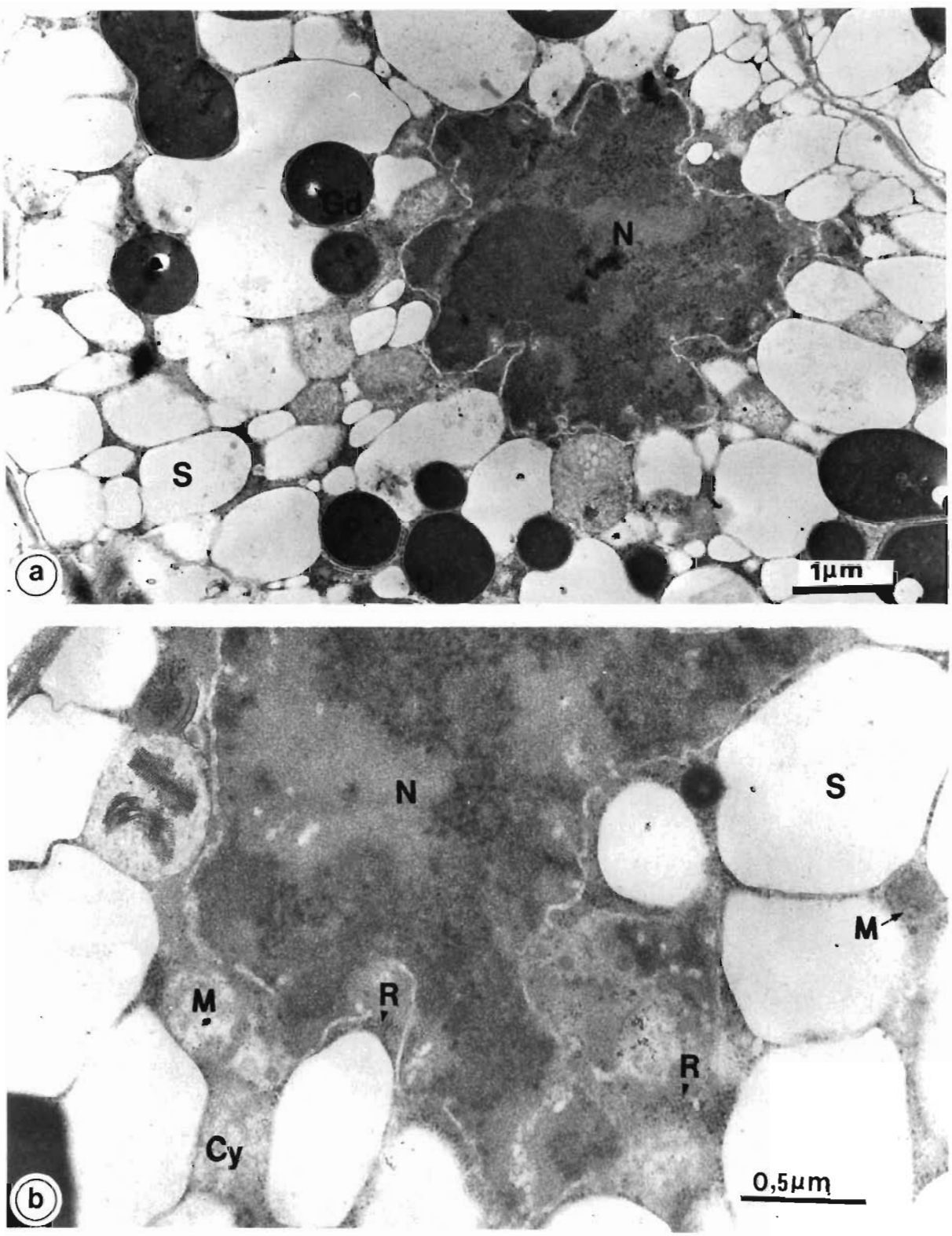
The protein content of these unimbibed embryonic axes, on the other hand, has been estimated at 26 per cent on a dry weight basis (WEHMEYER, 1976). This protein is clearly visible in the form of dense osmiophilic protein bodies which are enclosed in a distinct single unit membrane. Morphologically they are similar to spherosomes and are spherical to ovoid in shape and vary from 0,5 to 1,5 micro-

Plate 3.1

THE ULTRASTRUCTURE OF THE RADICLE TIP OF DRY *RICINODENDRON RAUTANENII* SEEDS.

a: A typical cell from the meristematic region of dry radicle tip tissue. Food reserves are present in the form of lipid containing spherosomes (S) and protein bodies (P) with their associated globoids (Gd). The nucleus of this cell displays the distinctive lobing which is characteristic of dry *R. rautanenii* tissue.

b: A closer view of this dry material showing the unaggregated ribosomes (R) scattered throughout the scant cytosol (Cv) present. Mitochondria (M) are also just visible within the cytosol and are distinguishable only by their double unit membranes.


Gd
N
S
a
1µm
N
S
M
M
R
R
Cy
b
0,5µm

metres in diameter. Within these protein bodies, phytin reserves in the form of globoids can be recognized. These protein bodies were identified as such using the toluidine blue staining technique (BUTLER, 1975).

Imbedded in the scant cytosol present, and compressed between the dense lipid and protein reserves are organelles which resemble mitochondria. These mitochondria appear to lack any well defined cristae and resemble the promitochondria described by VIGEL (1970). Microbodies, on the other hand, are scarce and no endoplasmic reticulum or dictyosomes are visible. Ribosomes can be seen scattered throughout the cytosol and these are present in the form of unaggregated monosomes. These observations are in agreement with those made by numerous other authors on dry seed material. However, it should be borne in mind that dictyosomes, polysomes and endoplasmic reticulum may, in fact, be present in dry tissue but may have become so compressed and fragile during dehydration that they are no longer recognizeable (ABDUL-BAKI and BAKER, 1973). In addition, damage may also occur during fixation (LOENING, 1968).

The most striking feature of this tissue is the distinctly lobed appearance of the nucleus. This phenomenon is anomalous in the sense that it is more frequently associated with either intense metabolic activity (CLOWES and JUNIPER, 1968) or with cells which are undergoing senescence (BERJAK and VILLIERS, 1970). Neither of these is probable

in the present instance and BUTLER (1975) attributed a similar phenomenon in *Welwitschia mirabilis* Hooker fil. cells, to pressure from the reserve materials on the nuclear surface. Nucleoli were not easily visible in this material. However, in the dry seed tissue of other species they have been described as being made up of densely packed, darkly staining fibrillar material with no apparent granular zone (HYDE, 1967; JORDAN, 1971).

An interesting feature of dry manketti seed tissue was that the cellular fine structure was similar irrespective of whether the samples were taken from the endosperm cotyledon or axial components. Furthermore, the cells of these tissues appeared to be qualitatively similar to numerous other descriptions of dry seed material, despite the use of aqueous fixation methods in this study. Other authors have suggested that permanganate should be used instead, since aqueous fixatives may induce slight changes (PALEG and HYDE, 1964; YATSU, 1965; VILLIERS, 1971). However, when compared with observations made on properly imbibed tissues, aqueous fixatives can be regarded as having had only a very limited effect on dry *R. rautanenii* material, if any at all.

Earlier, it was shown that *R. rautanenii* seeds readily imbibe water when placed under moist conditions. Further investigations revealed that after 48 hours imbibition, the moisture content of the endosperm, cotyledons and embryonic axis increased by 9,4, 7,34 and 3,0 per cent

respectively (Figure 3.6a). In cells of the radicle tip, this was reflected in the more hydrated appearance of the cytoplasm (Plate 3.2). These micrographs also show that the spherosomes had become more dispersed and this was probably the result of both rehydration and some utilization. The protein bodies are still in evidence but there are now clear indications that hydrolysis of this reserve has commenced. The degree of this breakdown varies between cells and examples of both internal fragmentation and peripheral erosion can be seen. The result of this is the development of ever enlarging vacuoles. Swelling of the protein bodies, a feature which is commonly associated with protein mobilization, is not visible in these tissues. Cavities within the protein bodies indicate that globoid hydrolysis had altogether commenced and in many cases, this reserve is absent.

The number of microbodies does not appear to have increased during this period of rehydration, nor does there seem to be any proliferation of the endoplasmic reticulum membrane system. Polysomes, however, can be observed and the micrographs also illustrate the apparent aggregation of ribosomes on the protein membrane surface, the significance of which is not clear. Mitochondria were scarce but those detected, were virtually unchanged from those observed in the dry tissue, although respiration, as measured by CO_2 evolution, had commenced (Figure 3.7a). The nuclei of these imbibed cells have lost their lobed appearance and there is a considerable increase in the granular content of

Figure 3.6a Increase in the fresh mass, expressed as a percentage per seed, of the embryonic axis (⬢), cotyledonary (●) and endosperm (■) tissues of ethylene treated *Ricinodendron rautanenii* seeds. The open symbols represent the moisture contents of the respective tissues of untreated, imbibed seeds incubated for 156 hours at 30°C. The bars represent the maximum 95 per cent confidence limits and the arrow the first day germination was recorded.

Figure 3.6b Changes in the dry mass of the embryonic axis (⬢), cotyledonary (●) and endosperm (■) tissues of ethylene treated *Ricinodendron rautanenii* seeds. The open symbols represent the dry mass of the respective tissues of untreated, imbibed seeds incubated for 156 hours at 30°C. Dry mass was determined by placing the tissue in an oven set at 100°C for 24 hours. The results are expressed in milligrammes per seed for endosperm tissue and milligrammes per 10 seeds for the embryonic axes and cotyledonary tissue. The bars represent the maximum 95 per cent confidence limits and the arrow the first day germination was recorded.

Fig 3.6a

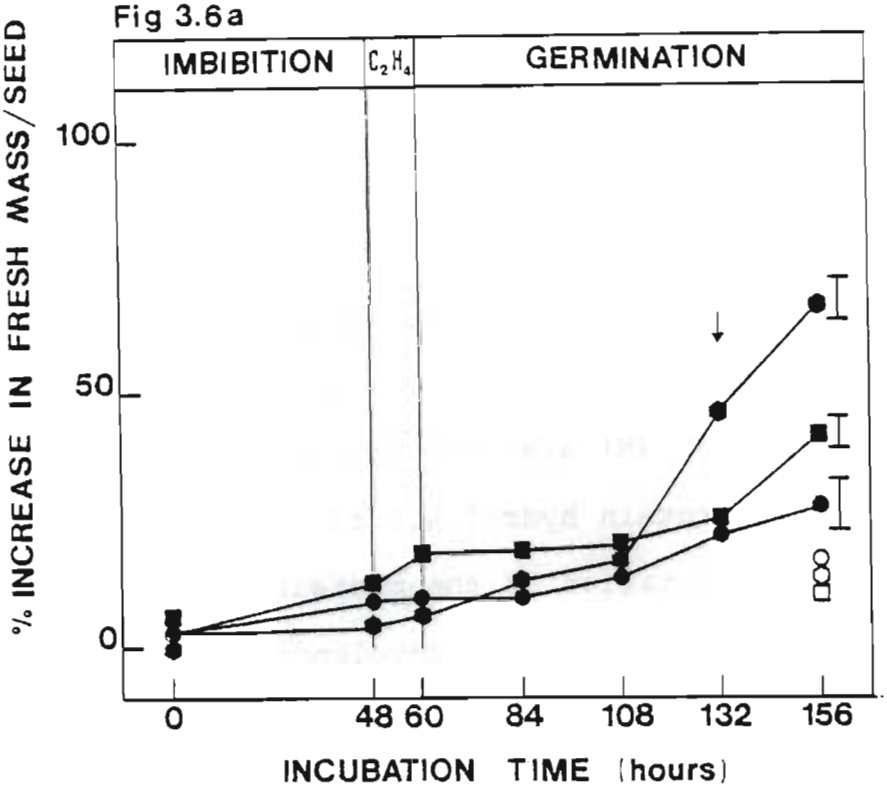


Fig 3.6b

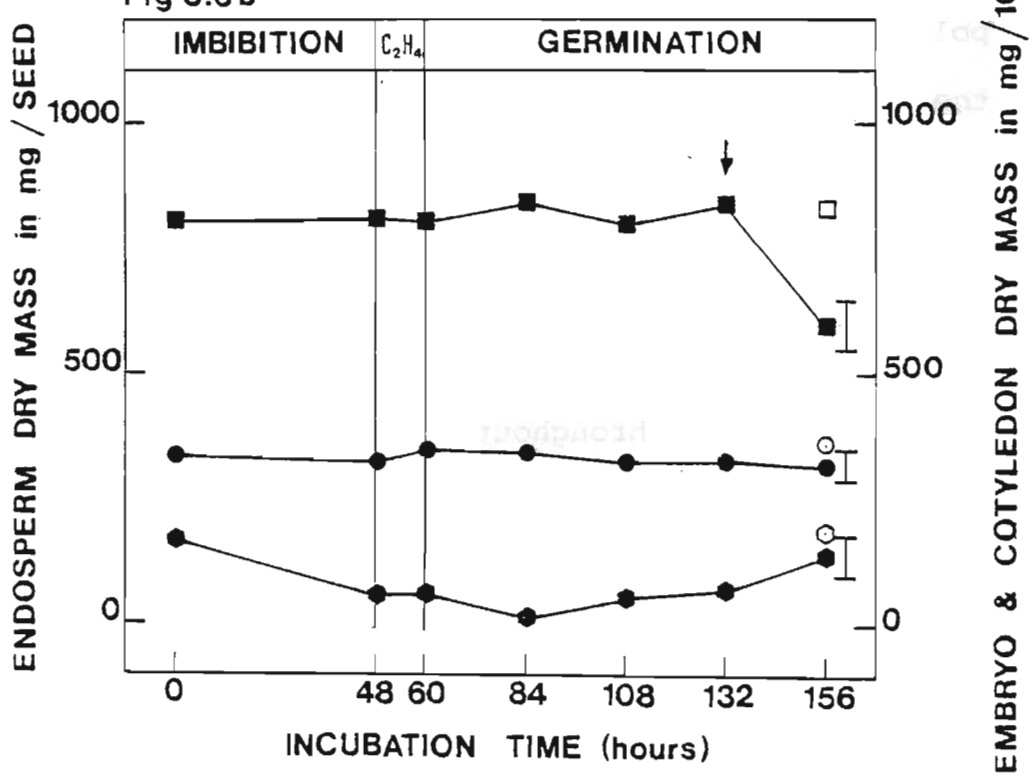


Plate 3.2

DETAILS OF THE FINE STRUCTURE OF RADICLE TIP CELLS FROM *RICINODENDRON RAUTANENII* IMBIBED IN DISTILLED WATER FOR 48 HOURS.

a: The cytoplasm of these cells has rehydrated and the spherosomes (S) are now more widely dispersed. Nucleoli (Nu) with distinctive nucleolar vacuoles (Nv) are clearly visible. Also as a result of rehydration, the mitochondria (M) are somewhat more distinctive. The onset of protein hydrolysis is reflected in the internal fragmentation of the protein bodies (P), ultimately resulting in the development of vacuoles (V).

b: In this cell the protein bodies (P) exhibit peripheral erosion. The aggregation of ribosomes into polysomes (Pr) has now commenced. At this stage the nucleoli (Nu) are still relatively small.

c: A characteristic feature of many of these rehydrated cells is the aggregation of ribosomes on the protein membrane surface (R arrowed). Numerous ribosomes can also be seen throughout the cytoplasm. Microbodies (Mb) such as the one visible in this cell are generally scarce.

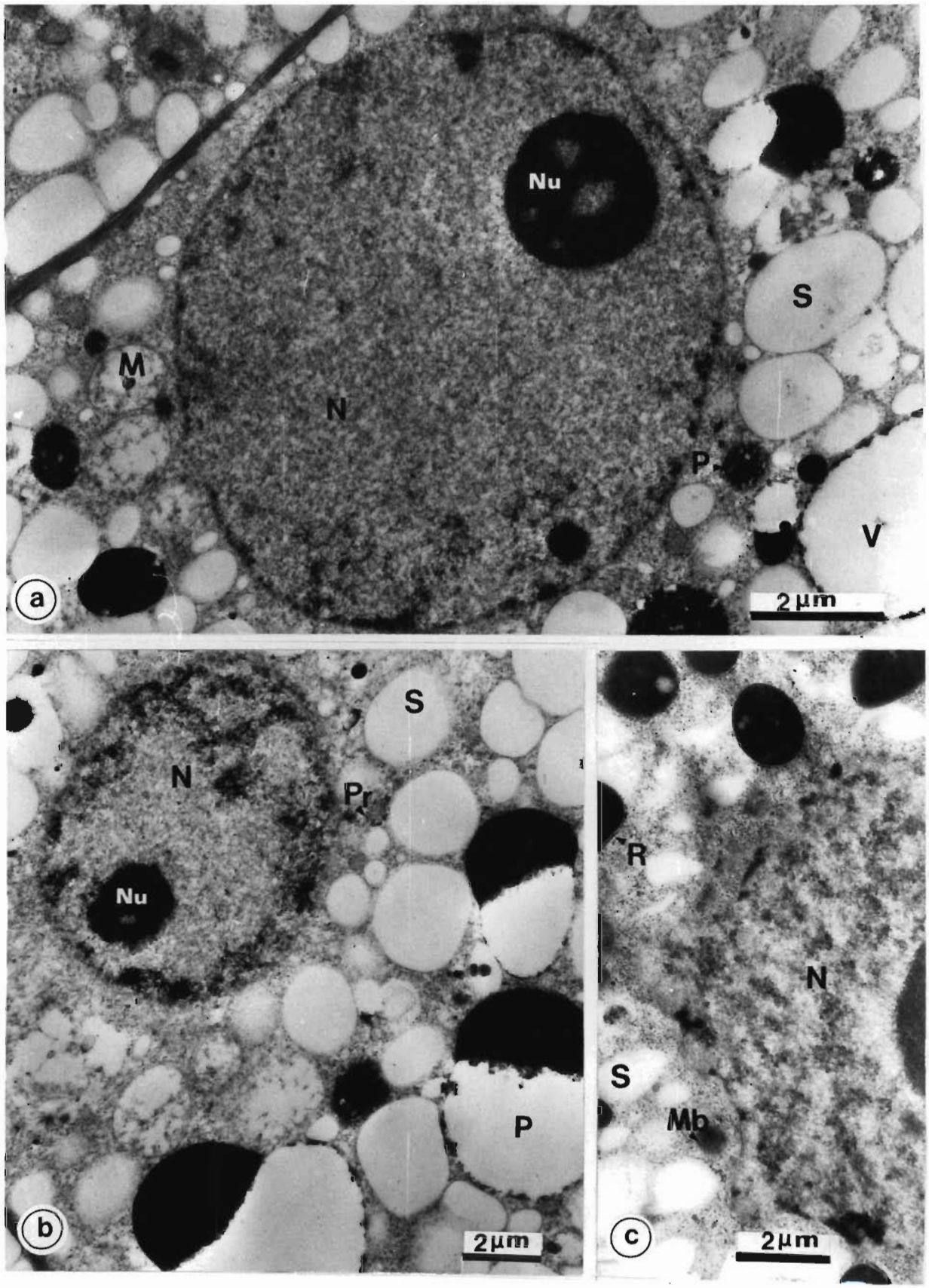

Nu
S
M
N
P
V
a
2 µm
S
N
Pr
Nu
P
b
2µm
R
N
S
Mb
c
2µm

Figure 3.7 Carbon dioxide evolution by ethylene (a) and ethrel (b) treated *Ricinodendron rautanenii* seeds. Control seeds (■) were incubated under ethylene-free conditions on vermiculite moistened with distilled water only. Treated seeds are represented by ⬢ . Carbon dioxide was measured with a Perkin-Elmer F11 gas chromatograph fitted with a poropak S column. The results are the means of three separate samples. The bars represent the maximum 95 per cent confidence limits. Arrow denotes the time at which germination was first recorded.

Fig 3.7a

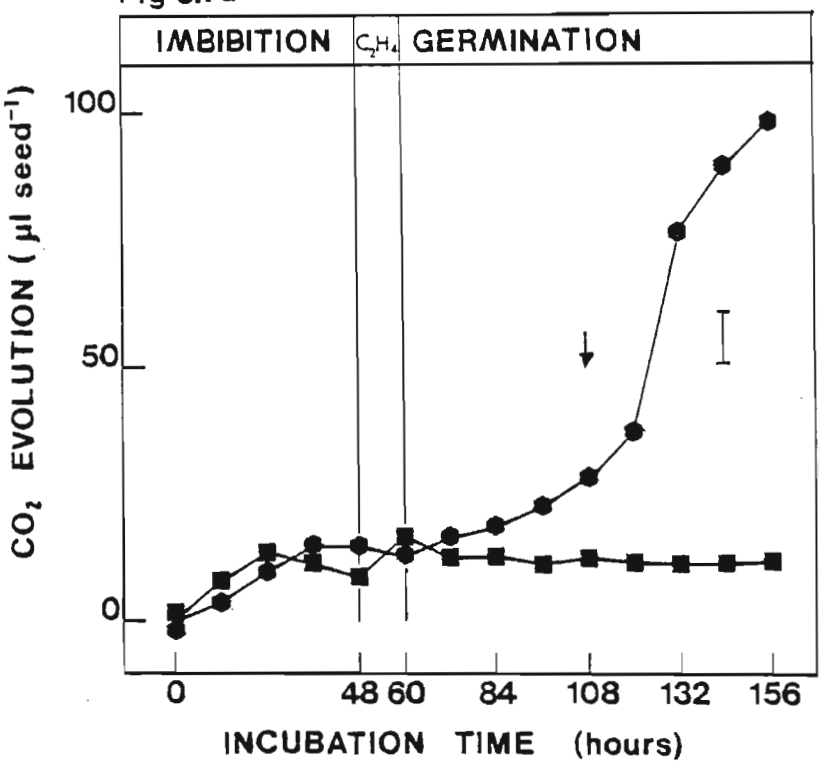
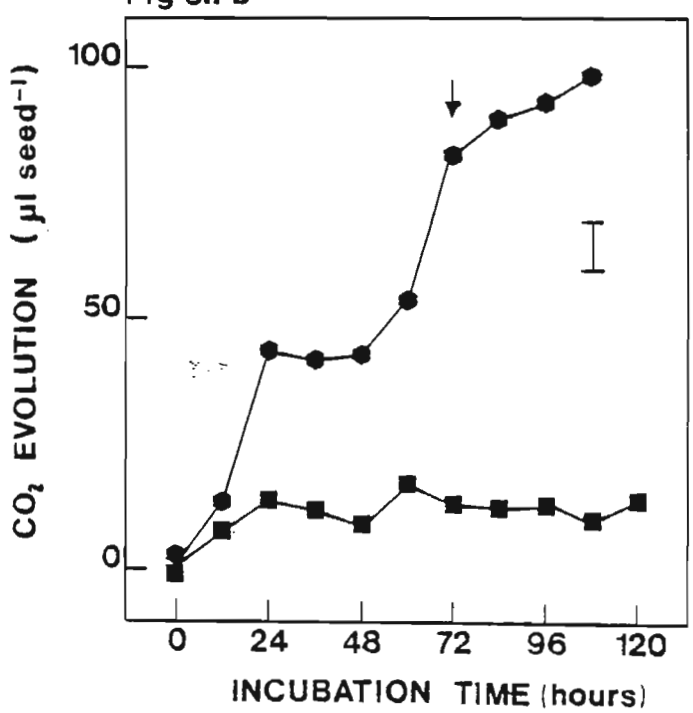


Fig 3.7b



the nucleoplasm. In addition, nucleoli are now clearly visible suggesting that the apparatus for ribosomal RNA synthesis had been reactivated. The ultrastructure of rehydrated endosperm and cotyledonary tissues was essentially the same as that just described and these findings are in agreement with those reported by ABDUL-BAKI and BAKER (1973) and VIGEL (1970) for other species.

The apparent visible decrease in the lipid and protein reserves of the embryonic axis during imbibition was supported by a measurable drop in the dry matter content of this tissue during the same period (Figure 3.6b). The dry matter content of the endospermal and cotyledonary tissues, on the other hand, remained unchanged. This finding that reserve hydrolysis may take place in the absence of any dormancy breaking treatments is not unique. VILLIERS (1971) found that lipid levels decreased and protein levels increased during the maturation of *Fraxinus excelsior* seeds. In *Setaria lutescens*, however, lipid reserves only declined in the non-dormant caryopses, whereas protein hydrolysis appeared to occur irrespective of the dormancy status (ROST, 1972). Thus, although definite patterns of reserve hydrolysis could be observed, no general rules exist with regard to the dormant and non-dormant seeds of different species.

During imbibition, there appeared to be some utilization of reserve materials for the production of energy rich compounds since carbon dioxide production of these seeds in-

creased during this period. Thus, the mitochondria in these dormant cells are apparently capable of producing ATP despite the disorganized appearance of their cristae. The enzymes necessary for respiratory activity and reserve hydrolysis may have been present in the dry seed tissues as this has been recorded in other species (BEWLEY and BLACK, 1978). On the other hand, *de novo* synthesis of certain enzymes from materials retained in the dry seed tissues may also have taken place. This is suggested by the changes observed in the nuclei of these radicle tip cells. However, the small size of these nuclei in relation to the rest of the cell, as well as the presence of nucleolar vacuoles, indicated that this metabolic activity was limited and that meristematic activity was unlikely at this stage. From this data, and from results obtained by other authors, it thus appeared that both the formation of cellular organelles and synthetic activities (as suggested by the presence of polysomes) could take place in dormant embryo meristems even when there was no indication of germination.

The dormancy of *R. rautanenii* seeds was broken by incubating the seeds in a saturated ethylene atmosphere for 12 hours. The micrographs presented in Plate 3.3 illustrate the ultrastructure of radicle tip cells sampled directly after this treatment. It can be seen that the spherosomes have decreased considerably in size and now measure between 0,1 and 1,1 micrometres in diameter, compared with their earlier mean diameter of 1,5 micrometres. Protein hydrolysis is

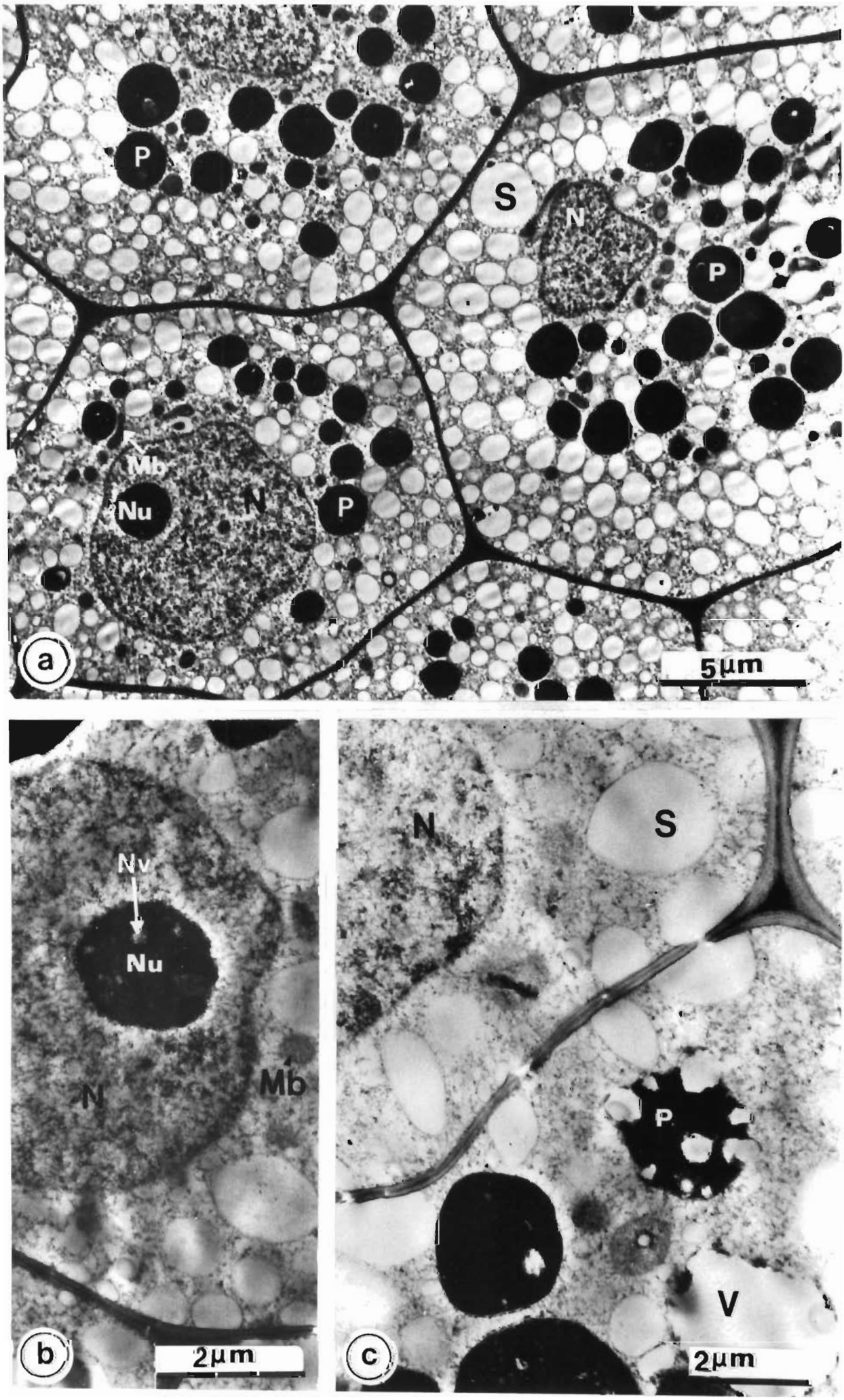
Plate 3.3

THE ULTRASTRUCTURE OF RADICLE TIP CELLS FROM SEEDS SUBJECTED TO A 12 HOUR ETHYLENE TREATMENT.

a: A number of cells from the meristematic region showing an overall reduction in the size and number of spherosomes (S). The protein reserves in these cells (P) are still intact. Microbodies (Mb) are more clearly visible and now resemble glyoxysomes in many respects.

b: The nucleoli (Nu) of these cells are still relatively small and the nucleolar vacuoles (Nv) have not yet disappeared. This latter feature is usually indicative of a low level of metabolic activity.

c: Digestion of the protein reserves (P) varies from cell to cell. In this instance some protein bodies remain intact whereas adjacent ones exhibit peripheral erosion. The development of vacuoles (V) as a result of protein hydrolysis is clearly evident.


P
S
N
P
Mb
Nu
N
P
a
5µm
Nv
Nu
N
Mb
b
2µm
N
S
P
V
c
2µm

continuing and this process is far from complete since many of the protein bodies are still in various stages of digestion. Globoid hydrolysis is also incomplete. However, this apparent continued breakdown of the lipid and protein reserves was not reflected in the actual dry mass determinations. Figure 3.6b shows that ethylene had no immediate effect on the dry matter content of the embryonic axes. Water uptake, on the other hand, increased slightly during this gasing period (Figure 3.6b).

Mitochondria remain scarce after the dormancy breaking treatment and this correlates well with the finding that no measurable change in respiratory activity took place during this period (Figure 3.7a).

The number of microbodies increased slightly during the treatment period, but the nuclei of these cells appear to be unaffected. In addition, nucleolar vacuoles within these nucleoli suggest that the synthesis of new ribosome fractions was proceeding at a slow rate. With regard to the other tissues examined, ethylene treatments also appeared to have no immediate effect on the ultrastructure and dry matter content of the cotyledons and endosperm (Figure 3.6b). Water uptake, on the other hand, varied and the endosperm continued to imbibe during this period, whereas the cotyledonary tissues remained unchanged.

After 24 hours incubation at 30°C, ethylene treated manketti

seeds showed no visible signs of germination. However, water uptake by the embryonic axes and cotyledons continued during this period, whereas the endosperm tissues appeared to have entered the lag phase (Figure 3.6b). With regard to ultrastructure, it seems that in cells of the radicle tip, extensive hydrolysis of the protein reserves is taking place and that the globoids have now almost disappeared (Plate 3.4). Correlated with this is the fact that the dry matter content of these cells decreased during this period (Figure 3.6b), possibly as a result of increased metabolic activity. In spite of this, the micrographs suggest that the lipid bodies have actually increased in size. This observation may reflect either an increase in lipid synthesis, or merely a coalescence of the smaller spherosomes. Mitochondria are still scarce, although respiratory activity seemed to be on the increase (Figure 3.7b). This was probably the result of improved efficiency due to membrane repair. Proliferation of the endoplasmic reticulum, however, is still not visible, although polysomes (which are normally associated with this membrane system) are present in considerable numbers.

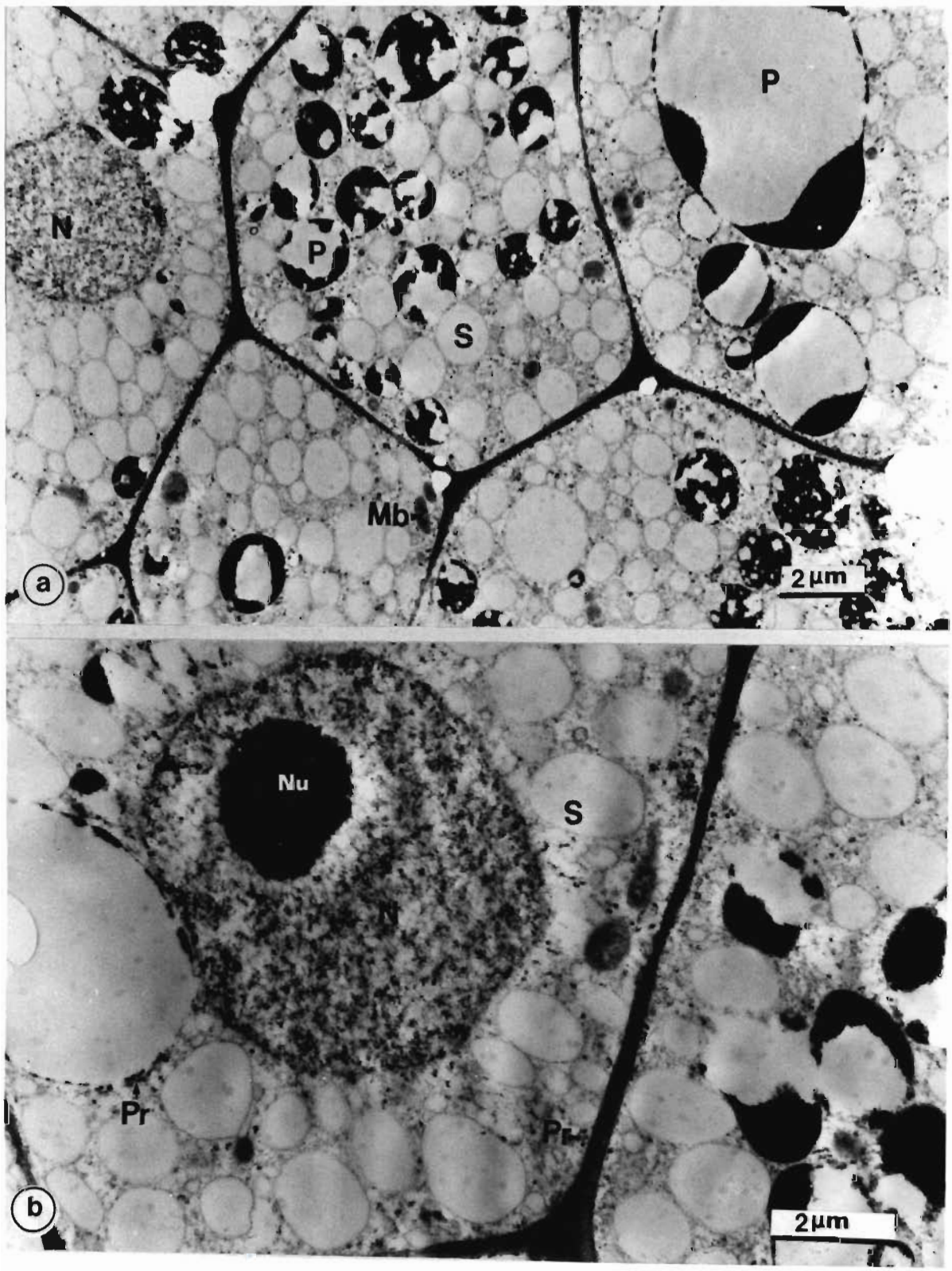
The nuclei of these radicle tip cells remained relatively unchanged 24 hours after the dormancy breaking treatment and they still occupy only a small proportion of the overall cell volume. In addition, only slight increases in the size and granular content of the associated nucleoli are visible, but the nucleolar vacuoles have disappeared. This suggests that little change had occurred in the overall

Plate 3.4

THE FINE STRUCTURE OF THE RADICLE TIP CELLS of *RICINODENDRON RAUTANENII* SEEDS 24 HOURS AFTER THE ETHYLENE TREATMENT.

a: Protein hydrolysis has increased markedly and examples of both internal fragmentation and peripheral erosion are visible (P). When compared with the previous sample, the spherosomes (S) appear to have increased in size. The number of microbodies (Mb arrowed) has remained unchanged.

b: The nuclei (N) of these cells has not changed much during the first 24 hours after ethylene treatment except that the nucleoli now lack any distinctive nucleolar vacuoles. Polyribosomes (Pr) are clearly visible scattered throughout the cytoplasm.


N
P
P
S
Mb
a
2 µm
Nu
S
Pr
Pr
b
2µm

metabolic activity of these cells during this period. In addition, the cytology and dry matter content of the endosperm and cotyledonary tissues continued to remain unchanged.

After 48 hours incubation, ethylene treated seeds began to show the first signs of germination. This took the form of a splitting of the testa in the region of the radicle. Figure 3.6a shows that this splitting was probably the result of increased water uptake by the cotyledons and embryonic axes only since imbibition by the endosperm tissues had not changed. In cells of the radicle tip, food reserves are still clearly visible (Plate 3.5). The number and size of spherosomes has remained the same although the microbodies have proliferated considerably. Many of these microbodies bear a close resemblance to the so-called glyoxysomes described by BREIDENBACH, KHAN and BEEVERS (1968). Glyoxysomes are usually associated with the breakdown of lipid reserves and normally form close spatial relationships with the spherosomes during this process (CHING, 1968). Such relationships appear to be visible. At this stage there is also little evidence of any hydrolysis of the remaining protein reserves taking place.

These observations concur with the dry mass data and no change was recorded in this parameter at this point in time (Figure 3.6b). One of the most notable features of these cells is the increased volume occupied by the nuclei. In addition, the nucleoli of these cells no longer contain any fibrous inclusions and the granular content of these regions

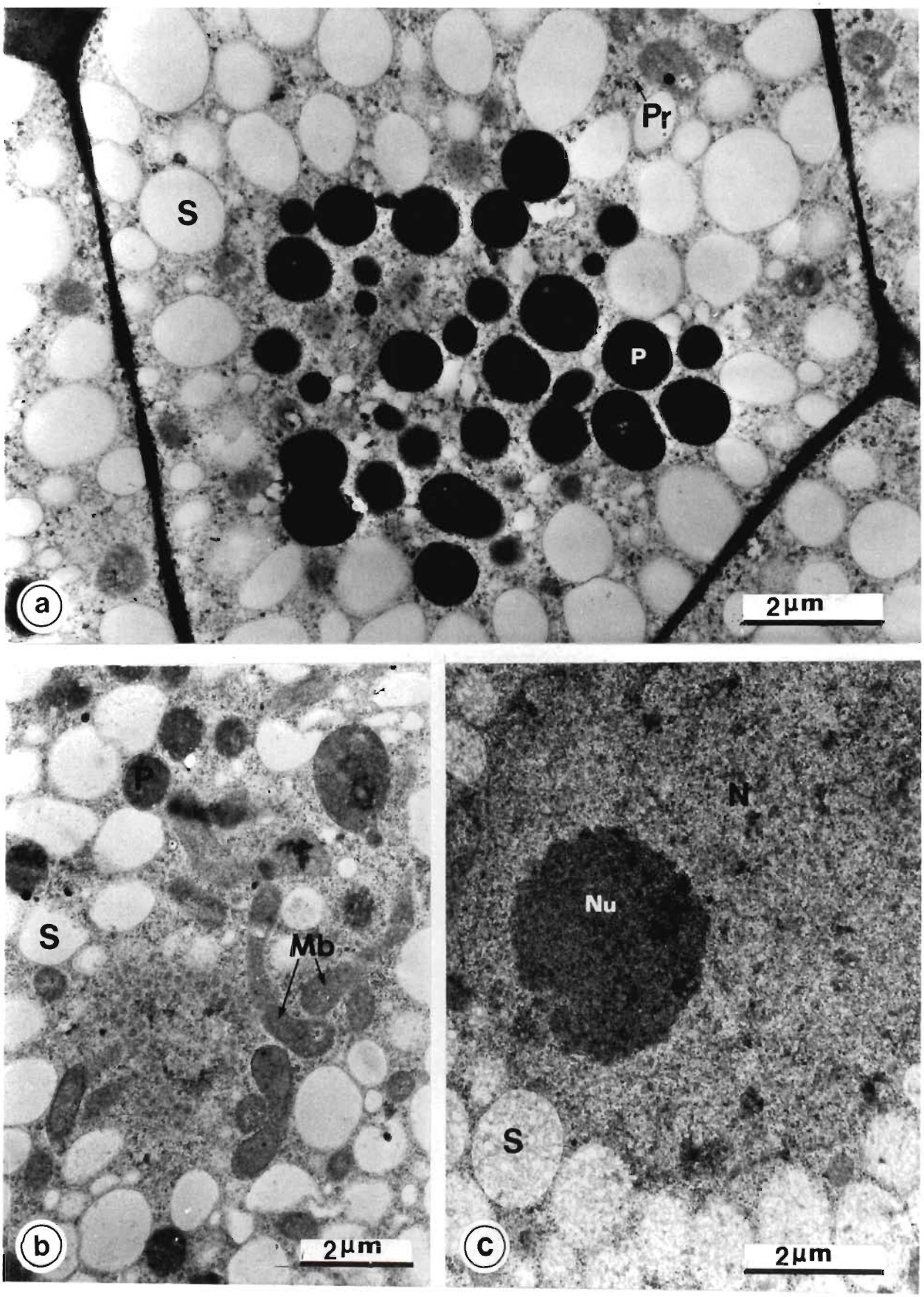
Plate 3.5

THE ULTRASTRUCTURE OF MERISTEMATIC ROOT TIP CELLS TAKEN FROM SEEDS 48 HOURS AFTER THE DORMANCY BREAKING TREATMENT. IN MANY CASES SPLITTING OF THE TESTA IN THE REGION OF THE RADICLE WAS VISIBLE.

a: The spherosomes (S) of these cells show no change from the previous sample whereas hydrolysis of the protein reserves (P) appears to have ceased. Polyribosomes (Pr) can in some instances be seen to form 'chains'.

b: Microbodies (Mb), which resemble glyoxysomes, continue to proliferate and in some cells, such as the one illustrated here, they can be seen to aggregate in considerable numbers.

c: During this period, the nuclei (N) have definitely increased in volume. In addition, the nucleoli (Nu) also increased in both size and granular content. No nucleolar vacuoles are present and the high ribosomal (R) content suggests that rRNA synthesis is increasing.


Pr
S
P
a
2µm
P
S
Mb
b
2µm
N
Nu
S
c
2µm

has increased considerably. It is well known that there is a relationship between nucleolar size and cellular metabolic activity, particularly protein synthesis (HYDE, 1967). On activation, the nucleolus usually swells in size, simultaneously becoming less compact especially in the developing outer granular zone. At the same time, the chromosomal organizing regions become deeply embedded in the inner fibrillar zone. As the peripheral granular zone enlarges and becomes interspersed in the central fibrillar core, there is a corresponding dramatic increase in cellular activity. Thus, in *R. rautanenii* tissue, it appears that there has been an upsurge in ribosomal RNA synthesis. Mitochondria, although still not clearly visible, appeared to be increasing their activity since the CO_2 output by these seeds, as shown in Figure 3.7a, had now increased considerably. This increase may be attributable to an improvement in mitochondrial efficiency, but it may also be due to the increased availability of certain substrates. No significant changes were observed in the ultrastructure and dry mass of the cotyledonary and endosperm cells (Figures 3.6a and b).

By the third day after the application of ethylene, the radicle of germinating *R. rautanenii* seeds had protruded by approximately one millimetre. The moisture content of this organ had also increased and was now estimated at approximately 45 per cent of the total mass (Figure 3.6a). An increase in moisture content for endosperm and cotyledonary tissues was also recorded but the rates of increase

were considerably below that of the embryonic axis. The dry matter content of the axes increased during this period (Figure 3.6b) and the micrographs in Plate 3.6 suggest that this could have been due to an increase in the size of the spherosomes. However, it is possible that this could also have resulted from lipid coalescence, and the presence of numerous microbodies in fact suggests that lipid hydrolysis had commenced. Protein bodies, on the other hand, have almost disappeared completely. In any event, reserve utilization for the production of energy rich compounds must have been taking place since a sharp increase in carbon dioxide production was recorded at this time (Figure 3.7a). Further signs of increased metabolic activity are present in the cytoplasm and nuclei. The cytoplasm is densely populated by ribosomes and polysomes and the nucleoli can be seen to have enlarged considerably. In addition, the presence of newly formed cell walls suggests that cell division has now commenced. An upturn in metabolic activity is also suggested by the presence of dictyosomes, but there is still a conspicuous lack of any endoplasmic reticulum. The increase in dry matter content of the embryonic axes does not appear to have taken place at the expense of reserves present in the cotyledons or endosperm tissues since these remained unchanged during this period.

Dormancy may be regarded as broken once radicle emergence has taken place. Thus, by the third day of incubation, ethylene treated manketti seeds are clearly no longer dor-

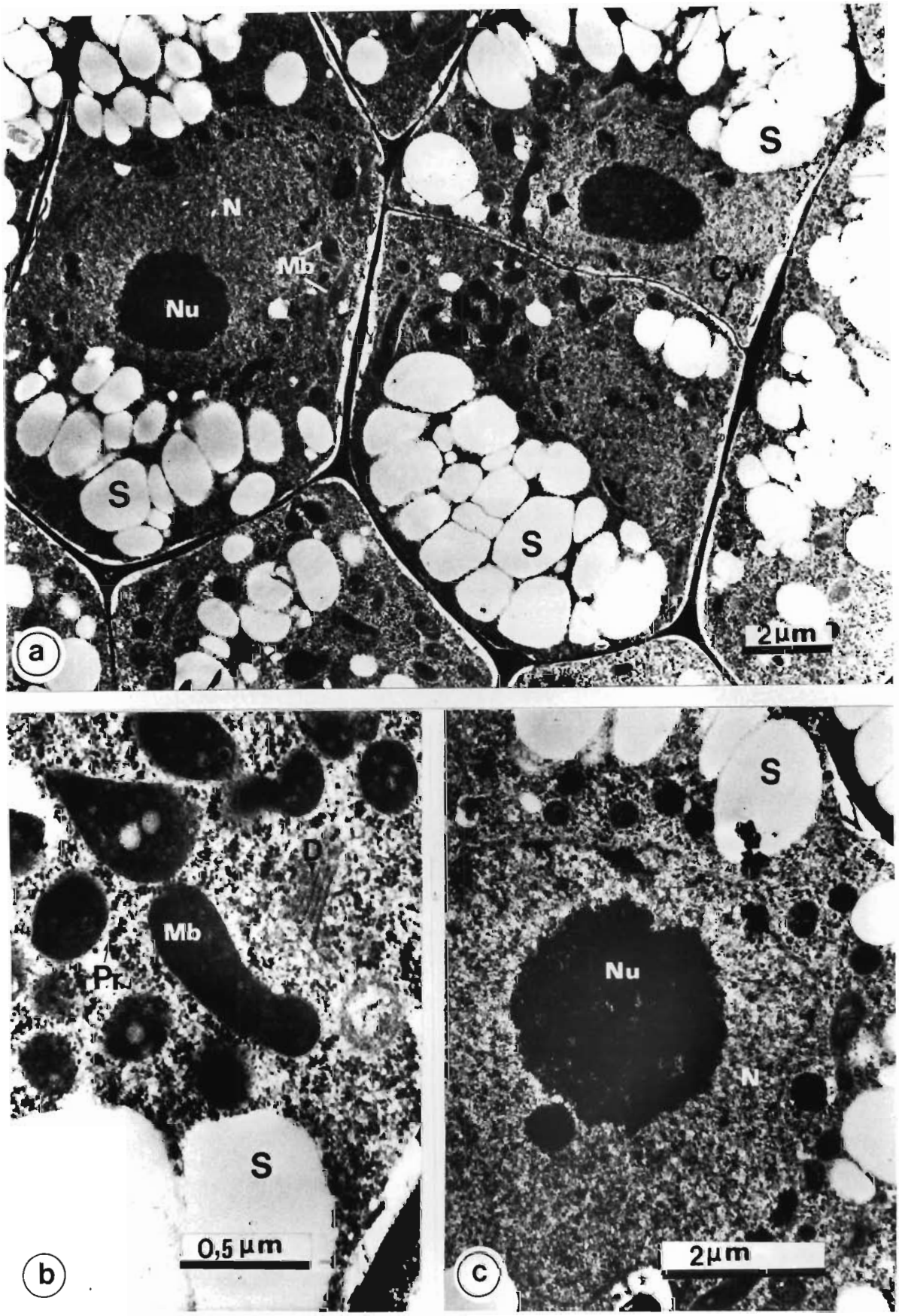
Plate 3.6

THE FINE STRUCTURE OF RADICLE TIP CELLS TAKEN FROM SEEDS THREE DAYS AFTER THE APPLICATION OF ETHYLENE. AT THIS TIME 40 PER CENT OF THE SEEDS HAD GERMINATED.

a: The spherosomes (S), although fewer in numbers, generally appear to have increased in size. Meristematic activity is now under way as suggested by the presence of a newly formed cell wall (CW).

b: As in the previous sample, microbodies (Mb) are much in evidence and this feature is usually associated with mobilization of lipid reserves. The cytoplasm is dense with polyribosomes (Pr) and dictyosomes (D) are now visible for the first time.

c: The nucleoli (Nu) of these cells have now expanded considerably and this, together with the increased granularity of this region, suggests that rRNA has increased considerably.


N
Mb
Nu
S
S
S
CW
2µm
a
D
Mb
Pr
S
0,5 µm
b
S
Nu
N
2µm
c

mant. However, for the sake of completeness, samples were also taken from ethylene treated seeds after four and five days incubation. By the fourth day, germination was well under way and the moisture content of the embryonic axis had increased by a further 21 per cent. In addition, the dry mass of these organs also increased considerably. This latter increase appeared to be at the expense of the endosperm tissues since the dry mass of these organs had decreased by more than 200 milligrammes per seed (Figure 3.6b). In cells of the radicle tip, the lipid reserves are still very much in evidence (Plate 3.7). Many of the spherosomes have, however, coalesced into large pools and the glyoxysomes, although few in numbers, have now formed close spatial relationships. This coalescence is similar to the situation observed in the cotyledons of germinating *Carya illinoensis* seeds (VAN STADEN, GILLILAND and DIMALLA, 1975). Protein bodies are still present and digestion is clearly incomplete. Endoplasmic reticulum and dictyosomes are now visible, the former for the first time, and this is unlike the findings in *Ricinus communis* (a species closely related to *R. rautanenii*) where these organelles appeared within 24 to 72 hours after the commencement of incubation (LORD, 1978). Starch reserves are also visible for the first time and appear in the form of amyloplasts. This starch accumulation probably results from the mobilization of endosperm reserves. The nuclei of these cells are distinctly lobed but, unlike the situation in the dry tissue, this is probably due to the increased metabolic activity as suggested by CLOWES and JUNIPER (1968).

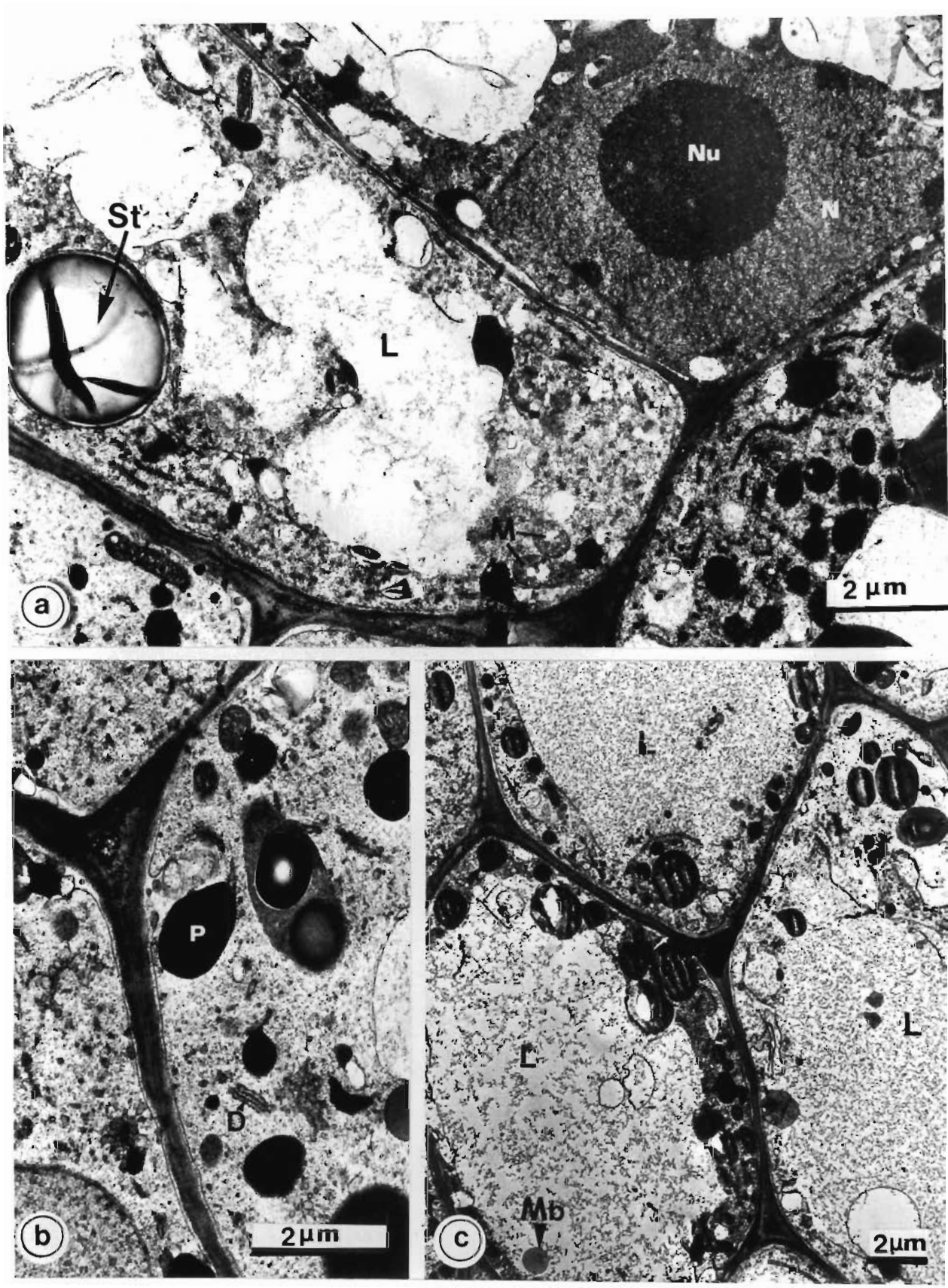
Plate 3.7

THE ULTRASTRUCTURE OF THE MERISTEMATIC REGION OF ROOT TIP CELLS TAKEN FROM SEEDS FOUR DAYS AFTER THE APPLICATION OF ETHYLENE. EIGHTY PER CENT OF THE SEEDS HAD GERMINATED BY THIS TIME.

a: Coalescence of the spherosomes has taken place resulting in the formation of large lipid vacuoles (L). In the cells the mitochondria (M), and in particular their internal membrane structures, are well developed. Starch granules (S) are now visible for the first time. The reappearance, at this stage, of a lobed nuclear envelope (Ne arrowed) suggests that metabolic activity is proceeding rapidly.

b: A close up view of one of the cells showing a dictyosome (D) and starch granule (S) contained within an amyloplast. Some protein reserves (P) are still present.

c: The lipid vacuoles (L) formed by the coalescence of spherosomes are large and in some instances almost fill the entire cell. In one of the cells a microbody (Mb), possibly a glyoxysome, can be seen to lie in close association with the lipid reserves.


Nu
N
St
L
M
2 µm
a
P
D
2µm
b
L
L
L
Mb
2µm
c

After five days incubation, the radicle had developed into a well defined root. The ultrastructure of cells from the tips of these roots is illustrated in Plate 3.8. Most of the food reserves have now disappeared and sequestration of the microbodies appears to be taking place. Many of these features are, in fact, typical of meristematic root tissue.

From the results obtained, and from the observations made, it was concluded that it was those events which occurred during the first 48 hours after ethylene treatment that reflected the processes involved in dormancy breaking. Furthermore, it appeared that the endosperm and cotyledonary tissues contributed little to the actual release from dormancy. This was based, to a large extent, on the observation that mobilization of the endosperm reserves was a fairly late event and took place after radicle emergence was already evident. The cotyledons, on the other hand, appeared to act as the means by which these endosperm reserves reached the growing axis.

During the critical 48 hour post-treatment period, a number of interesting changes were noted in cells of the embryonic axis. However, to place these in perspective, the features associated with hydrated, dormant *R. rautanenii* seeds of the same age should be considered first. The ultrastructure of cells from the radicle tip of seeds incubated without ethylene for six days is illustrated in Plate 3.9. From these micrographs, it appears that the lipid and protein

Plate 3.8

MICROGRAPHS SHOWING THE ULTRASTRUCTURE OF *RICINODENDRON RAUTANENII* ROOT TIP CELLS FIVE DAYS AFTER THE APPLICATION OF ETHYLENE.

a: The meristematic nature of these cells is reflected in the presence of large nuclei (N) which themselves have large, granular nucleoli (Nu). Apart from a few spherosomes (L), most of the food reserves have now been depleted. Intense metabolic activity is also indicated by the even more lobed appearance of the nuclear envelope (Ne arrowed) and presence of rough endoplasmic reticulum (RER). Microbodies (Mb), although scarce, are still present in some cells.

b: In a closer view of these cells it can be seen that some sequestration of microbodies (Mb) (possibly) glyoxysomes) may be taking place. The microbodies become incorporated into vacuoles (V) and this phenomenon represents a means of inactivating and removing these organelles.

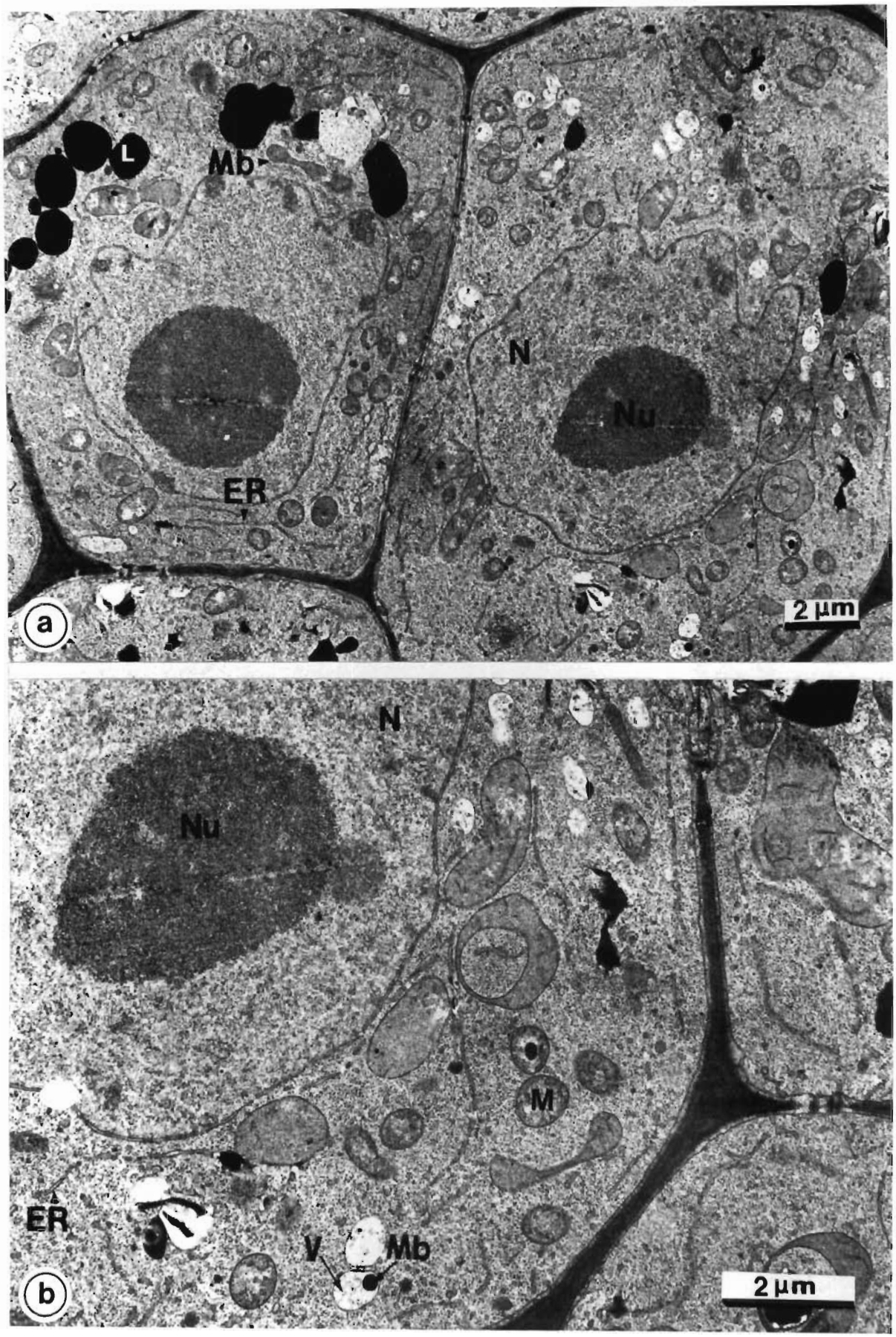

L
Mb
N
Nu
ER
2 µm
a
N
Nu
M
ER
V
Mb
2 µm
b

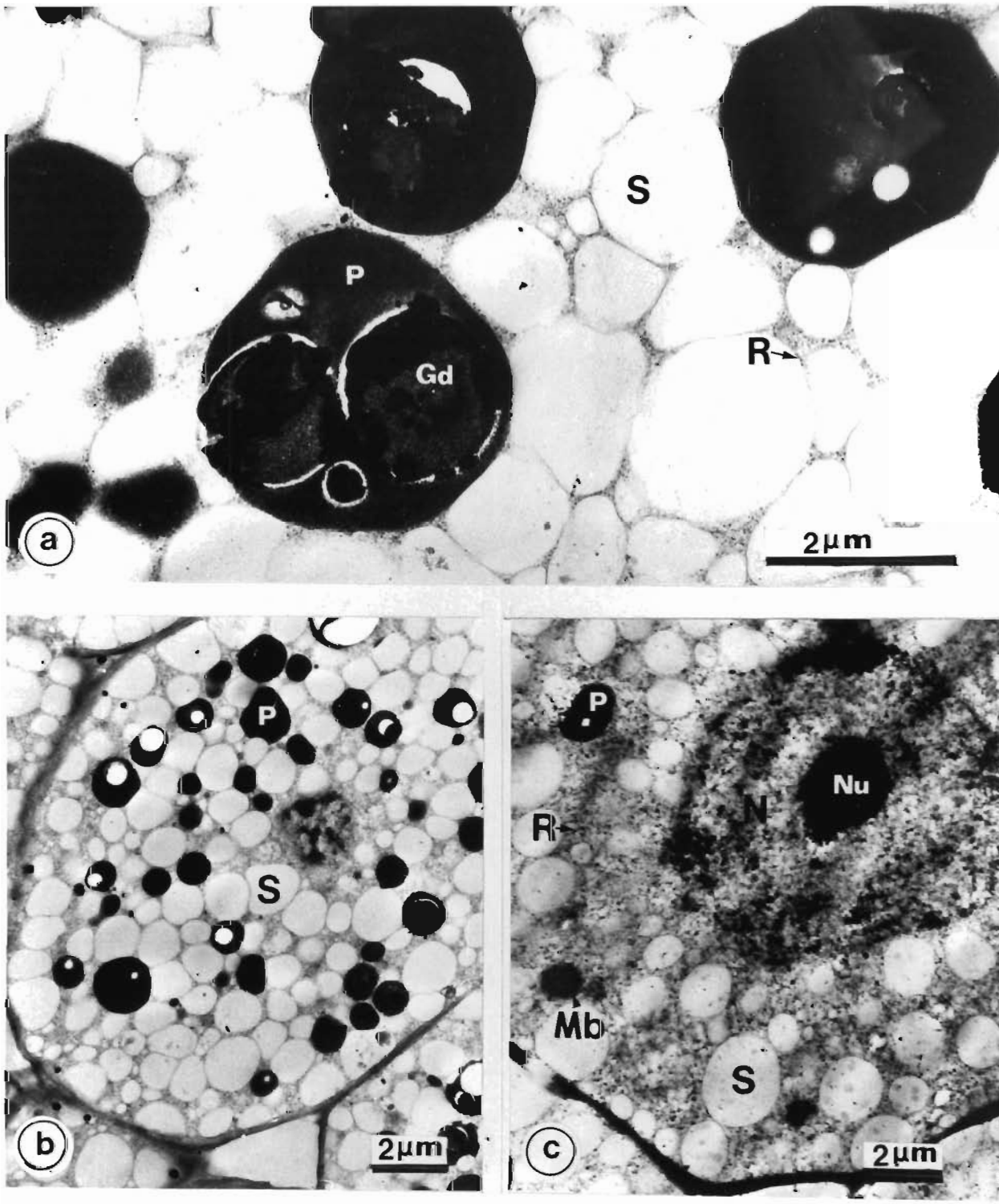
Plate 3.9

THE ULTRASTRUCTURE OF THE RADICLE TIP TAKEN FROM DORMANT SEEDS IMBIBED IN DISTILLED WATER FOR SIX DAYS.

a: The food reserves present in these cells resemble those observed in dry tissue. It seems that the protein reserves (P) have been resynthesized and there are no longer any signs of digestion taking place. Globoids (Gd) are also intact. Lipid reserves also appear to have been resynthesized since, compared with seeds imbibed for 48 hours, both their numbers and size have increased. Ribosomes (R) are also present but they are not aggregated into polysomes.

b: At lower magnifications the apparent resynthesis of reserves is more evident. Lipid bodies (S) are no longer dispersed and there are no indications of protein hydrolysis. The nucleus (N) shown in this cell is typically small. Mitochondria (M) are present but the internal membranes of these organelles are still poorly developed.

c: A close-up view of a somewhat larger nucleus containing a relatively small nucleolus (Nu). Most important is the lack of granular material in the nucleolus which is usually associated with a low level of ribosomal RNA synthesis.


S
P
Gd
R
a
2µm
P
S
b
2µm
P
Nu
N
R
Mb
S
c
2µm

reserves have been resynthesized. The fact that the dry matter content of these seeds was similar to that obtained for dry seeds (Figure 3.6b) also suggested that no further utilization had occurred and that, in fact, any reserves which were hydrolyzed had been resynthesized. In addition to this, there is no longer any visible evidence of protein hydrolysis taking place. The nuclei of these cells, by virtue of their size, appear to be quiescent and the nucleolar regions lack the distinctive granular appearance typical of active nuclei. These results suggested that the reserve hydrolysis which occurred during the first 48 hours after ethylene treatment was significant in terms of dormancy breaking. However, the changes observed in the appearance of the nuclei were probably of greater significance in this respect. This organelle was found to increase in size and the granular content of the associated nucleoli was generally greater than that observed in untreated seeds. VILLIERS (1972) found that the only features which could be associated with dormancy breaking in *Fraxinus excelsior* seeds was an increase in nucleolar activity. Similarly, the breaking of *Helianthus tuberosus* L. (Jerusalem artichoke) tuber dormancy was also correlated with increases in the activity of the nucleolus (JORDAN, 1971). The importance that may be attached to changes occurring in this region of the nucleus becomes evident when it is considered that the nucleolus is the site of ribosomal RNA synthesis. It therefore plays an important role in determining the nature of the proteins synthesized within the cells. Further support for the view that dormancy

breaking may be linked to changes in nuclear activity came from the results obtained with seeds incubated with ethrel.

The germination of ethrel treated seeds was first visible after three days and, as with the gas treatment, was characterized by a splitting of the testa in the region of the radicle. By the fourth day of incubation, approximately 75 per cent of these seeds had germinated (see Figure 1.6, Chapter 1). The fresh and dry mass changes which occurred in the various seed components during the period investigated are presented in Figures 3.8a and b. Of these, it is probably only those which occurred during the first 48 hours which would represent the period of dormancy breaking. In this regard, water uptake by cells of the embryonic axis increased rapidly during the first 24 hours, after which a lag phase became evident. Dry mass, on the other hand, decreased during the same period (Figure 3.8b) and this was similar to the initial decrease observed in seeds prior to the application of ethylene. Thus, the decline in dry mass which occurred during the first 24 hours was probably a passive event in which ethrel played little part. It therefore appeared that dormancy breaking could rather be associated with the subsequent increase in dry mass which occurred over the following 24 hour period (Figure 3.8b). This implies that a number of anabolic reactions must be taking place, the substrate source for which is not immediately clear. However, CO_2 evolution measured during the first and second days of incubation showed that there was an initial marked increase in respiratory activity

Figure 3.8a Increase in the fresh mass, expressed as a percentage per seed, of the embryonic axis (⬢), cotyledonary (●) and endosperm (■) tissues of ethrel treated *Ricinodendron rautanenii* seeds. The bars represent the maximum 95 per cent confidence limits and the arrow the first day germination was recorded.

Figure 3.8b Changes in the dry mass of the embryonic axis (⬢), cotyledonary (●) and endosperm (■) tissues of ethrel treated *Ricinodendron rautanenii* seeds. Dry mass was determined by placing the tissue in an oven set at 100°C for 24 hours. The results are expressed in milligrammes per seed for the endosperm tissue and milligrammes per 10 seeds for the embryonic axes and cotyledonary tissue. The bars represent the maximum 95 per cent confidence limits and the arrow the first day germination was recorded.

Fig 3.8a

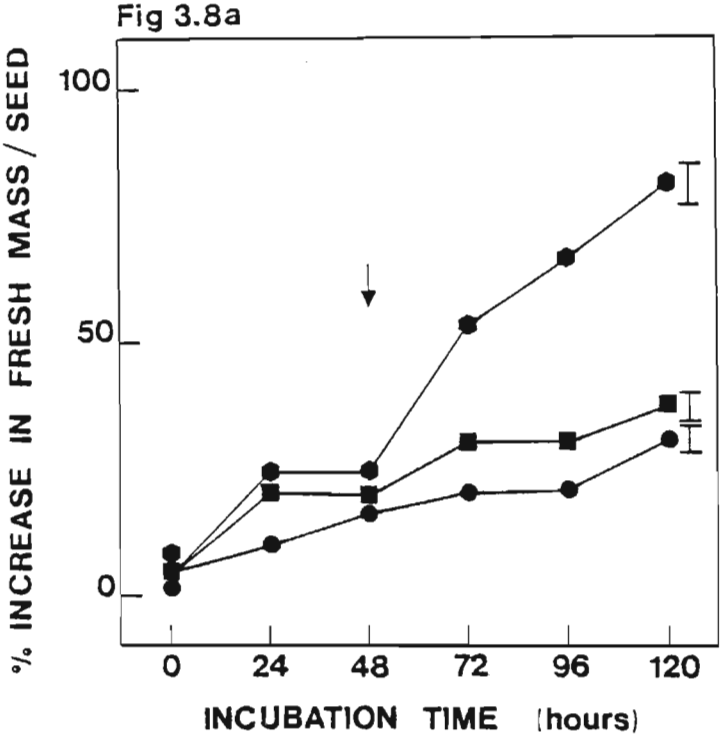
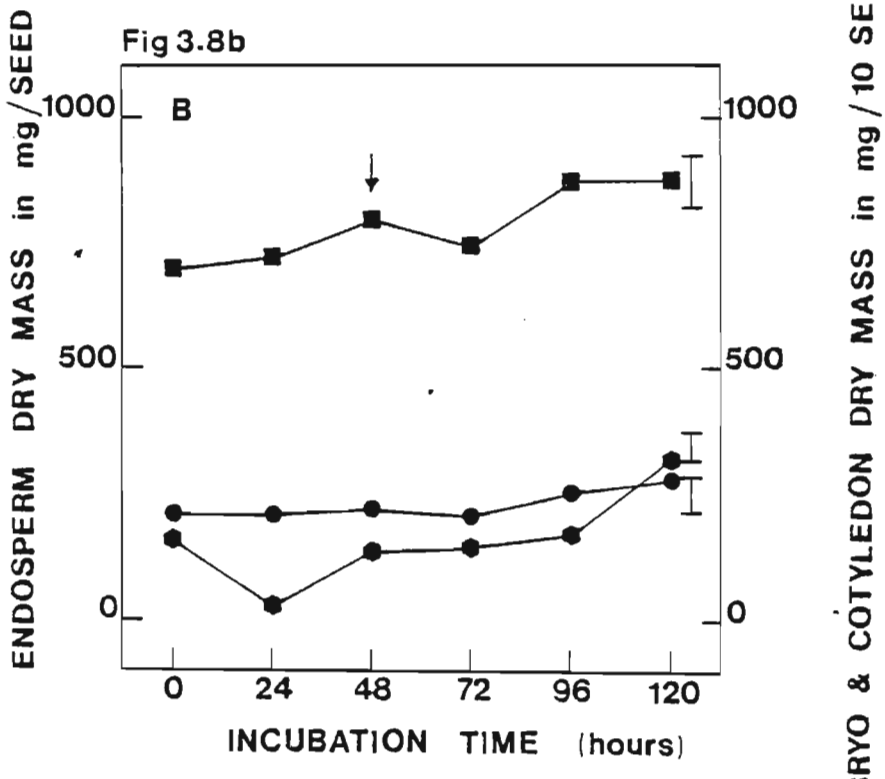


Fig 3.8b



during the first 24 hours. After this, an asymptote was reached and the only major subsequent increase in respiration corresponded to the protrusion of the radicle. From this it was concluded that sufficient production of energy rich compounds took place during dormancy breaking.

An ultrastructural investigation on cells from the radicle tip of ethrel treated seeds showed that the overall pattern of reserve mobilization was similar to that observed in gas treated seeds (Plates 3.10 and 3.11). Protein body digestion was visible after the first 24 hours of imbibition and these reserves continued to be depleted throughout the period of germination. Spherosomes, on the other hand, appeared to decrease in size during the first 24 hour period followed by an apparent increase. This latter observation correlates well with the increase in dry matter recorded during this period.

The major similarity between ethrel and gas treated seeds during dormancy breaking lay in the appearance of the nuclei (Plate 3.10). The nuclei of cells from the radicle tip of ethrel treated seeds sampled after 24 and 48 hours are considerably larger than those from imbibed, dormant tissue of the same age. Furthermore, the nucleoli associated with these cells lack vacuoles and are more granular than their dormant counterparts. These observations, together with the increased population of ribosomes in the nucleoplasm and cytoplasm of these cells, suggested that considerable nuclear activity was taking place. In

Plate 3.10

THE FINE STRUCTURE OF RADICLE TIP CELLS TAKEN FROM *RICINODENDRON RAUTANENII* SEEDS AFTER 24 AND 48 HOUR INCUBATION ON VERMICULITE MOISTENED WITH ETHREL. THE EFFECTIVE ETHYLENE CONCENTRATION OF THE ETHREL USED WAS 200 $\mu\ell\ \ell^{-1}$.

a: After 24 hours incubation, rehydration of the cytoplasm is clearly visible. When compared with dry tissue, the spherosomes (S) in these cells are more widely dispersed indicating that mobilization had occurred. In addition, protein hydrolysis has also commenced as suggested by the internal fragmentation visible in the protein bodies (P). The nucleus visible in this micrograph shows that rehydration of the nucleoplasm (N) has taken place although the nucleolus (Nu) is small. Microbodies (Mb) are scarce at this stage.

b: After 48 hours incubation with ethrel the ribosome (R) content of the cells has increased considerably. The lipid reserves (S) have remained virtually unchanged. Protein bodies (P) are still visible indicating that hydrolysis of this reserve is incomplete.

c: The nuclei (N) of cells taken from seeds incubated with ethrel for 48 hours show a definite increase in nucleolar (Nu) size suggesting the onset of nuclear activity.

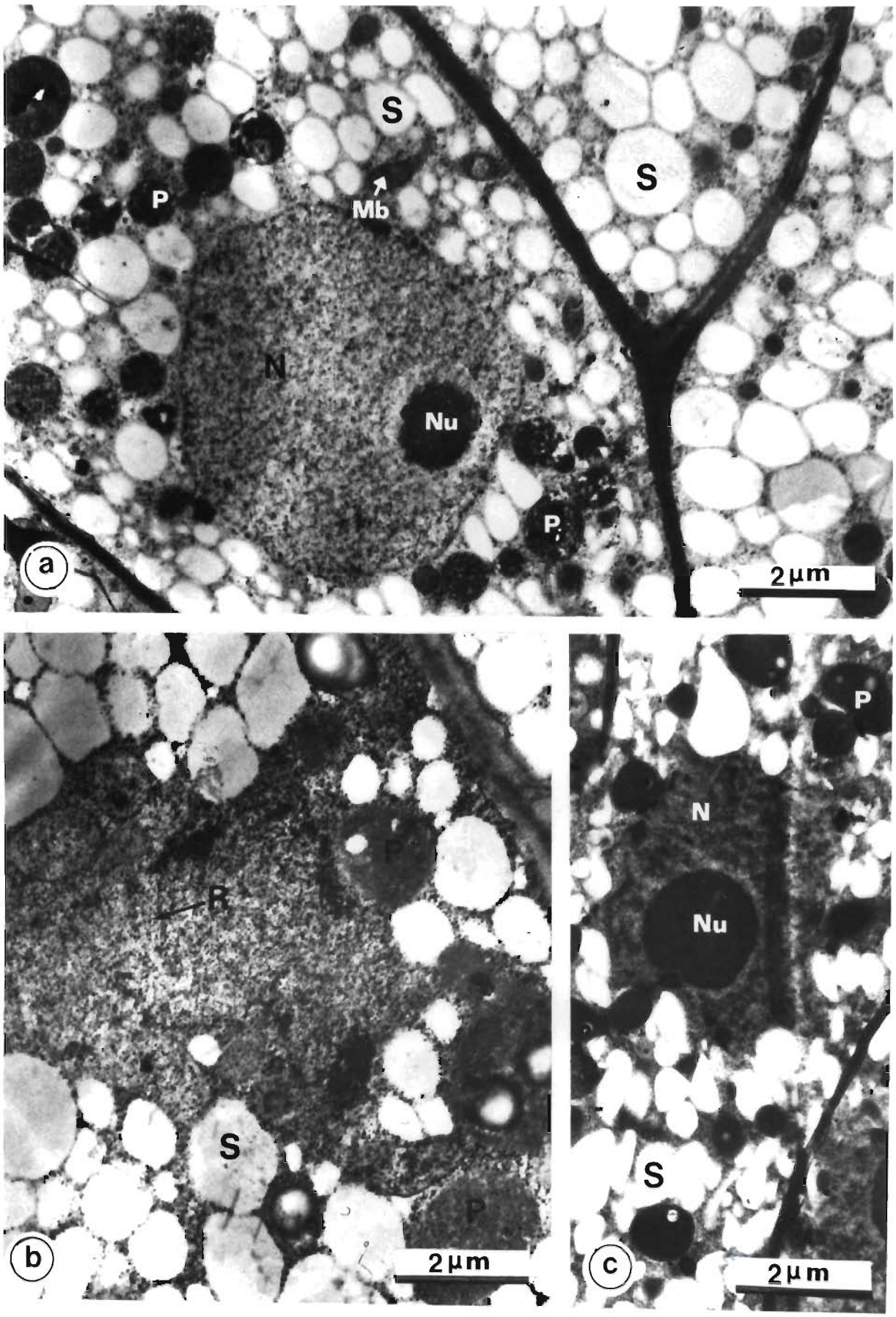
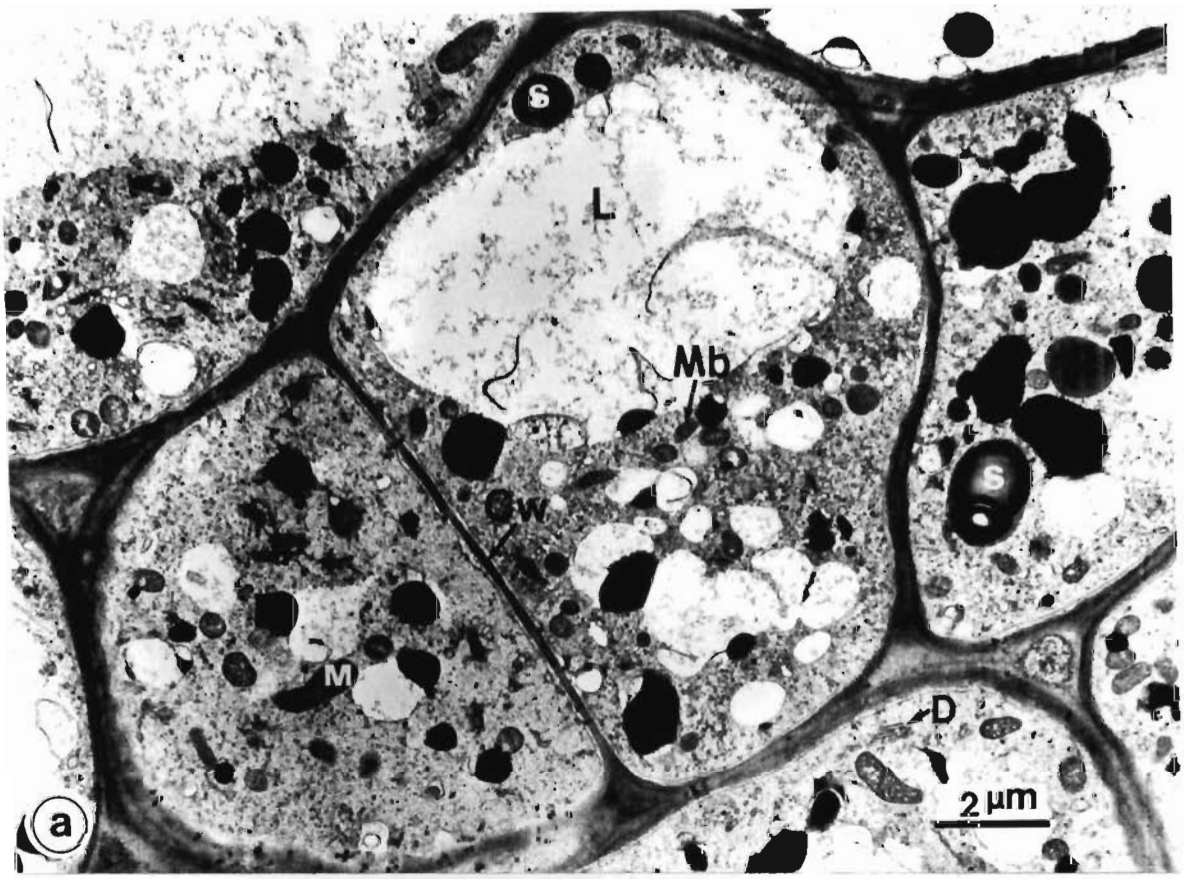

S
S
P
Mb
N
Nu
P
a
2µm
P
R
P
S
P
b
2µm
P
N
Nu
S
c
2µm

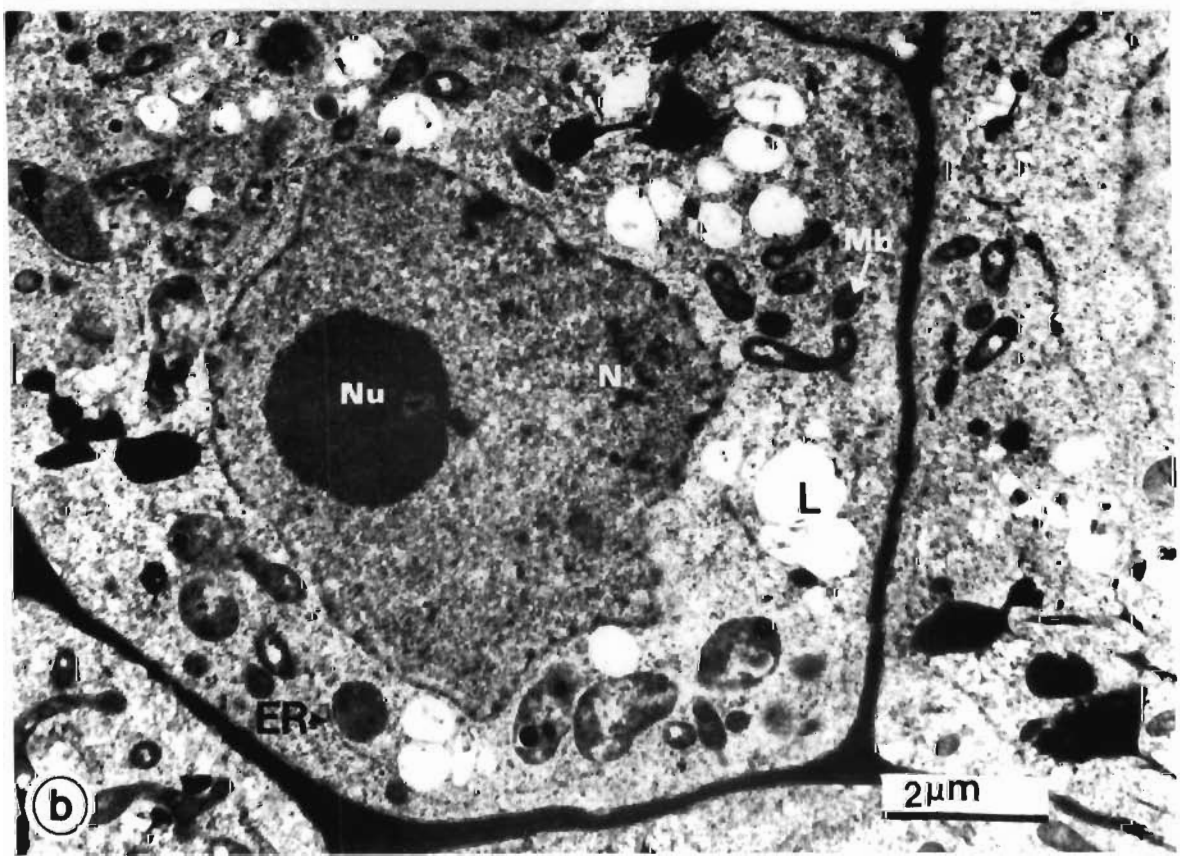
Plate 3.11

MICROGRAPHS SHOWING THE ULTRASTRUCTURE OF RADICLE TIP CELLS TAKEN FROM SEEDS AFTER 72 AND 96 HOURS INCUBATION ON VERMICULITE MOISTENED WITH ETHREL. GERMINATION HAD COMMENCED BY THE THIRD DAY AND AFTER FOUR DAYS 75 PER CENT OF THE SEEDS HAD GERMINATED.

a: After 72 hours incubation the cells show the typical coalescence of lipid bodies into large vacuoles (L). Microbodies (Mb) are present and appear to be in close proximity to the lipid reserves. The presence of starch grains (S) suggests that lipid mobilization has commenced. Mitochondria (M) are now fairly well developed and this applies particularly to the internal membrane systems. A newly formed cell wall (CW) and the presence of dictyosomes indicates that meristematic activity has commenced.

b: After 96 hours incubation the cells resemble those of the meristematic tissues of other species. Apart from a small quantity of lipid (L) most of the food reserves have been depleted. Intense metabolic activity is also suggested by the lobed appearance of the nuclear envelope (Ne arrowed), the granular nature of the nucleolus (Nu) and the presence of rough endoplasmic reticulum (RER).


S
L
Mb
CW
S
M
D
2 µm
a


Mb
Nu
N
L
ER
2µm
b

cells of the cotyledon and endosperm tissues, however, no significant ultrastructural changes were noted during the dormancy breaking period.

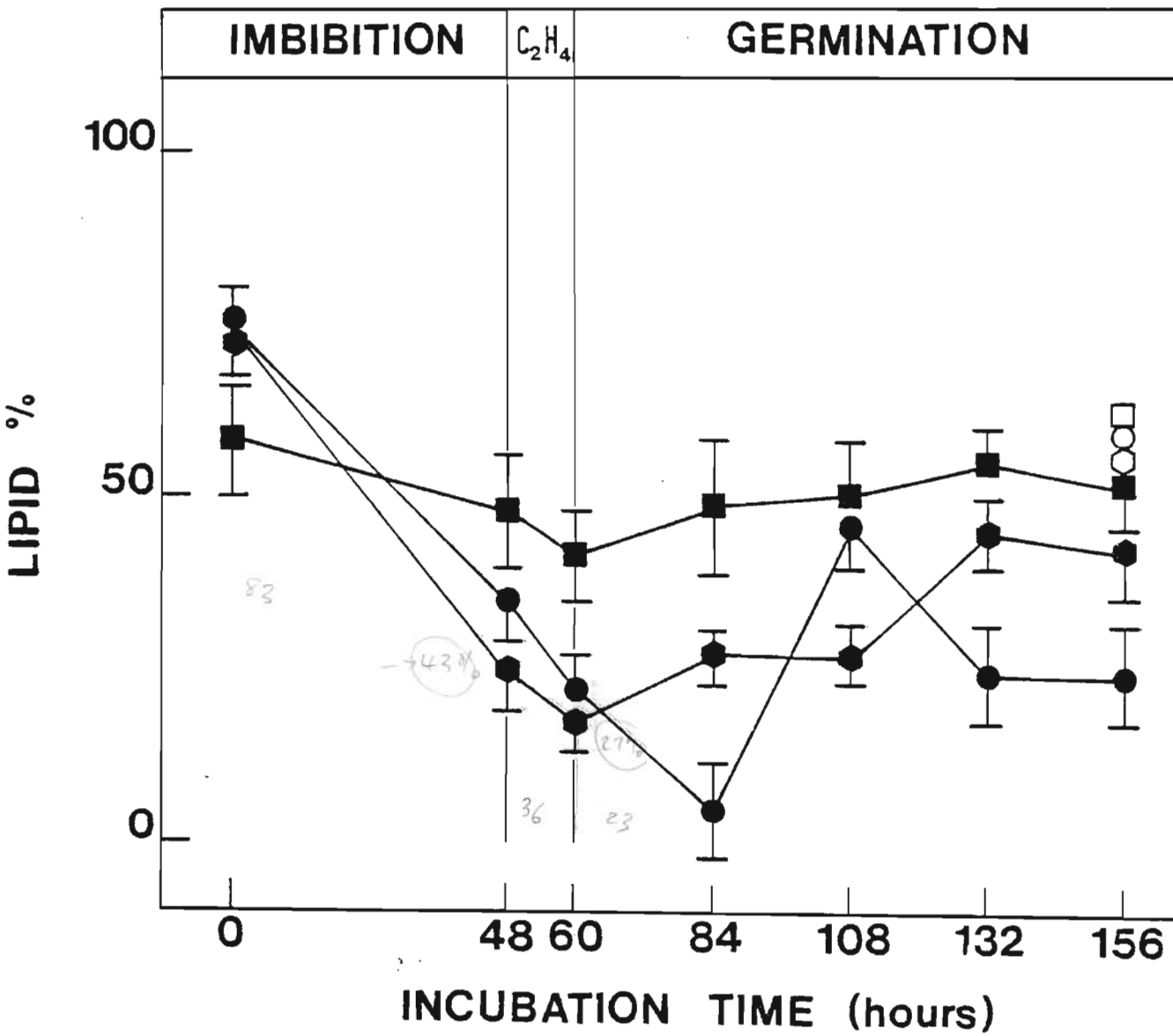
The fine structure of radicle tip cells sampled after three and four days incubation with ethrel is typical of meristematic tissue. In addition, the same coalescence of lipids, as that observed in gas treated seeds, is visible. These features are clearly unrelated to the actual period of dormancy breaking and merely reflect the consequence of this phenomenon.

The electron microscope studies carried out on *R. rautanenii* seeds provided valuable information on the ultrastructural changes associated with germination. In addition, although it was not possible to pin-point the principle vehicle for ethylene action in these seeds, two factors could clearly be associated with the dormancy breaking treatment. The first was a characteristic change in the appearance of the nucleus which preceded visible germination. This took the form of an increase in the granular content of the nucleolus which suggests that ethylene may, in some way, be responsible for stimulating the production of certain RNA fractions. The result of this would probably be a change in protein synthesis. The other factor was the notable increase in dry mass which occurred directly after the ethylene treatment. This suggested that ethylene may also be involved in the regulation of certain anabolic and catabolic reactions. Alternatively, this could be a re-

sult of dormancy breaking rather than a cause of it. In a recent study, EVANS, DODDS, LLOYD, AP-GWYNN and HALL (1982), using high resolution autoradiography, reported that the binding site for ethylene in *Phaseolus vulgaris* L. seeds was located on the endoplasmic reticulum and protein body membranes. Thus, if the ethylene binding sites are similar in manketti seeds, then ethylene may be more closely related to reserve hydrolysis than previously thought, since protein bodies were extremely well represented in *R. rautanenii* seed tissues.

The biochemical studies carried out on dormant and germinating *Ricinodendron rautanenii* seeds are complementary to the ultrastructural studies and concur to a large extent with the fine structural observations. The lipid content of the various components of these seeds is shown in Figure 3.9. An interesting observation is that during imbibition, the levels of this reserve, on a dry weight basis, decreased in all three tissues examined. This decrease continued for the duration of the ethylene treatment. After 24 hours incubation, however, this trend was reversed in the endosperm and embryonic axes. Cotyledonary tissues required a further 24 hours before a similar increase was observed. These data provided clear evidence that metabolism of the lipid reserves took place prior to the application of any dormancy breaking treatment. In addition, the breaking of dormancy was associated with an overall increase in lipid levels. The significance of this latter observation is difficult to assess in view of

Figure 3.9 The lipid content, expressed as a percentage, of the embryonic (⬢), cotyledonary (●) and endosperm (■) tissues of ethylene treated *Ricinodendron rautanenii* seeds. The lipid was extracted in a Soxhlet apparatus using petroleum ether (40-60^{o}C). The bars represent the 95 per cent confidence limits and the arrow the first day germination was recorded.


IMBIBITION
C_2H_4
GERMINATION
100
50
0
LIPID %
0
48
60
84
108
132
156
INCUBATION TIME (hours)

the fact that the lipid content of all three components also increased in untreated seeds incubated for a total of six days (Figure 3.9, open symbols). Nevertheless, a similar increase in lipid levels was noted during the germination of *Pinus ponderosa* (CHING, 1966) and *Corylus avellana* (SHEWRY, PINFIELD and STOBBART, 1973). In the latter, this increase was attributed to the renewed synthesis of phospholipids. The concomitant increase in endosperm lipid content of *R. rautanenii* seeds is unusual, since embryo growth usually proceeds at the expense of this organ. An example of this may be found in *Ricinus communis* seeds (YAMADA, 1955).

In ethrel treated seeds, a similar trend in lipid mobilization was observed in cells of the embryonic axis (Figure 3.10). However, unlike the gas treatment, lipids in the endosperm and cotyledonary tissues of these samples remained unchanged during the initial stages of germination.

The data presented in Table 3.4 show that the lipids in dry *R. rautanenii* embryonic axes were comprised mainly of linoleate (38 per cent), α eleostearate (28 per cent), oleate (16 per cent) and palmitate (8 per cent). It is interesting to note that these fatty acids are of similar importance in many other oil seeds (MEARA, 1957). The levels of linoleate and α eleostearate were found to decrease during the 48 hour period of imbibition prior to gasing, whereas oleate and palmitate levels increased.

Figure 3.10 The lipid content, expressed as a percentage, of the embryonic (⬢), cotyledonary (●) and endosperm (■) tissues of ethrel treated *Ricinodendron rautanenii* seeds. The lipid was extracted in a Soxhlet apparatus using petroleum ether (40-60°C). The bars represent the 95 per cent confidence limits and the arrow the first day germination was recorded.

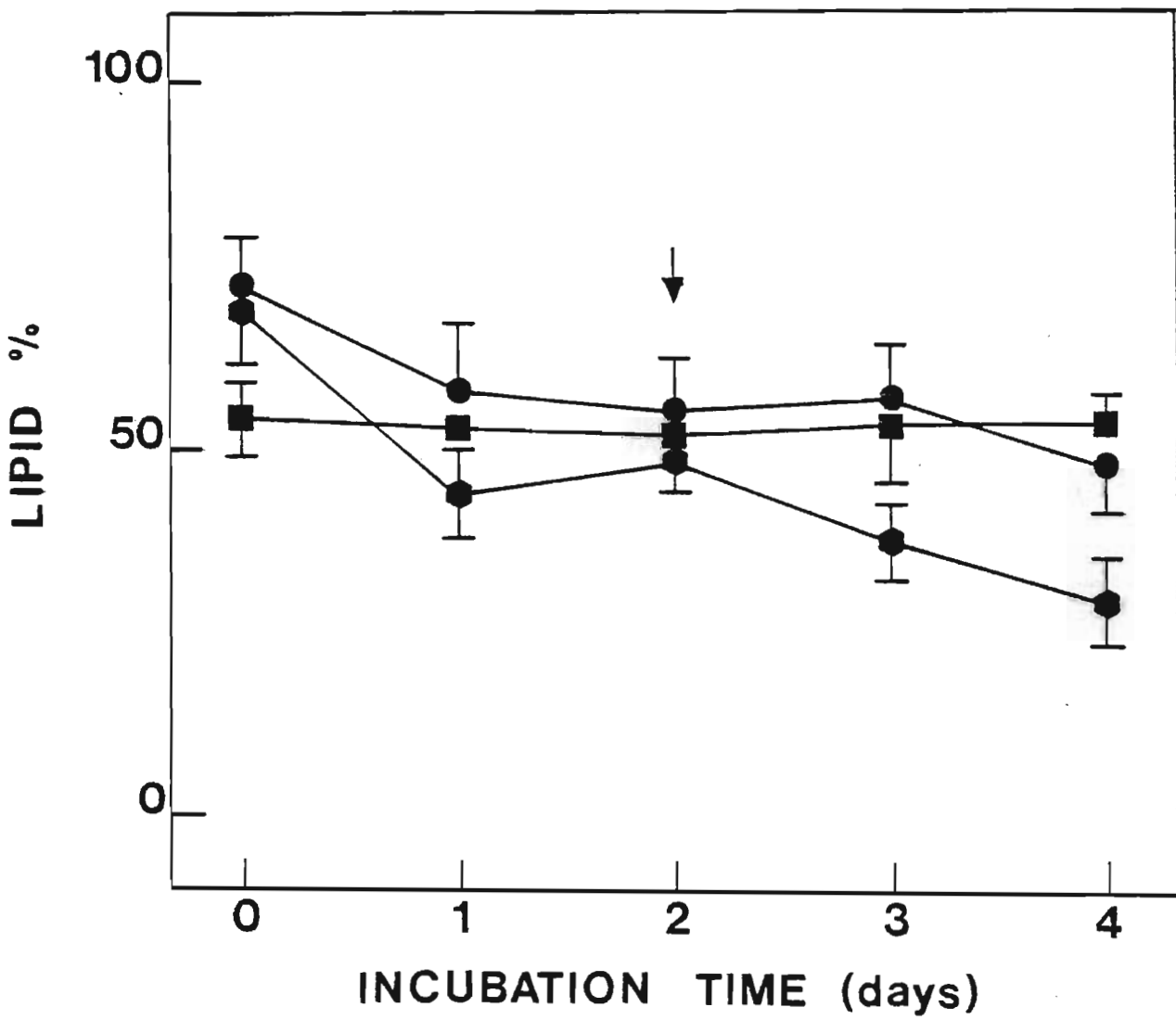

100
50
0
LIPID %
0
1
2
3
4
INCUBATION TIME (days)

Table 3.4 Changes in the constituent fatty acids of *Ricinodendron rautanenii* embryonic axes during imbibition and dormancy breaking. The results are expressed as percentages of the total fatty acid content. 'T' denotes trace amounts.

Fatty Acid	Symbol	Embryonic Axis							
		Sample Time (hours)							
		0	48	60	84	108	132	156	156 (untreated)
Myristic	14:0	T	0,3	0,5	T	0,3	0,3	0,4	T
Palmitic	16:0	7,7	10,7	9,1	8,2	8,2	9,0	7,5	8,0
Stearic	18:0	6,6	8,0	7,8	8,0	7,3	7,7	7,1	7,2
Oleic	18:1	16,0	19,2	19,0	18,8	16,9	18,1	15,2	15,4
Linoleic	18:2	38,6	36,2	34,6	35,2	36,1	35,5	34,1	33,0
Linolenic	18:3	T	0,3	0,3	T	T	0,4	1,0	T
Nonadecanoic	19:0	T	0,4	0,5	T	T	0,3	0,3	0,3
Nonadecadienoic	19:2	T	0,3	0,4	T	0,3	0,4	0,6	T
Arachidic	20:0	0,4	0,7	0,5	0,5	0,5	0,5	0,5	0,4
Gadoleic	20:1	T	0,6	0,4	0,6	0,5	0,5	0,6	0,6
α Eleostearic	18:3	28,3	19,3	17,8	26,2	21,8	21,3	24,3	30,2
β Eleostearic	18:3	0,9	2,4	7,0	1,4	6,5	4,7	5,0	3,3
Total % fatty acid/sample		82,9	51,9	54,1	63,7	66,7	69,2	74,4	83,5

It is not clear whether or not this represents a preferential metabolism of these fatty acids during this period of rehydration. That metabolism was in fact taking place, was suggested by the finding that there was a considerable decline in the percentage of total fatty acids per sample. When incubation of these seeds without ethylene was continued for a further six days, the relative proportions of these fatty acids, as well as the total percentage fatty acids per sample, were restored to almost their original values. This indicated that further metabolism of these compounds had taken place and that resynthesis of reserves may occur if a dormancy breaking stimulus was not forthcoming. However, during the period of dormancy breaking and germination, the fatty acid content of the axes remained virtually unchanged in spite of the general increase in extractable lipid. This suggested that during germination, no preferential metabolism of these fatty acids occurred and it seemed more likely that lipid biosynthesis was taking place, since the total fatty acid content of each sample increased during this period. These findings are consistent with the observations made in many other species and therefore probably represent a general phenomenon in germinating oil seeds (HITCHCOCK and NICHOLS, 1977). (An exception to this, however, is the preferential metabolism of oleate by watermelon seedlings (CROMBIE and COMBER, 1956)). These findings are important in terms of the fact that ethylene does not seem to have its effect by stimulating the preferential metabolism of a specific fatty acid.

Lipid breakdown commences with the action of lipase enzymes which may be present in the dry seed tissue depending on the species (BEWLEY and BLACK, 1978). This enzyme splits the fat molecules into their constituent fatty acid and glycerol components. The glycerol molecules are then converted to hexoses within the cytosol, whereas the free fatty acids are thought to pass into glyoxysomes where they undergo β-oxidation. Gluconeogenesis from free fatty acids is illustrated in Figure 3.11. The electron microscope studies carried out on *R. rautanenii* embryonic axes revealed that glyoxysomes were, in fact, rare during the period of maximum lipid decline. This suggested that gluconeogenesis may be different in this species and a similar finding was reported for seeds of *Podocarpus henkelii* Stapf. (DODD, 1981). This was also supported by the fact that no starch accumulation was visible in these axes during imbibition and such an accumulation is frequently associated with lipid hydrolysis. However, the absence of starch may also indicate that the sugars generated during this period are rapidly utilized. In spite of this uncertainty, the results clearly show that it is unlikely that ethylene stimulates lipase activity, since the levels of extractable lipids declined (and for that matter, also increased) in the absence of any dormancy breaking treatments.

The changes in the ethanol soluble fractions from the three tissue components of both gas and ethrel treated seeds showed similar trends (Figures 3.12a and b). These extracts usually contain the soluble sugars and free amino

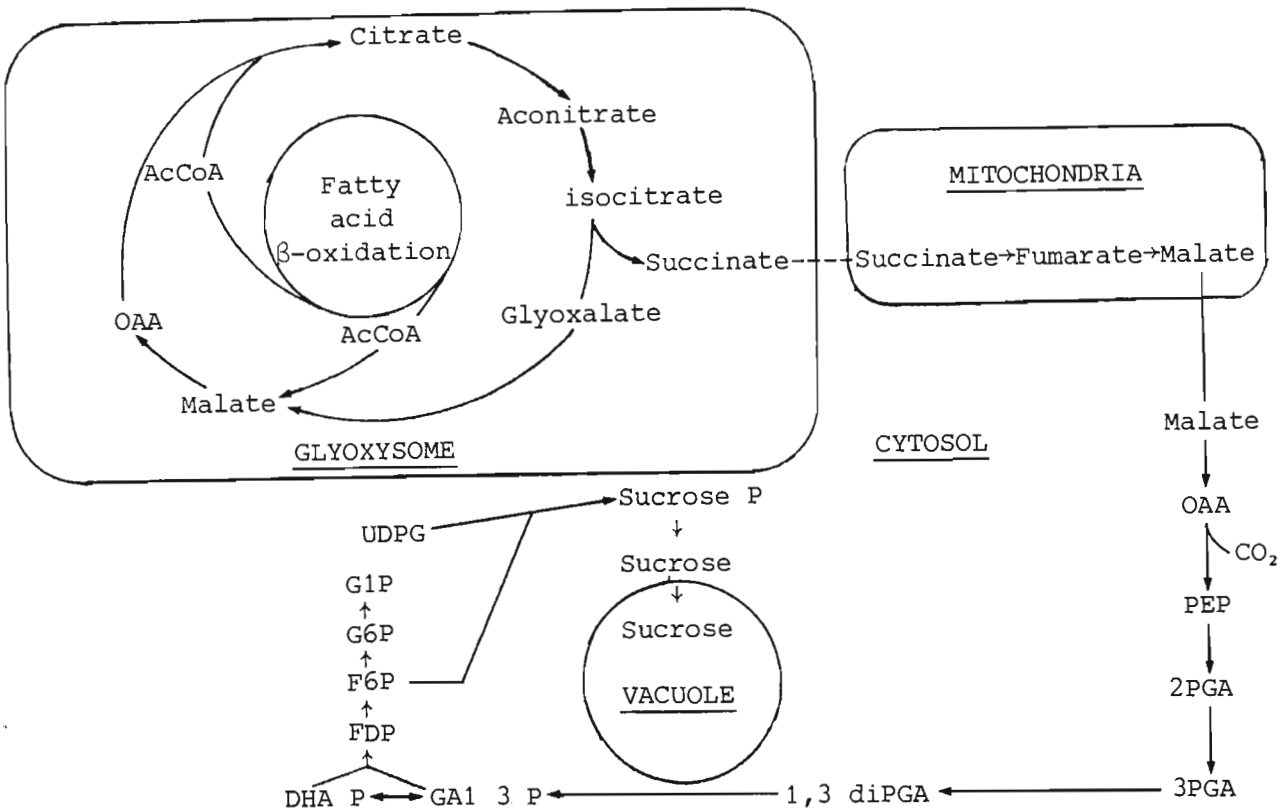
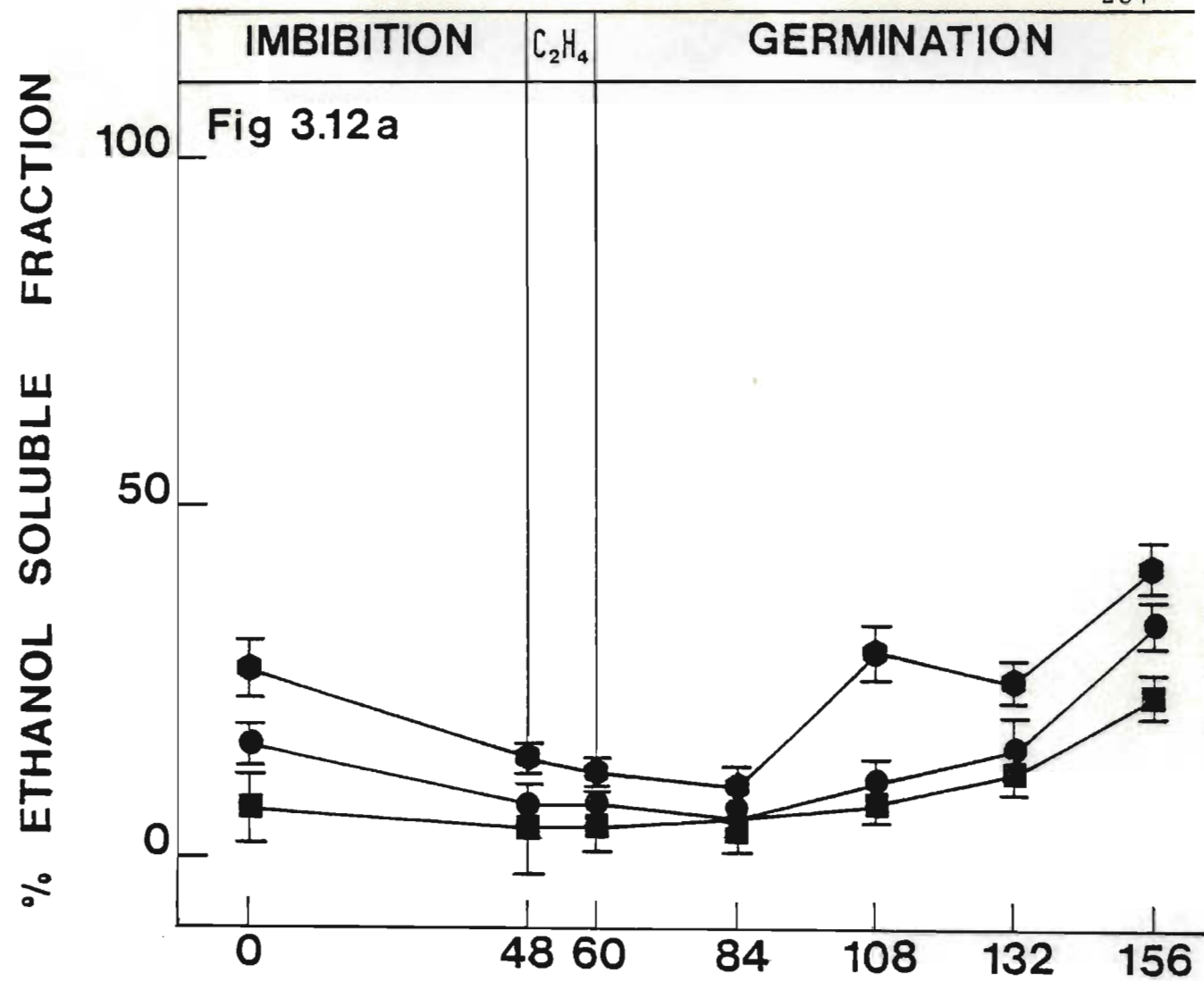


Fig. 3.11 Intracellular distribution of enzyme systems responsible for the conversion of fatty acid to sucrose in castor bean endosperm. In this representation malate leaves the mitochondria and is oxidized in the cytosol, yielding the NADH required for reduction of 1,3 diphosphoglycerate to triose-P. The vacuole is shown as a repository of sucrose (taken from NISHIMURA and BEEVERS, 1979).

acids. In the embryonic axes it was noted that, as with the lipids, these substances decreased during imbibition. This suggested that the decrease in the ethanol soluble fraction observed in the ethrel treated axes during the first 24 hours may be unrelated to the presence of ethrel.

The sugars were separated from the amino acids using a cation exchange resin and the results of this are presented in Figures 3.13a and b. Figure 3.13a shows that dry *R. rautanenii* seed material contained considerable amounts of free sugars and that some utilization of these compounds


IMBIBITION
C_2H_4
GERMINATION
Fig 3.12a
% ETHANOL SOLUBLE FRACTION
100
50
0
0
48
60
84
108
132
156

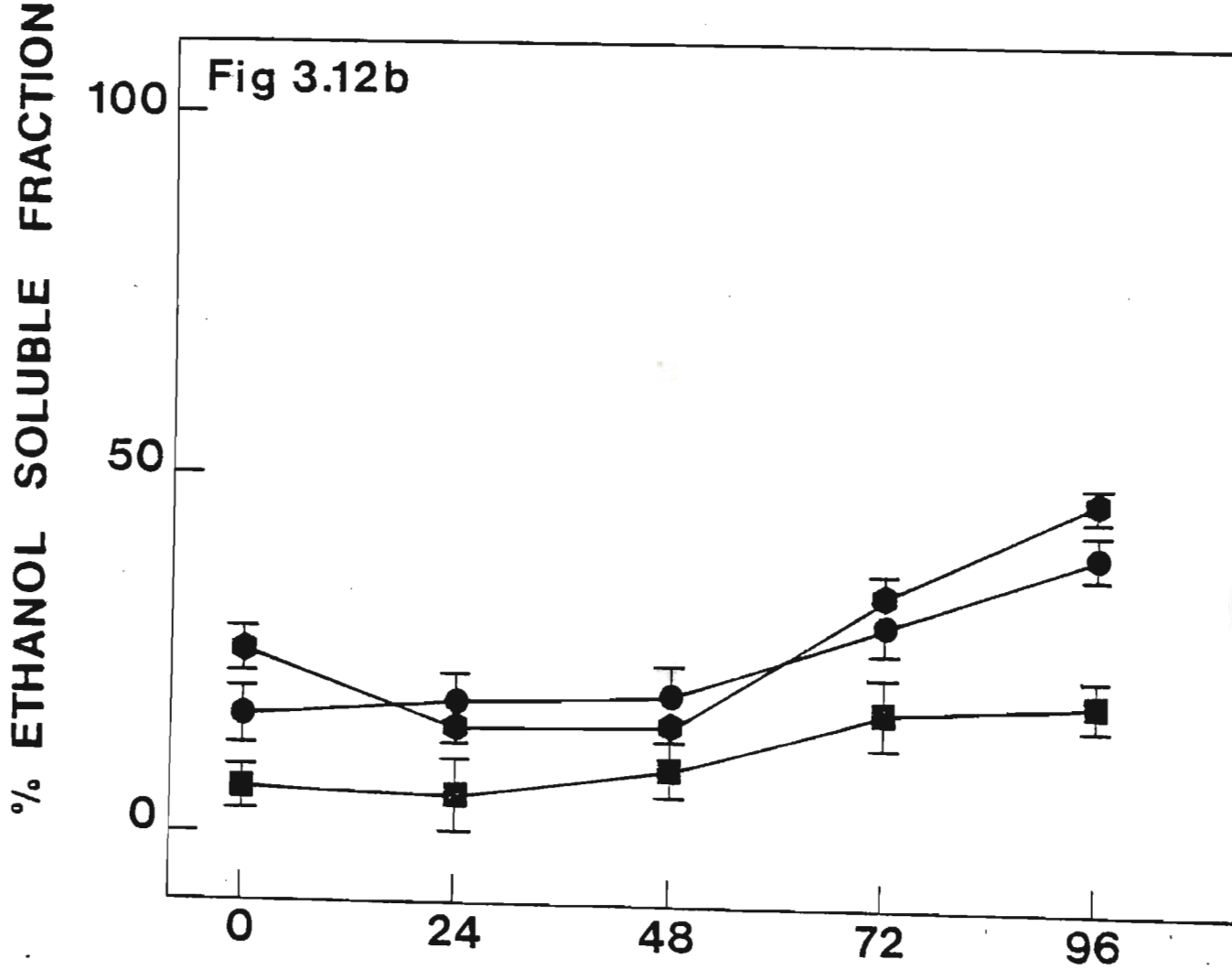
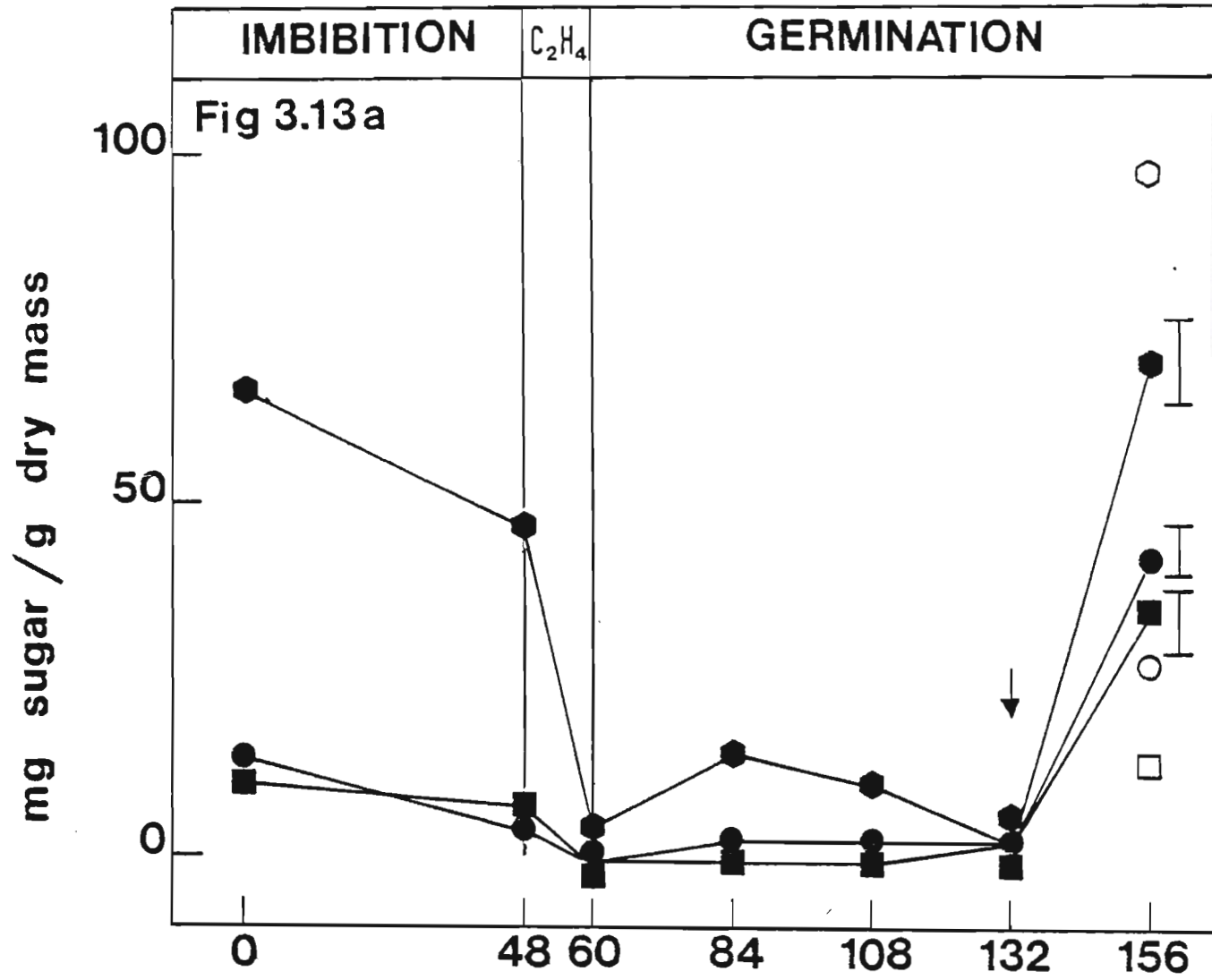


Fig 3.12b
% ETHANOL SOLUBLE FRACTION
100
50
0
0
24
48
72
96

appeared to take place during the period of imbibition preceding ethylene treatment. However, these substances were drastically reduced in samples taken immediately after the dormancy breaking treatment and this was most notable in the embryonic axes. Following this, the only other significant change occurred once mobilization of the endosperm reserves appeared to have commenced. Seeds imbibed without ethylene for six days (Figure 3.13a, open symbols), showed a reaccumulation of soluble carbohydrates and these levels in fact exceeded the values recorded in the dry tissue. In ethrel treated seeds, the overall trend in sugar utilization was essentially the same, although the initial decrease in sugar levels could possibly, in this instance, be attributed to the presence of ethrel (Figure 3.13b). These results indicated that any sugars which may have resulted from the hydrolysis of lipid reserves prior to ethylene treatment, were not accumulated in these tissues. On the contrary, a modest decline in soluble carbohydrates was observed during this period. Application of the dormancy breaking treatment resulted in an almost complete disappearance of the free sugars and whether this was due to an increase in metabolism, was not clear since carbon dioxide evolution remained unchanged (Figure 3.7a). Three days after the application of the dormancy breaking treatment, the sugar levels again increased. This correlated well with the increase in water uptake, which according to CHING (1972) would result from the increased osmotic potential caused by these sugars.

Figure 3.13 The total free sugars extracted with ethanol from the embryonic axis (⬢), cotyledonary (●) and endosperm (■) tissues of ethylene (a) and ethrel (b) treated *Ricinodendron rautanenii* seeds. The open symbols in Fig. 3.13a represent the sugars present in the respective tissues of untreated, imbibed seeds after 156 hours incubation at 30°C. The results are expressed as milligrammes of sugar per gramme dry mass. The sugars were separated and quantified on a high pressure liquid chromatogram. The bars represent the maximum 95 per cent confidence limits and the arrows the first day germination was recorded.


IMBIBITION
C_2H_4
GERMINATION
Fig 3.13a
100
50
0
mg sugar /g dry mass
0
48
60
84
108
132
156

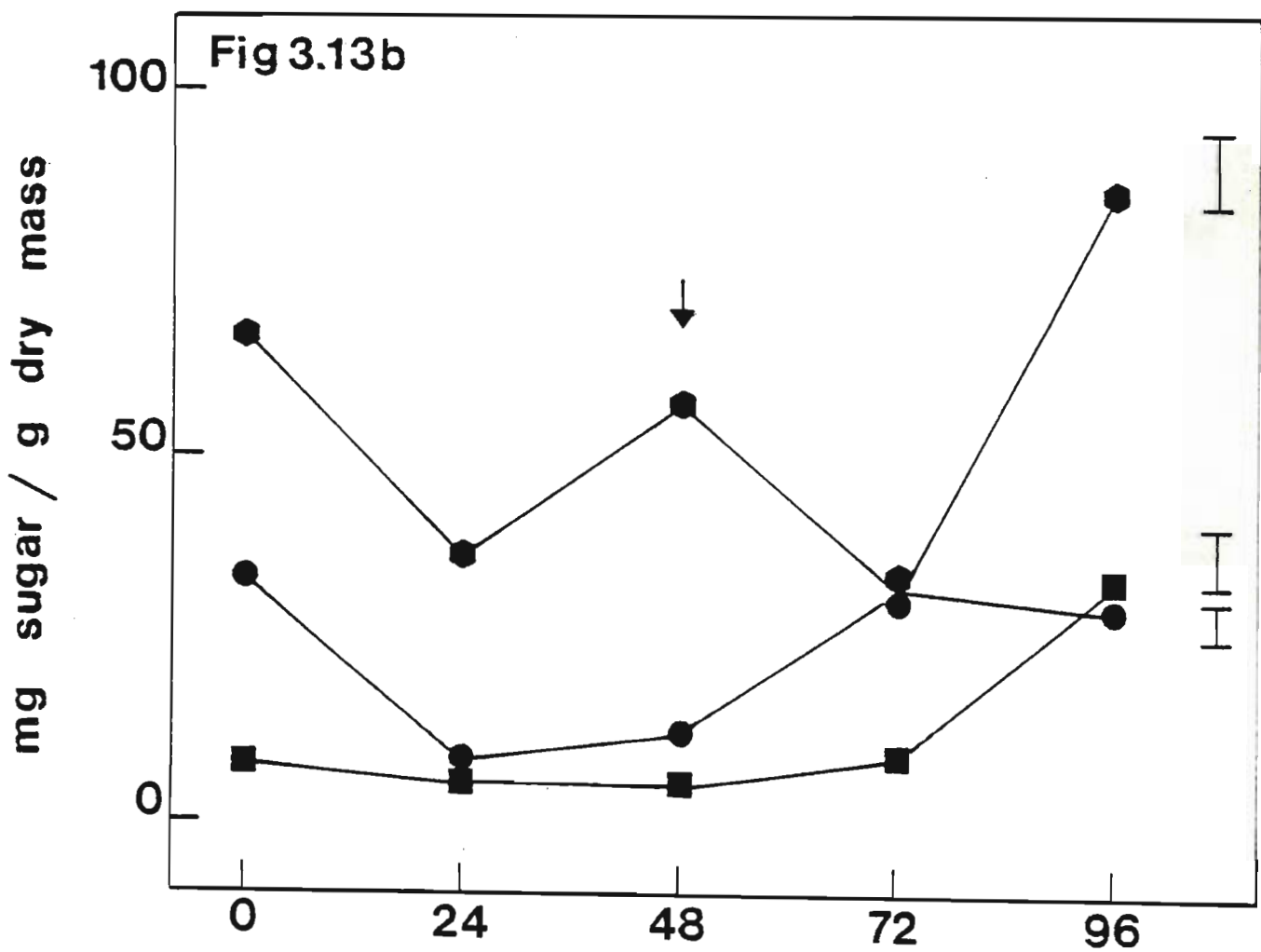


Fig 3.13b
100
50
0
mg sugar / g dry mass
0
24
48
72
96

Separation and identification of the sugars present in the extracts from gas treated seeds was achieved using high pressure liquid chromatography. From this it was found that ribose, glucose, fructose, sucrose and maltose were the most important sugars present in these seeds (Table 3.5). Ribose only made its appearance in the embryonic axes 24 hours after the ethylene treatment and no amount was detected in the dry tissue. This finding may be significant in terms of dormancy breaking since ribose could be involved in nucleic acid synthesis. Unlike the situation in the embryonic axes, ribose was detected in samples from dry cotyledonary and endosperm tissues and imbibition resulted in a decline in these levels. However, after treatment with ethylene this sugar disappeared altogether in these tissues. Subsequent to dormancy breaking, the pattern of ribose metabolism in the cotyledons was similar to that observed in the embryonic axis, possibly indicating that, here too, nucleic acid synthesis had recommenced. In endosperm tissues, on the other hand, this sugar was absent from all remaining samples.

Glucose and fructose seldom accumulate in seed tissues since they are relatively unstable (BEWLEY and BLACK, 1978). However, small quantities of these sugars were detected in the dry embryonic axes and endosperm tissues of *R. rautanenii* seeds. In these organs the levels of both compounds were found to decline after 48 hours imbibition, whereas increases were recorded in the cotyledonary material. These fluctuations probably reflect the initial rapid

Table 3.5 Ethanol soluble sugars extracted from the embryonic axes, cotyledons and endosperm of dormant and germinating *Ricinodendron rautanenii* seeds. Quantification was carried out on a high pressure liquid chromatogram.

Sugar Detected	Embryonic Axis							
	Sample Time (hours)							
	0	48	60	84	108	132	156	156 (Untreated control)
Ribose	0	0	0	6,7	3,94	0,73	0	0
Fructose	1,36	0	0	0	0	0,61	19,88	95,4
Glucose	1,65	0	0	1,24	0	0,55	4,85	0
Sucrose	19,17	6,19	0	6,65	0	0	25,2	6,19
Maltose	1,01	5,06	0	0	6,10	1,13	4,01	2,51
	Cotyledons							
	Sample Time (hours)							
	0	48	60	84	108	132	156	156 (Untreated control)
Ribose	4,87	0,51	0	0,10	0,72	0,55	0	0
Fructose	0	1,39	0,13	0	0,71	0	24,55	14,59
Glucose	0	0,01	0	0	0,3	1,85	0	0
Sucrose	0	0,47	0	0,55	0,55	0	15,28	7,66
Maltose	6,44	10,55	0	1,8	0	0	0,78	0,77
	Endosperm							
	Sample Time (hours)							
	0	48	60	84	108	132	156	156 (Untreated control)
Ribose	1,27	0,54	0	0	0	0	0	0
Fructose	2,86	0,57	0,112	0	0,53	0,64	8,28	5,15
Glucose	2,34	0,71	0	0	1,14	1,181	5,32	4,56
Sucrose	3,83	5,13	0	0,48	0	0	11,76	2,09
Maltose	0	0,14	0	0	0,32	0,46	0,95	0

utilization of reserves as a result of renewed cellular activity. Twenty four hours after the ethylene treatment, a transient increase in glucose levels was observed in the embryonic axis, followed by another increase 48 hours later. The former peak may reflect the increase in metabolic activity resulting from dormancy breaking, whereas the latter is probably the normal accumulation of sugars associated with germination (METIVIER and PAULILO, 1980). With regard to the endosperm and cotyledonary material, the only other important changes which occurred in the levels of fructose and glucose, took place when the first signs of germination were already visible.

Sucrose is an important sugar in that it is the means by which carbohydrates are transported in plant tissues (BEWLEY and BLACK, 1978). This is probably due to its stability and relative ease with which it can be hydrolyzed. In dry *R. rautanenii* tissue, considerable quantities were detected in the embryonic axis, somewhat less in the endosperm and none in the cotyledons. In the embryonic axes, sucrose levels decreased after imbibition and then disappeared altogether after the ethylene treatment. This was probably also related to the renewed cellular activity. Furthermore, the transient reappearance of sucrose 24 hours after the application of ethylene may also, like glucose, reflect the activities associated with dormancy breaking. The large amounts measured after four days, on the other hand, were undoubtedly due to endosperm hydrolysis.

Like the monosaccharides, maltose seldom accumulates in plant tissues and is more frequently associated with starch synthesis and hydrolysis (DEVLIN, 1975). Thus, the changes observed in the levels of this sugar were most likely related to changes occurring in starch metabolism.

An interesting finding to emerge from these results was that dormant seeds showed considerable changes in the carbohydrate status between the second and sixth days of imbibition. In this respect, considerable quantities of fructose and somewhat lesser amounts of sucrose were found to accumulate in the imbibed but dormant tissues after a period of time. The significance of this is not clear, but it provides additional support for the view that reserves are resynthesized in imbibed seeds if a dormancy breaking stimulus is not forthcoming.

In this study, the changes observed in the levels of specific sugars in the embryonic axis and endosperm tissues, initially seemed unlike those observed in other species such as *Pseudotsuga manziesii* Mirb. (CHING, 1966) and *Phaseolus vulgaris* (METIVIER and PAULILO, 1980). In these instances the levels of sugars increased in the embryos during germination. However, it must be borne in mind that *R. rautanenii* seeds are different in the sense that they first undergo a period of dormancy breaking. Thus, the early stages of germination in non-dormant species probably corresponds to the third and fourth days after ethylene treatment in manketti seeds. Samples of *R. rautanenii*

tissue taken on these days showed that the sugar levels were on the increase.

The most important observation made in this study was that catabolism of the soluble carbohydrate reserves occurred prior to the application of ethylene. This suggested that; (a) the enzymes necessary for this catabolism were already present in dry *Ricinodendron rautanenii* tissue or were synthesized soon after the onset of imbibition; and (b) ethylene had no effect on the activity of these enzymes since there was a considerable reduction in the levels of the major sugars prior to the application of this gas. In addition, the main source of these carbohydrates appeared to be the axis itself, which is unlike the situation in *Lactuca sativa* seeds where the endosperm provides the initial stored reserves for early axis growth (BEWLEY and HALMER, 1981).

The starch content of dry *R. rautanenii* seed tissues was found to be low and disappeared altogether after 48 hours imbibition (Figure 3.14a). In the embryonic axes, this starch was most probably associated with cells found in the peripheral regions of the radicle (as noted by VILLIERS (1971) in *Fraxinus excelsior* seeds) since there was no evidence of amyloplasts in the meristematic tissues examined under the electron microscope. The only significant effect of the dormancy breaking ethylene treatment on starch content was observed after 48 hours incubation. At this time the starch content of embryonic axes increased to 200 milli-

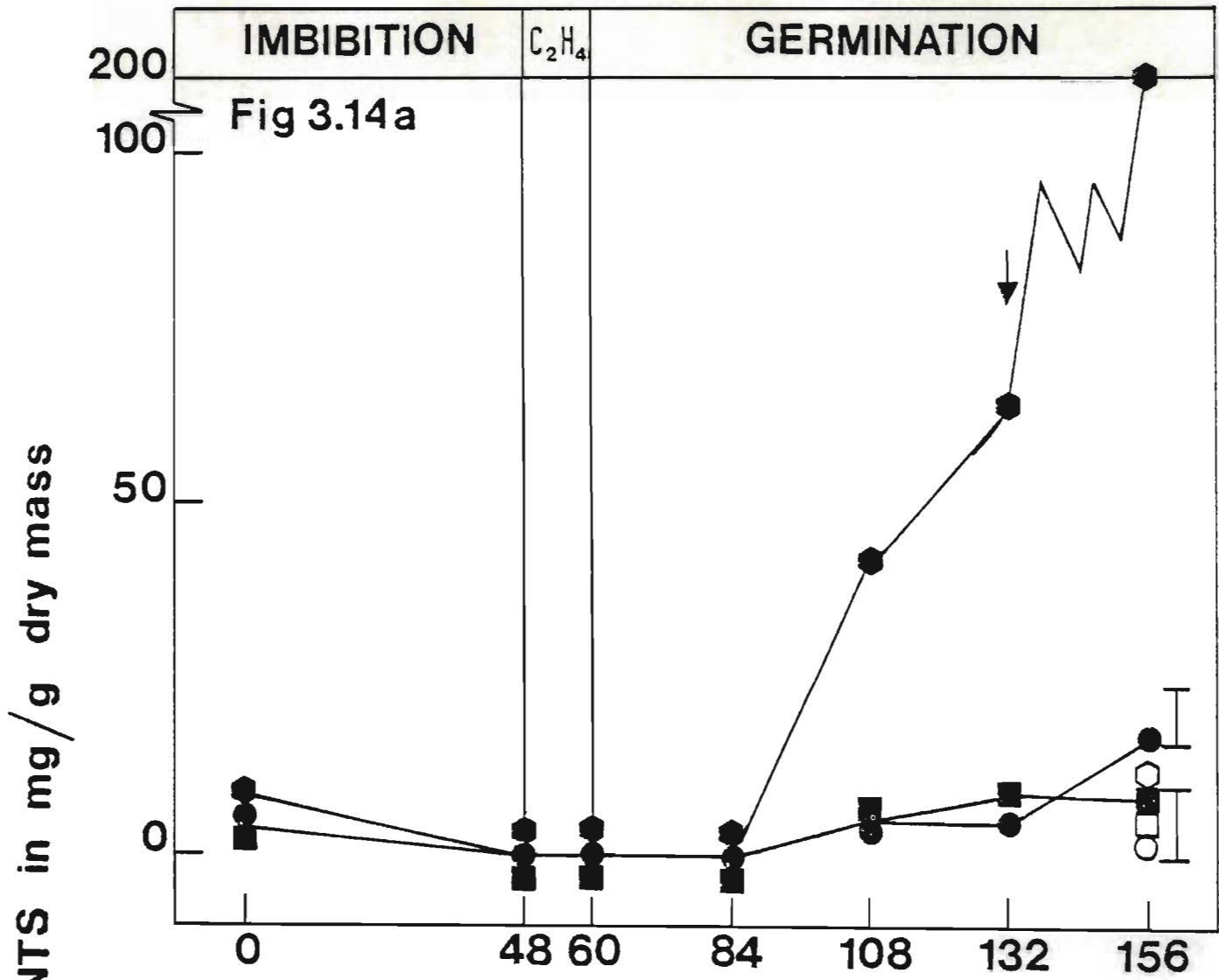
grammes per gramme dry mass. This also coincided with the first visible signs of germination, and the probable onset of lipid hydrolysis in the endosperm. A similar trend was observed in the embryos of the gymosperm *Pseudotsuga manziesii* (CHING, 1966). However, amyloplasts themselves were only visible in cells of the radicle tip three days after the application of ethylene.

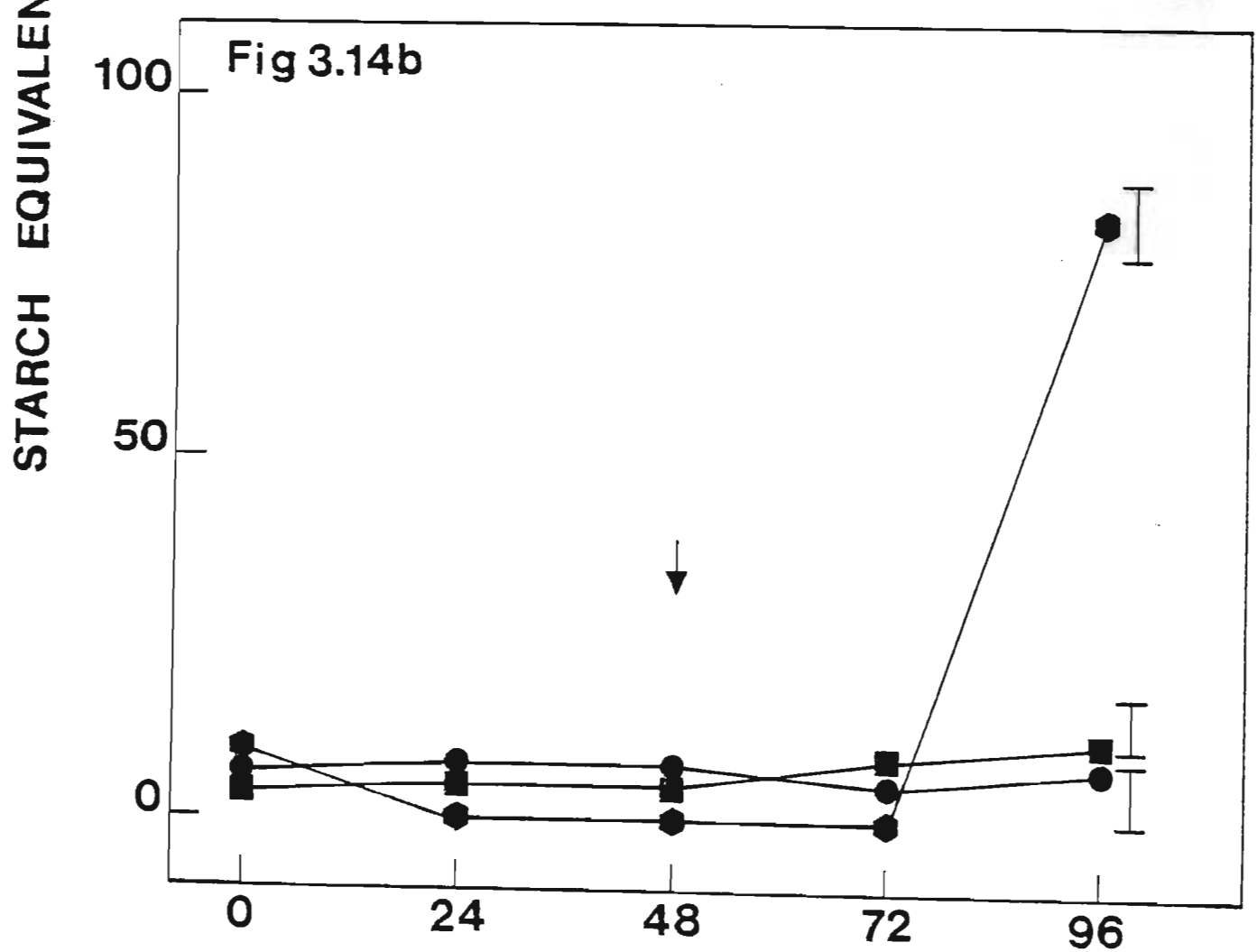
It is interesting to note that, like the other reserves discussed so far, starch also reappeared in dormant tissues after a further period of imbibition (Figure 3.14a, open symbols). This indicated that both hydrolysis and synthesis of this reserve can occur in the dormant seeds. A similar trend in starch hydrolysis was observed in the embryonic axes of ethrel treated seeds (Figure 3.14b). However, the levels of this reserve remained virtually unchanged in the cotyledons and endosperm tissues.

These results indicated that in *R. rautanenii* seeds, starch metabolism was probably not directly related to dormancy breaking and only becomes important once germination is already under way. In many other non-starchy seeds, this carbohydrate is a somewhat transitory reserve and is mostly associated with lipid hydrolysis (BEWLEY and BLACK, 1978).

The protein content of the endosperm and cotyledonary tissues of ethylene and ethrel treated *R. rautanenii* seeds remained virtually unchanged throughout the experimental period (Figures 3.15a and b). In the embryonic axis, however, a

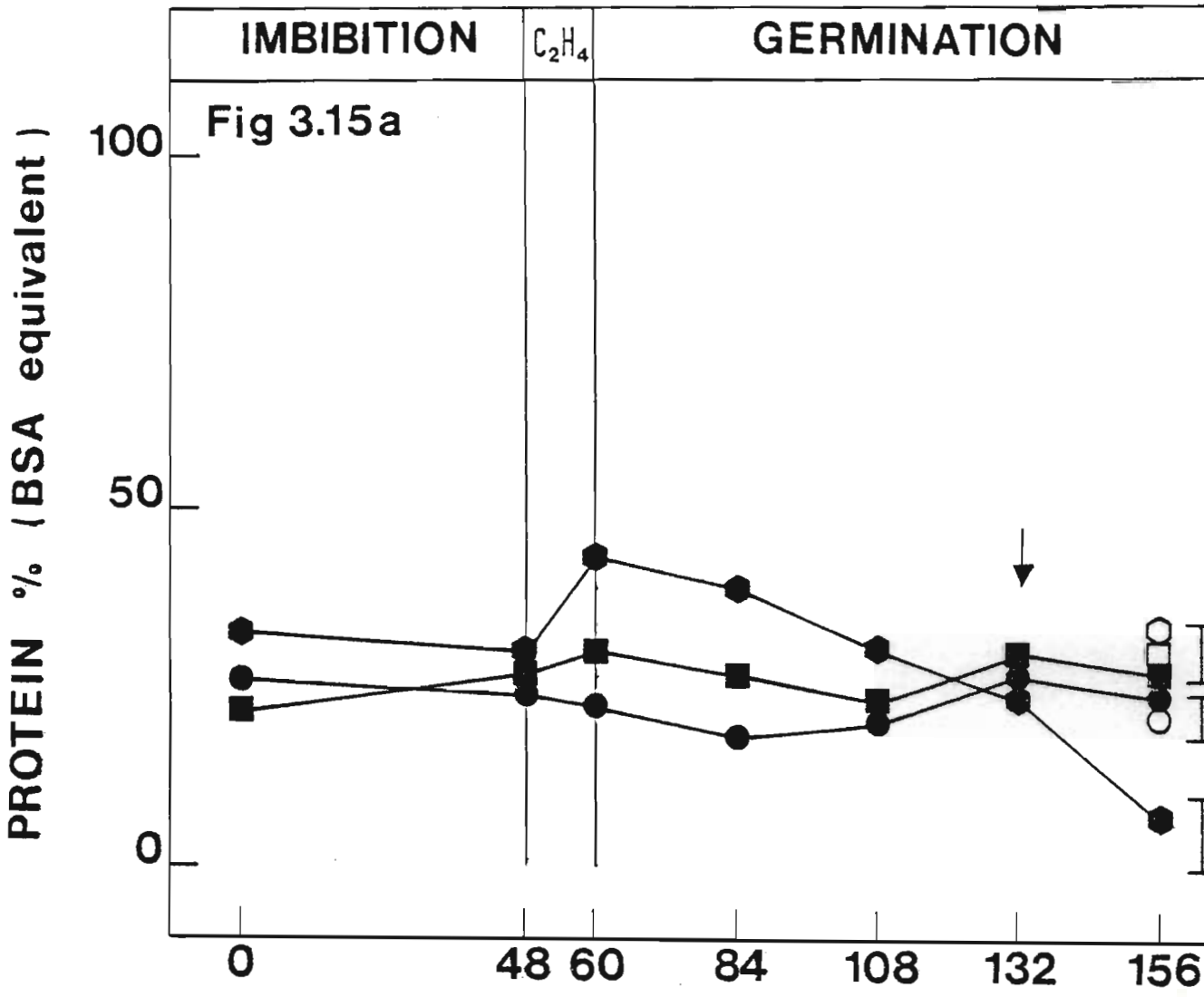
Figure 3.14 The starch content of the embryonic axes (⬢), cotyledonary (●) and endosperm tissues (■) of ethylene (a) and ethrel (b) treated *Ricinodendron rautanenii* seeds. The open symbols in Fig. 3.14a represent the starch content of the respective tissues of untreated, imbibed seeds after 156 hours incubation at 30°C. The starch extracts were converted to glucose with enzymes and quantified using a high pressure liquid chromatogram. The results are expressed in milligrammes of starch equivalents per gramme dry mass. The bars represent the maximum 95 per cent confidence limits and the arrows the first day germination was recorded.


IMBIBITION
C_2H_4
GERMINATION
200
100
50
0
Fig 3.14a
0
48 60
84
108
132
156


STARCH EQUIVALENTS in mg/g dry mass
100
50
0
Fig 3.14b
0
24
48
72
96

slight decline in protein levels occurred as a result of imbibition but a further four day incubation of these dormant seeds had no additional effect (Figure 3.15a, open symbols). Ethylene treatment, on the other hand, resulted in an immediate increase in the total protein levels followed by a gradual reduction in this component during the subsequent period of dormancy breaking and radicle emergence (Figure 3.15a). These results concur with the fine structural observations, except that no increase in protein bodies was noted as a result of ethylene treatments. This pattern of protein hydrolysis was not as marked in the axes of ethrel treated seeds (Figure 3.15b). One possible reason for this could be that a transitory 12 hour increase would not be detected in the sampling periods used. The overall reduction in protein levels is not typical of germinating axes, since germination is more frequently associated with an accumulation of protein. However, ROST (1972) reported a similar decline in protein levels during the very early stages of *Setaria lutescens* germination. Furthermore, it should be borne in mind that protein hydrolysis usually results in the release of free amino acids (BEWLEY and BLACK, 1978). Therefore, it is unlikely that there is a net loss in total nitrogen during the imbitition and germination of *R. rautanenii* seeds. Furthermore, it should be borne in mind that the technique used to detect these compounds would not distinguish between storage and structural proteins. It is interesting to note that during the period investigated, the endosperm made no measurable contribution to the protein content of the embryonic axis.

Figure 3.15 The protein content of the embryonic axes (⬢), cotyledonary (●) and endosperm tissues (■) of ethylene (a) and ethrel (b) treated *Ricinodendron rautanenii* seeds. The open symbols in Fig. 3.15a represent the protein content of the respective tissues of untreated, imbibed seeds after 156 hours incubation at 30°C. The protein was extracted with alcoholic Na OH and quantified using the Biuret technique with BSA as a standard. The results are expressed as percentage milligrammes protein per gramme dry mass. Bars represent the maximum 95 per cent confidence limits and the arrows the first day germination was recorded.


IMBIBITION
C_2H_4
GERMINATION
Fig 3.15a
PROTEIN % (BSA equivalent)
100
50
0
0
48
60
84
108
132
156

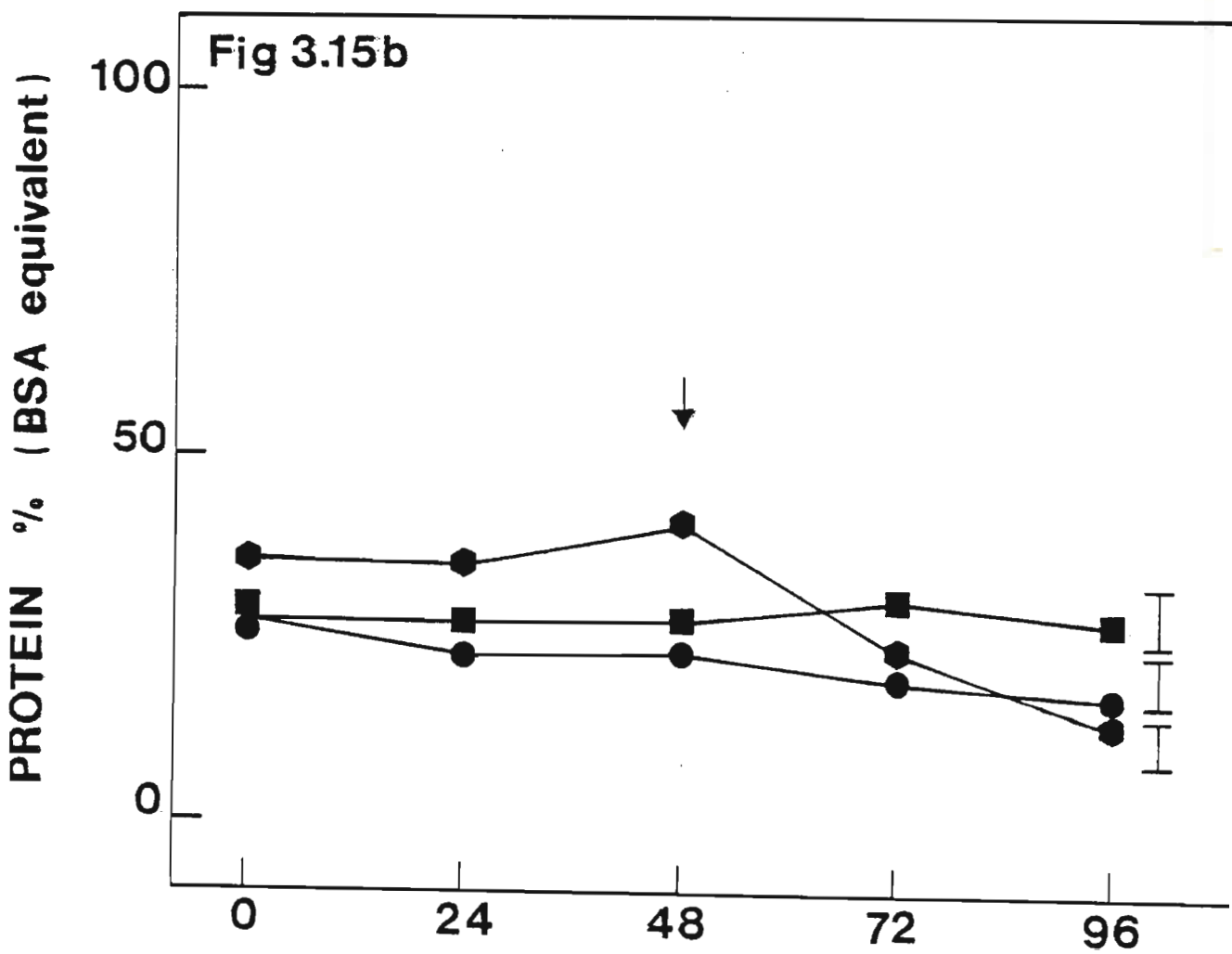


Fig 3.15b
PROTEIN % (BSA equivalent)
100
50
0
0
24
48
72
96

In other species, such as *Zea mays* (INGLE, BEEVERS and HAGEMAN, 1964) and *Pisum sativum* (BAIN and MERCER, 1966) the situation is rather different and the endosperm and cotyledons (respectively) do supply the growing axis with protein nitrogen. The results obtained in this study also suggests that dormancy breaking in this species may be associated with a transient increase in the protein content of the embryonic axes. Thus, it is possible that ethylene may be involved in stimulating the production of certain proteins, the absence of which would be responsible for the dormant condition.

The overall impression gained from this study on the mobilization of food reserves prior, and subsequent to ethylene treatments was that the cotyledons and endosperm play little or no role in the breaking of dormancy in this species. In addition, the fact that reserve hydrolysis occurred even in the absence of any dormancy breaking treatments, suggests that the enzymes necessary for this hydrolysis are either present in the dry tissue or are rapidly synthesized during imbibition. This latter possibility is supported to some extent by the fact that polysomes were visible in this tissue prior to the application of ethylene. In this respect, manketti seeds may resemble those of *Setaria lutescens* (ROST, 1972) and *Fraxinus excelsior* (VILLIERS, 1971) both of which exhibited reserve hydrolysis whilst still in the dormant condition. Thus, dormancy in *R. rautanenii* is probably not directly related to reserve hydrolysis. In his investigation on *Setaria*

lutescens seeds, ROST (1972) suggested that it was more likely that dormancy breaking was associated with those processes involved in the *de novo* synthesis of new proteins. A similar situation may exist in manketti seeds. This is supported by the finding that protein levels initially increased in embryonic axes in response to ethylene.

Another interesting and somewhat unusual finding which emerged from this study was that the various food reserves appear to reorganize themselves in dormant tissues which had been imbibed for a length of time. It thus seemed that after having been mobilized in 'anticipation' of a dormancy breaking treatment, the food reserves are then reassembled into more stable compounds if the stimulus is not forthcoming. This is the only known report of a situation where considerable metabolic interconversions take place in imbibed, dormant seeds.

The involvement of protein synthesis in the breaking of manketti seed dormancy was investigated further using inhibitors of protein and RNA synthesis. In addition, these compounds were also applied at various times during the overall germination sequence in an attempt to determine which period of protein synthesis was important for dormancy breaking. The results presented in Figure 3.16 show the effects of actinomycin D, an antibiotic which is thought to affect protein synthesis by suppressing DNA dependent RNA synthesis. When applied during imbibition, actinomycin D only caused a slight delay in the onset of germination.

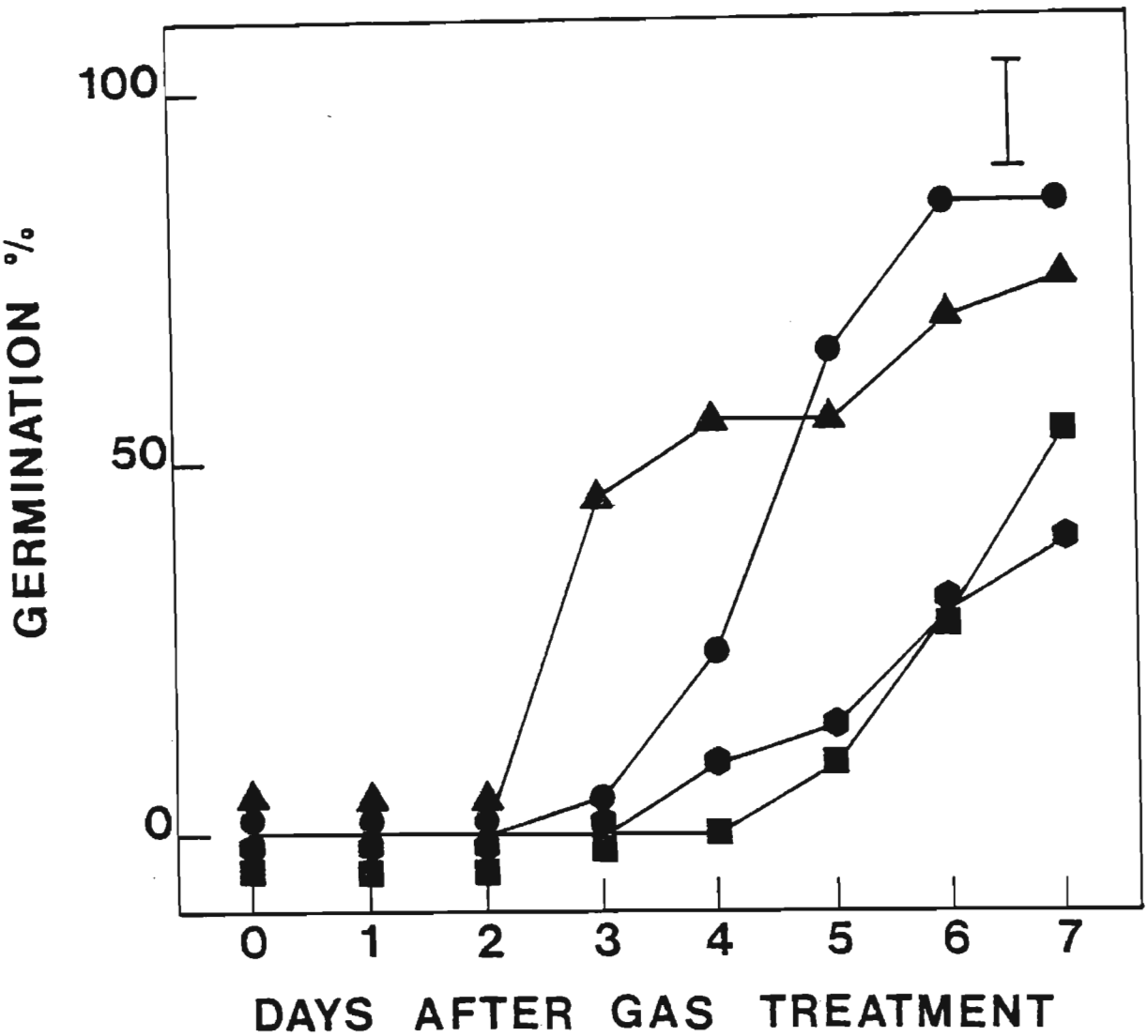


Figure 3.16 Germination response of *Ricinodendron rautanenii* seeds treated with an RNA synthesis inhibitor, actinomycin D, at various times during dormancy breaking. The inhibitor was applied to seeds either throughout the experimental period (■) or during the periods of imbibition (●) or gasing (▲) only. Control seeds (▲) were gased and incubated in the normal manner. Incubation was at 30°C. The bar represents the maximum 95 per cent confidence limit

An even greater delay in the onset of germination was observed when seeds were maintained in the presence of this inhibitor after the application of the dormancy breaking treatment. In addition, the final germination percentages obtained as a result of this treatment were considerably below the controls. Actinomycin D also affected subsequent seedling growth. Table 3.6 shows that, when compared with control seedlings of the same age, the root and shoot growth (as measured by dry matter content) was considerably reduced by this compound irrespective of when it was applied. Thus, both germination and seedling growth were affected by this inhibitor. This experiment was repeated using another antibiotic, cycloheximide. Cycloheximide differs from actinomycin D in that it specifically inhibits protein synthesis on extramitochondrial cytoplasmic ribosomes (DE KLOET, 1965). Figure 3.17 shows that no germination response was obtained from seeds incubated with this compound after the ethylene treatment. However, some germination did occur if the seeds were removed from the inhibitor immediately after imbibition. Cycloheximide treated tissues have also been reported to exhibit full recovery when thoroughly rinsed in distilled water (see BROOKER, CHEUNG and MARCUS, 1977). In the case of ungerminated, cycloheximide treated manketti seeds, only 10 per cent germination was recorded after seven days from seeds which had received a six hour rinsing treatment (Table 3.7), indicating that those processes which were associated with mediating the ethylene response had been blocked. That this was not of a permanent nature, however,

Table 3.6 Effect of actinomycin D, applied at various times, on the growth of ethylene treated *R. rautanenii* seeds. Dry mass determinations were made from seven day-old seedlings. Figures in parentheses represent the confidence limits where p = 0,05.

Period of Actinomycin D Treatment	Dry Mass Seed^{-1}		
	Roots	Shoots	Endosperm
Control (untreated)	0,04 (± 0,01)	0,24 (± 0,006)	0,61 (± 0,1)
During imbibition	0,02 (± 0,01)	0,12 (± 0)	0,83 (± 0,1)
During germination	0,01 (± 0)	0,073 (± 0,01)	0,84 (± 0,1)
Throughout	0,01 (± 0)	0,083 (± 0,007)	0,82 (± 0,1)

Table 3.7 Percentage germination(after seven days incubation) of cycloheximide treated *R. rautanenii* seeds which had been rinsed and re-treated with ethylene. These treatments were applied to seeds which failed to germinate when treated with ethylene the first time. Figures in parentheses represent the confidence limits where p = 0,05.

Period of Cycloheximide Treatment	Percentage Germination	
	Rinsed Only	Rinsed and Gased
During imbibition	10 (± 0,5)	25 (± 1,0)
During germination	10 (± 0,5)	25 (± 1,5)
Throughout	0	5 (± 1,5)

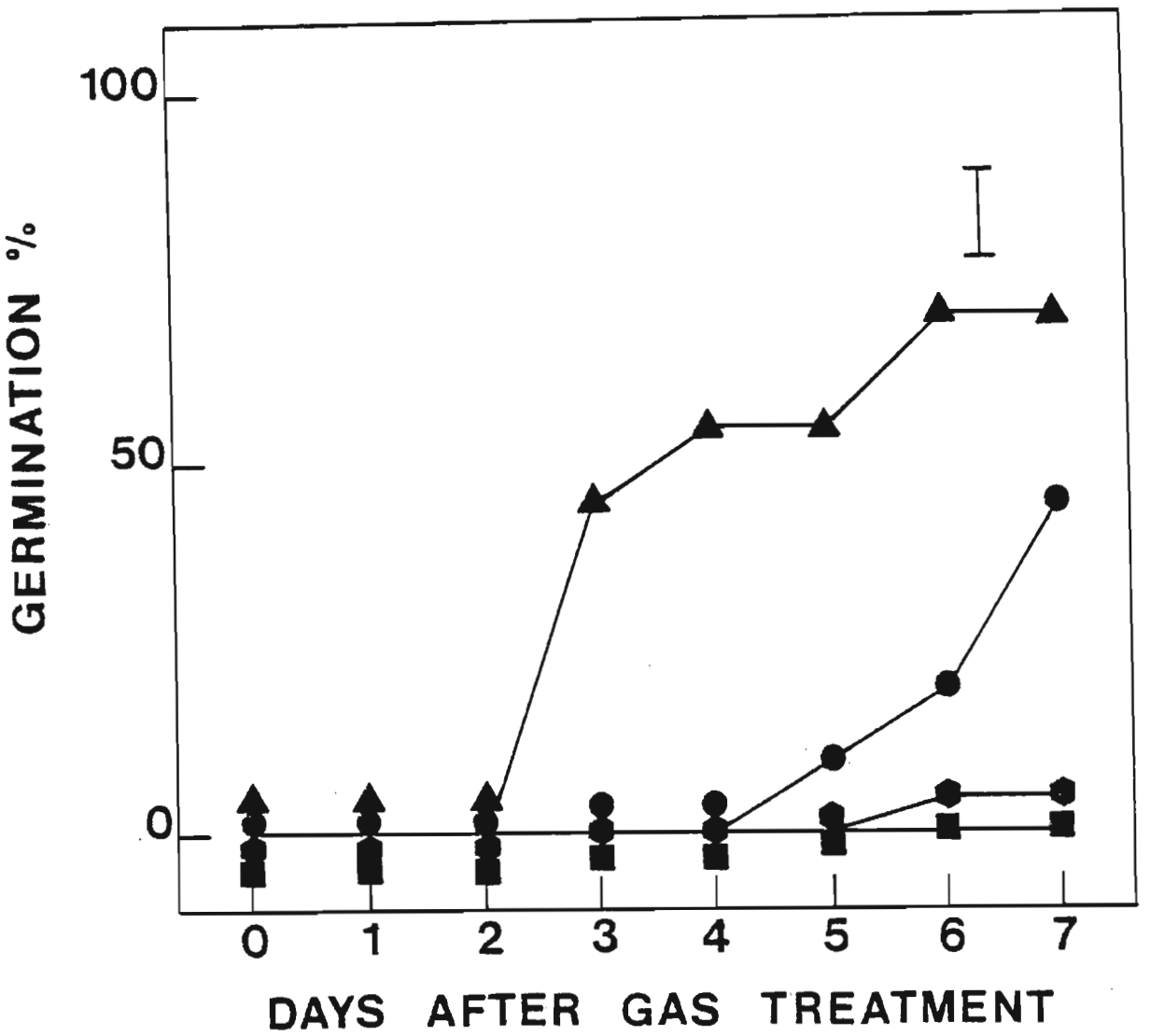


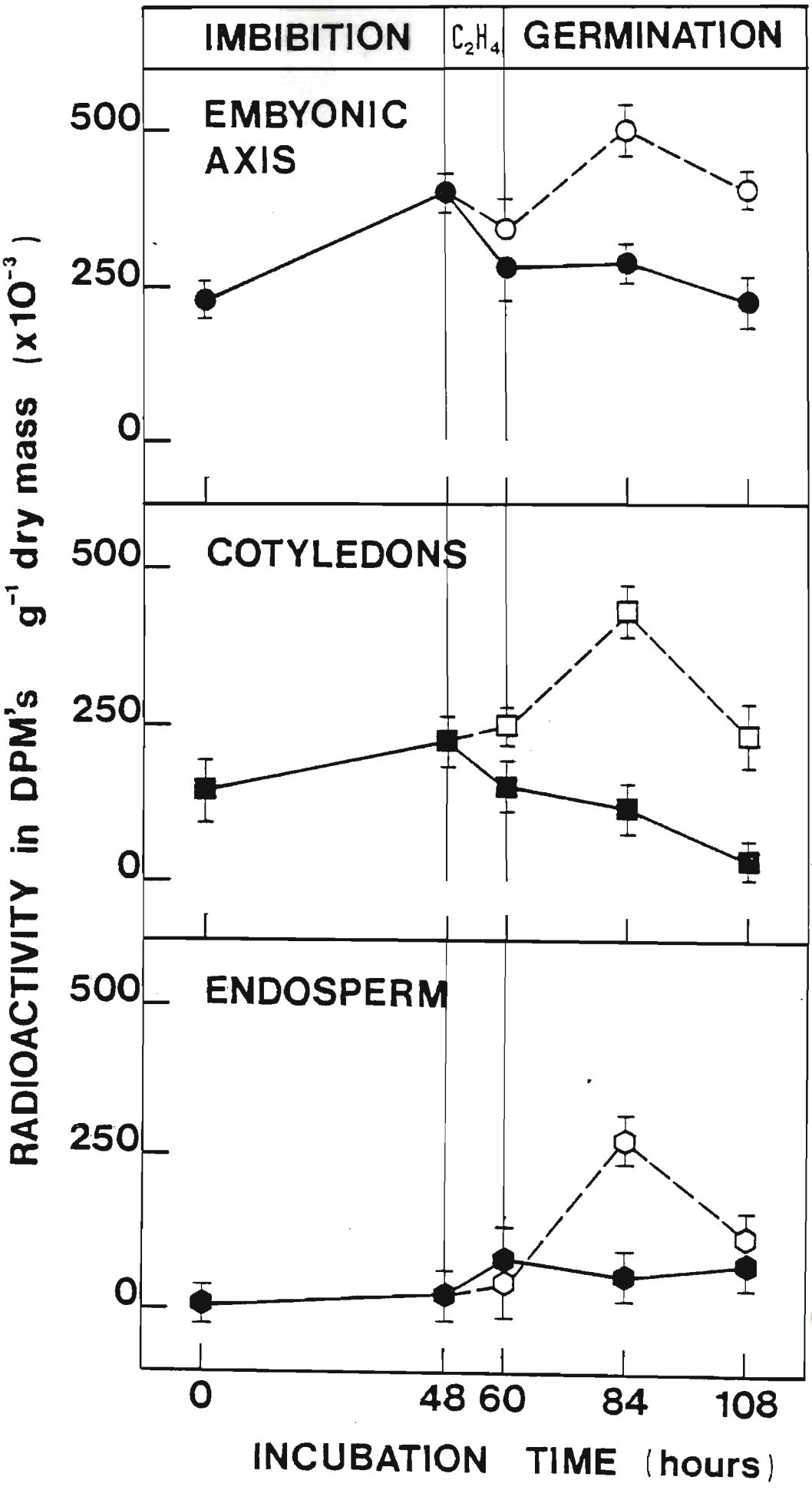
Figure 3.17 Germination response of manketti seeds treated with a protein synthesis inhibitor, cycloheximide, at various times during dormancy breaking. The inhibitor was applied to seeds either throughout the experimental period (■) or during the periods of imbibition (●) or gasing (⬢) only. Control seeds (▲) were gased and incubated in the normal manner. The bar represents the maximum 95 per cent confidence

was suggested by the finding that germination could be improved if rinsing was followed by a 12 hour ethylene treatment.

These results are consistent with the view that protein synthesis is a necessary pre-requisite for germination (BEWLEY and BLACK, 1978). Furthermore, this protein synthesis does not appear to rely, initially, on the synthesis of new RNA fractions, since inhibition of this aspect of metabolism with actinomycin D did not prevent germination entirely. In this respect, it is also possible that the concentration of actinomycin D was not sufficiently strong and that this, together with inadequate penetration of the compound, resulted in a muted response. Thus, it is not possible to state unequivocably that the ribosomes and mRNA's present in the dry seeds were sufficiently capable of supporting germination. In addition, it is not possible to conclude from these data that ethylene's effects are mediated via protein synthesis, since cycloheximide suppresses all protein synthesis and could, therefore, be affecting processes other than those related to ethylene.

For these reasons, actual measurements of protein synthesis were also carried out. This was achieved by recording the amount of ^{14}C-leucine incorporated by the three tissue components taken from intact seeds prior and subsequent to ethylene treatments. The total respective radioactivity recovered from these tissues is presented in Figure 3.18. These data indicated that 'dry' tissues, which only re-

FIGURE 3.18. The total radioactivity recovered from the embryonic axis, cotyledon and endosperm tissues of ethylene treated (open symbols) and untreated (closed symbols) *Ricinodendron rautanenii* seeds. The tissues were sampled at various times and incubated for 2 hours in a solution containing ^{14}C-leucine.


IMBIBITION
C_2H_4
GERMINATION
EMBYONIC AXIS
COTYLEDONS
ENDOSPERM
500
250
0
RADIOACTIVITY in DPM's g^{-1} dry mass ($x10^{-3}$)
0
48
60
84
108
INCUBATION TIME (hours)

ceived moisture during the two hour incubation period with ^{14}C-leucine, imbibed a considerable amount of the label. Furthermore, this uptake increased after 48 hours imbibition. Twenty four hours after the ethylene treatment, large increases in the amount of measurable radioactivity were recorded in all three tissues. However, it was not possible to determine from these data whether the increases were due to increased protein synthesis or whether it was the amino acid itself which had been affected. This was resolved by measuring the TCA (trichloroacetic acid) soluble and TCA insoluble fractions separately. These represented the amino acid and protein components respectively.

From this, the radioactivity detected in 'dry' endosperm and cotyledonary tissues could be attributed to the amino acid fraction and very little protein synthesis was, in fact, taking place (Figures 3.19 and 3.20). In the dry embryonic axes, on the other hand, a considerable proportion of the label had been incorporated into proteins suggesting that, in these organs, protein synthesis commenced rapidly (Figure 3.21). The protein synthetic activities of all three tissues increased as a result of imbibition as did the incorporation of the labelled amino acid. Ethylene treatments, however, had very little effect on the protein synthetic ability of the embryonic axes, whereas amino acid uptake by all three tissues increased markedly. Thus, the increase in total radioactivity observed after 24 hours incubation was not the result of increased protein synthesis.

Figure 3.19 Radioactivity recovered from the cotyledonary tissue of control (open symbols) and ethylene treated (closed symbols) *Ricinodendron rautanenii* seeds. Samples of cotyledonary tissue were taken at various times and incubated for two hours at 23°C in a buffered solution containing 0,4 µCi ^{14}C-leucine. Protein synthesis is represented by the amount of radioactivity present in the trichloroacetic acid insoluble fraction (○ , ●). Uninincorporated luecine is represented by the trichloroacetic acid soluble fraction (□ , ■). The bars represent the 95 per cent confidence limits.

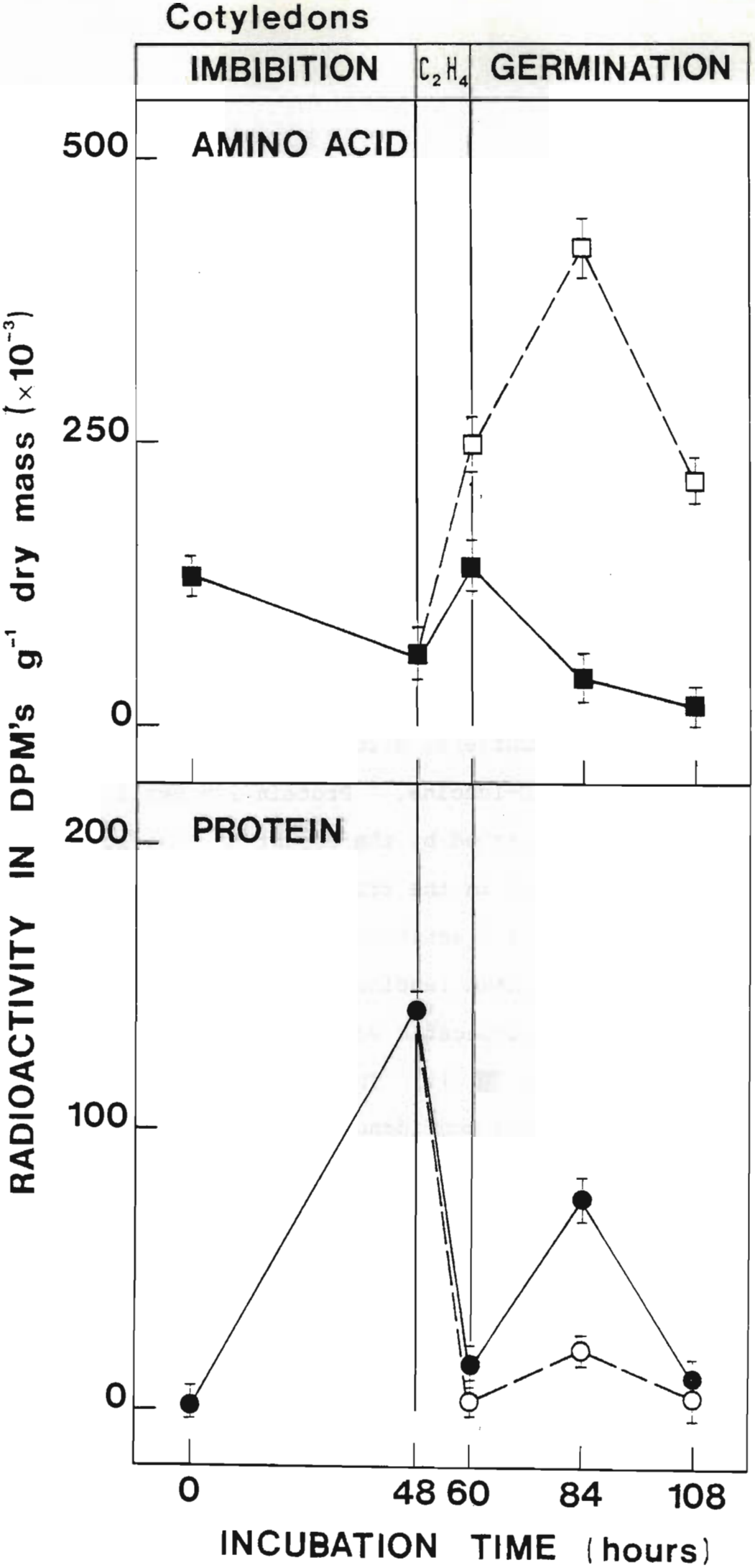

Cotyledons
IMBIBITION
C_2H_4
GERMINATION
AMINO ACID
PROTEIN
500
250
0
200
100
0
RADIOACTIVITY IN DPM's g^{-1} dry mass ($\times 10^{-3}$)
0
48
60
84
108
INCUBATION TIME (hours)

Figure 3.20 Radioactivity recovered from the endosperm tissue of control (open symbols) and ethylene treated (closed symbols) *Ricinodendron rautanenii* seeds. Samples of endosperm tissue were taken at various times and incubated for two hours at 23°C in a buffered solution containing 0,4 $\mu Ci\ ^{14}C$-leucine. Protein synthesis is represented by the amount of radioactivity present in the trichloroacetic acid insoluble fraction (○ , ●). Unincorporated leucine is represented by the trichloroacetic acid soluble fraction (□ , ■). The bars represent the 95 per cent confidence limits.

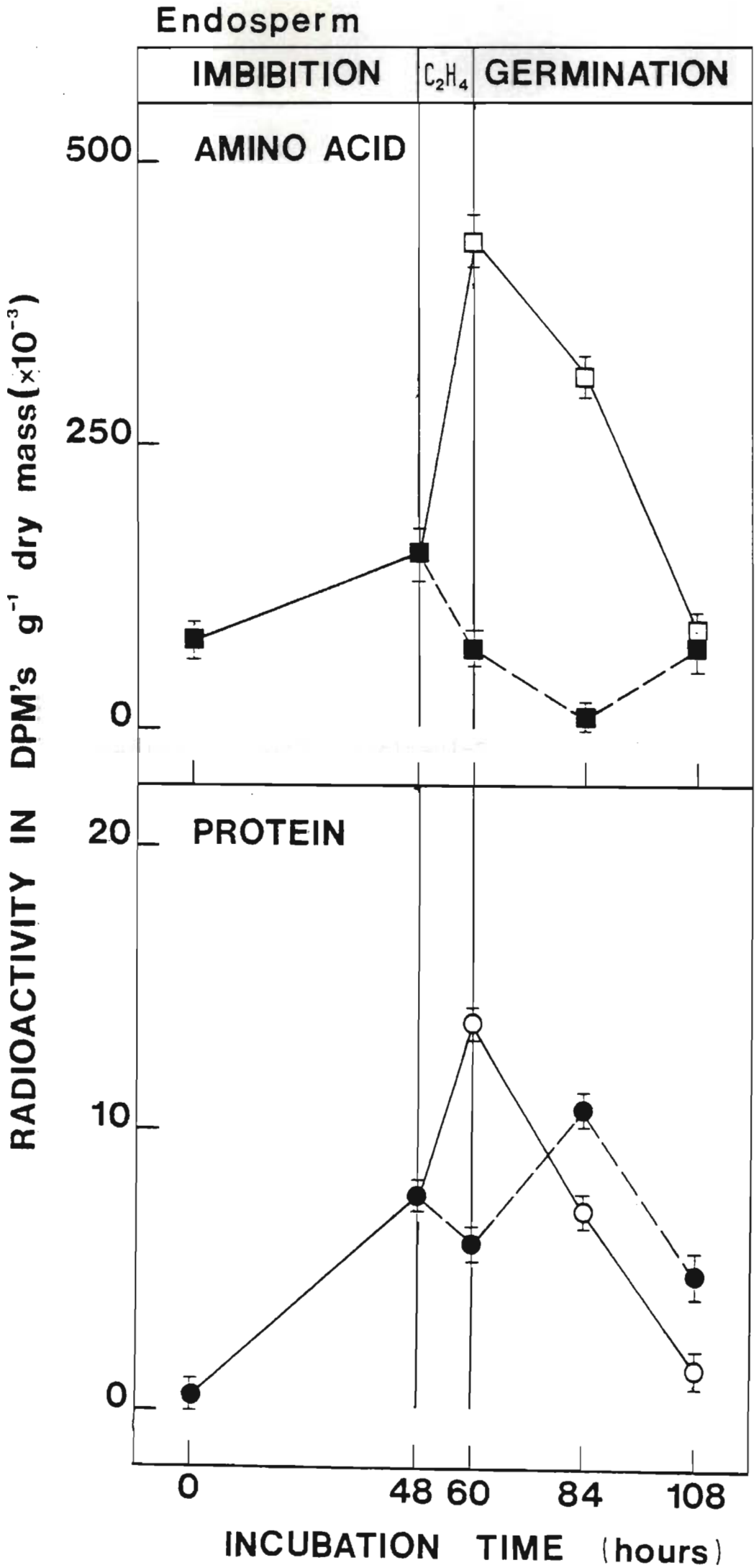
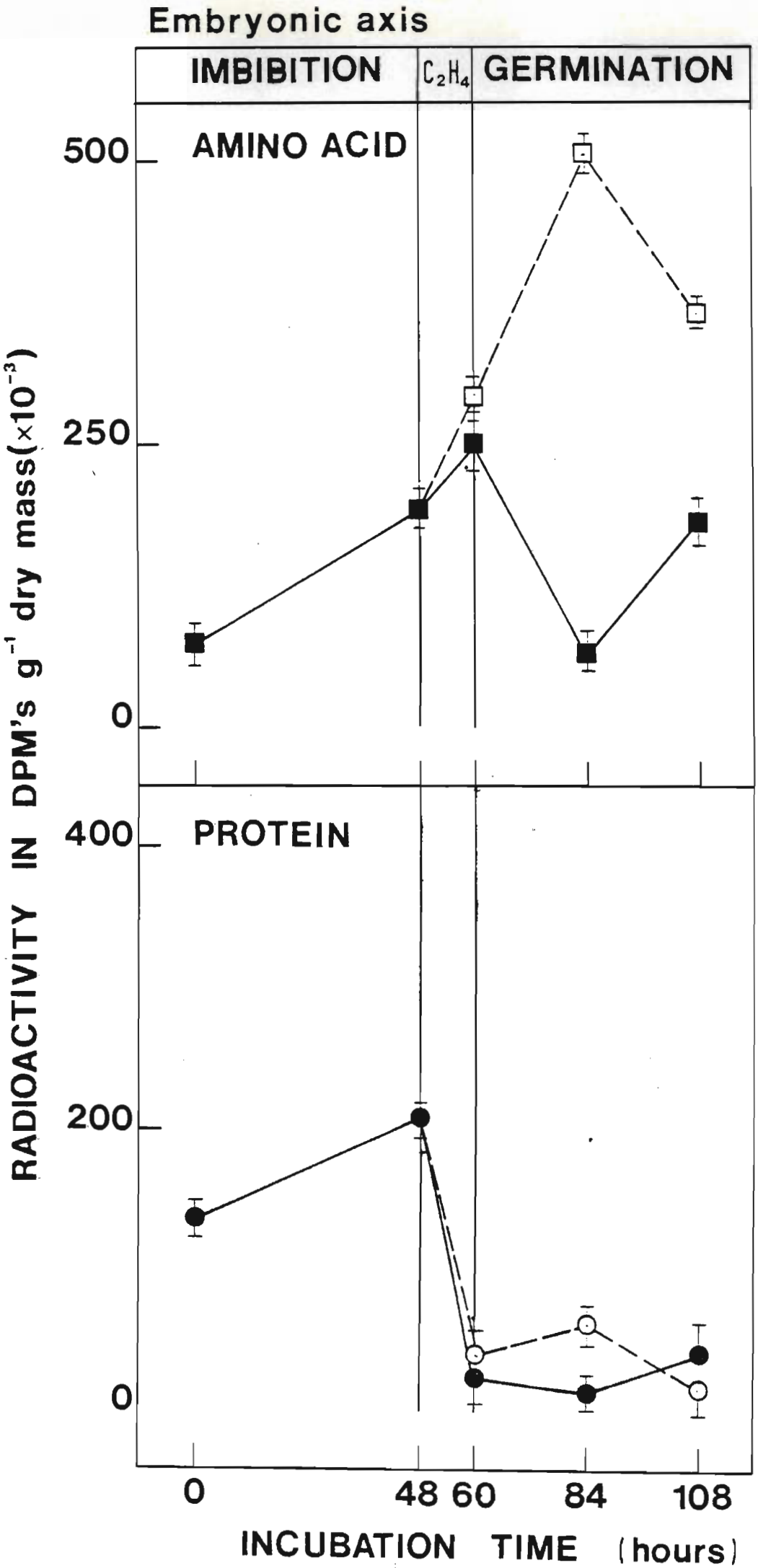

Endosperm
IMBIBITION
C_2H_4
GERMINATION
AMINO ACID
500
250
0
PROTEIN
20
10
0
RADIOACTIVITY IN DPM's g^{-1} dry mass ($\times 10^{-3}$)
0
48
60
84
108
INCUBATION TIME (hours)

Figure 3.21 Radioactivity recovered from the embryonic axes of control (open symbols) and ethylene treated (closed symbols) *Ricinodendron rautanenii* seeds. Embryonic axes were removed at various times and incubated at 23°C in a buffered solution containing 0,4 µCi ^{14}C-luecine. Protein synthesis is represented by the amount of radioactivity present in the trichloroacetic acid insoluble fraction (○ , ●). Unincorporated leucine is represented by the trichloroacetic acid soluble fraction (□ , ■). The bars represent the 95 per cent limits.


Embryonic axis
IMBIBITION
C_2H_4
GERMINATION
AMINO ACID
PROTEIN
500
250
0
400
200
0
RADIOACTIVITY IN DPM's g^{-1} dry mass($\times 10^{-3}$)
0
48
60
84
108
INCUBATION TIME (hours)

These results are in agreement with the view that all of the components for protein synthesis are already present in dry seeds (MAYER and SHAIN, 1974) and in this respect, *R. rautanenii* embryonic axes are similar to many other species. For example, in germinating rye embryos (*Secale cereale* L.), protein and RNA synthesis could be detected 5-10 minutes after the commencement of imbibition (SEN and OSBORNE, 1974). These data conflict with the hypothesis that dormant tissues are characterized by a general suppression of protein synthesis.

The increased protein synthesis observed at the end of imbibition was probably due to the increased efficiency of preformed components. This is based on the fact that DNA replication is usually a late event and according to OSBORNE, SHARON and BEN-ISHAI (1981) many of the synthetic and growth events occurring during early germination are apparently independent of DNA replication. In *R. rautanenii*, this is supported to some extent by the finding that actinomycin D had only a limited effect on germination and by the results obtained from the experiment carried out using acridine orange (Figure 3.22). These data clearly demonstrated that interference with early DNA replication did not inhibit germination (assuming that sufficient amounts of the active compound were taken up during imbibition).

The results obtained show that dormancy breaking in *R. rautanenii* axes was not associated with an increase in

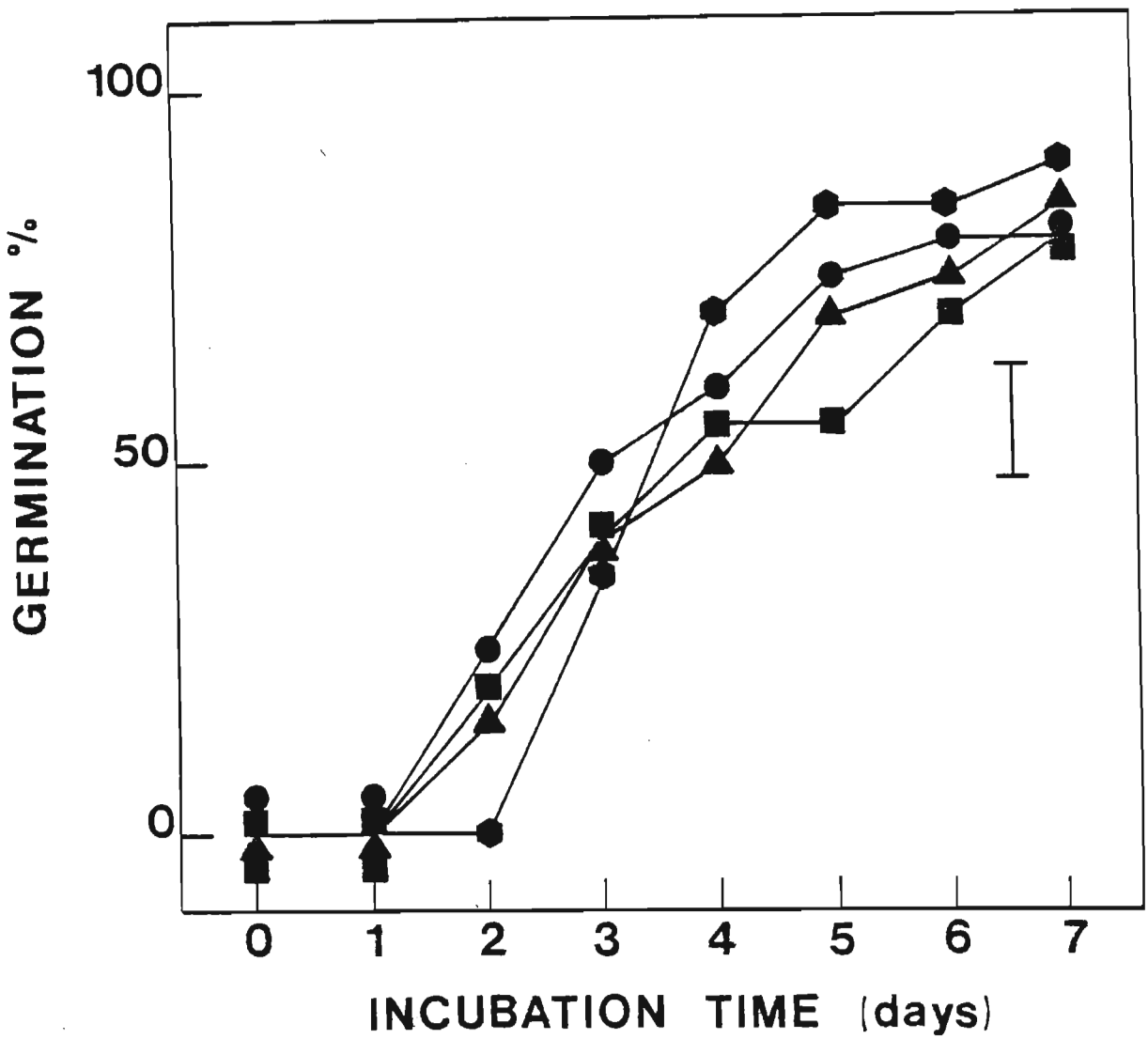
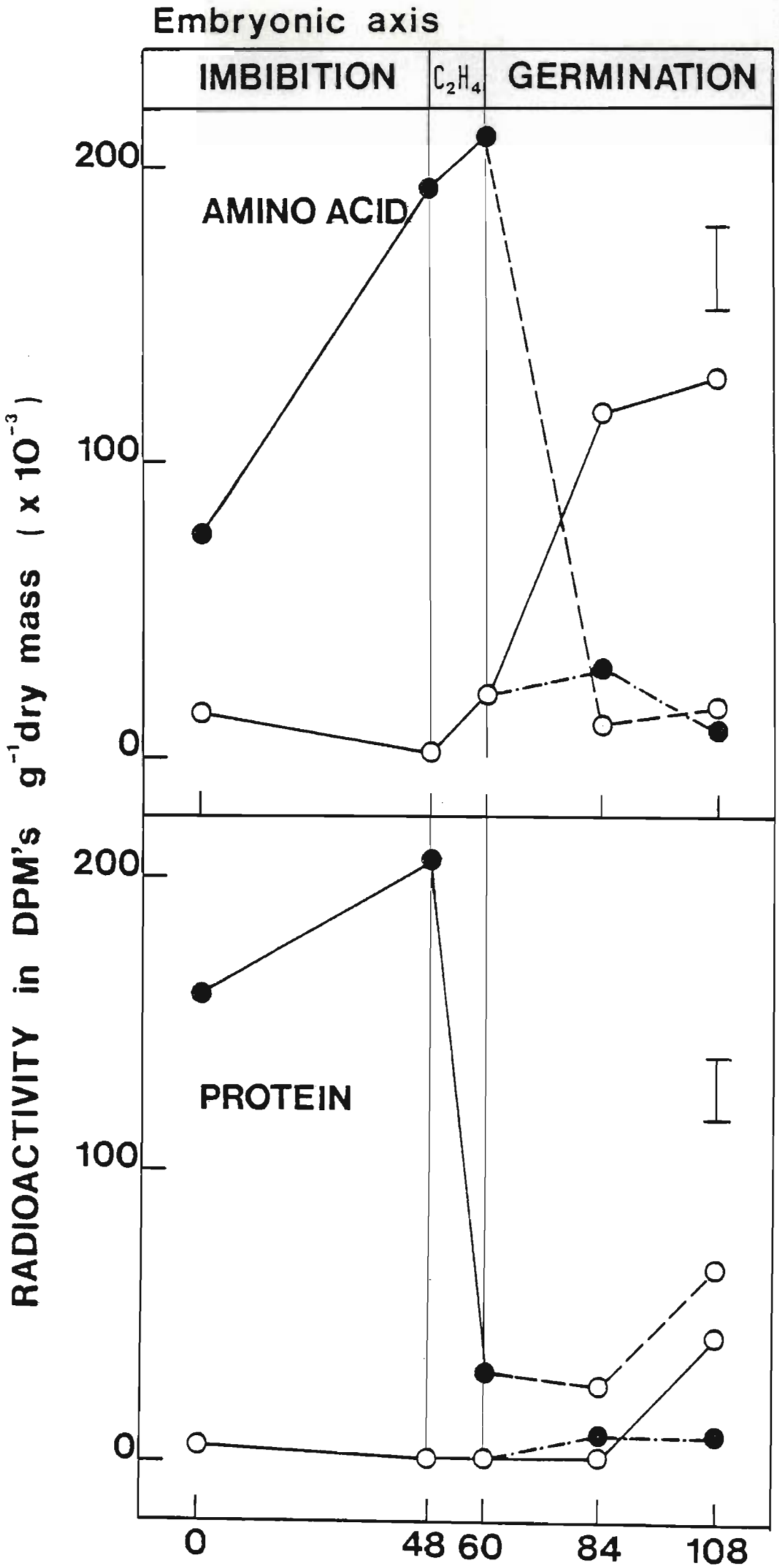


Figure 3.22 The germination response of *Ricinodendron rautanenii* seeds treated with acridine orange, a compound which interferes with DNA replication. The inhibitor was applied either throughout the experimental period (■) or during the periods of imbibition (●) or gasing (⬢) only. Control seeds (▲) were gased and incubated in the normal manner. The bar repre-

protein synthesis and similar rates of synthesis were observed in both treated and untreated axes. This appears to be similar to the situation reported for *Agrostemma githago* seeds, where no differences existed in the protein synthesis abilities of dormant and after-ripened embryos (HECKER and BERNHARDT, 1976). DE KLERK and LINSKENS (1979), however, disputed this and reported that after-ripened axes of this species did exhibit higher protein synthetic activities. A careful examination of their data shows that, initially, dormant seeds did synthesize more protein and that this only changed after 12 hours imbibition. In addition, SATOH and ESASHI (1979) found that dormant cocklebur axes also exhibited higher initial protein synthetic abilities than their non-dormant counterparts. These findings, to some extent, correlate with the results obtained in the present study, except that protein synthesis was usually found to increase as germination proceeded. It therefore seemed anomalous that in manketti seeds, protein synthesis did not increase as a result of ethylene treatments, whereas cycloheximide treatments showed that protein synthesis was nevertheless important. This was investigated further by examining the effects of cycloheximide and actinomycin D on the measurable rates of protein synthesis using ^{14}C-leucine.

It was found that protein synthesis was markedly suppressed in cycloheximide treated embryonic axes from dry and 48 hour imbibed tissue (Figure 2.23). In addition, the amount of label present in the amino acid fraction was also considerably reduced in these organs when compared with the controls. Ethylene treatments resulted in a slight in-

FIGURE 3.23 The effect of cycloheximide on the incorporation of ^{14}C-leucine into the amino acid (●) and protein fractions (●) of embryonic axes from ethylene treated seeds. Cycloheximide treatments (open symbols) were applied either through out the experimental period (——), after gasing only (----), or up until the end of the gas treatment (-·--·). Non-cycloheximide treated axes are represented by closed symbols. The bars represent the maximum 95 per cent confidence limits.


Embryonic axis
IMBIBITION
C_2H_4
GERMINATION
AMINO ACID
PROTEIN
200
100
0
RADIOACTIVITY in DPM's g^{-1}dry mass (x 10^{-3})
0
48
60
84
108

crease in the uptake of ^{14}C-leucine but subsequent increases in protein synthesis and amino acid uptake were only recorded in those seeds which did not germinate. In cotyledonary and endosperm tissues from the same seeds, the inhibitory effects of cycloheximide were also clearly visible (Figures 3.24 and 3.25). In these tissues, no trends in amino acid uptake or protein synthesis could be associated with dormancy breaking and germination. One exception in this regard, however, was the distinct increase in protein synthesis noted in the cotyledons from seeds continuously incubated with cycloheximide. The remarkable feature of this result is the fact that these cotyledons were from seeds which did not germinate. Actinomycin D, on the other hand, did not significantly reduce protein synthesis in dry axes, but had a marked effect on seeds imbibed for 48 hours (Figure 3.26). This result suggested that at this stage, protein synthesis was dependent on newly synthesized RNA. Ethylene treatments generally tended to increase the protein synthetic activities in these tissues, but a closer examination showed that the rate was generally lower in those seeds which germinated most quickly. Amino acid uptake by these organs was essentially the same irrespective of the treatment applied. In addition, as with axes not treated with protein synthesis inhibitors, ethylene treatments were associated with an increased uptake of the labelled leucine and the values obtained in this instance were generally much higher than those recorded in the cycloheximide treated seeds. A similar pattern of protein synthesis was ob-

FIGURE 3.24 The effect of cycloheximide on the incorporation of ^{14}C-leucine into the amino acid and protein fractions of the cotyledonary tissue from ethylene treated seeds. Cycloheximide treatments (open symbols) were applied either throughout the experimental period (——), after gasing only (----), or up until the end of the gas treatment (-·-·-). Non-cycloheximide treated tissue is represented by closed symbols. The bars represent the maximum 95 per cent confidence limits.

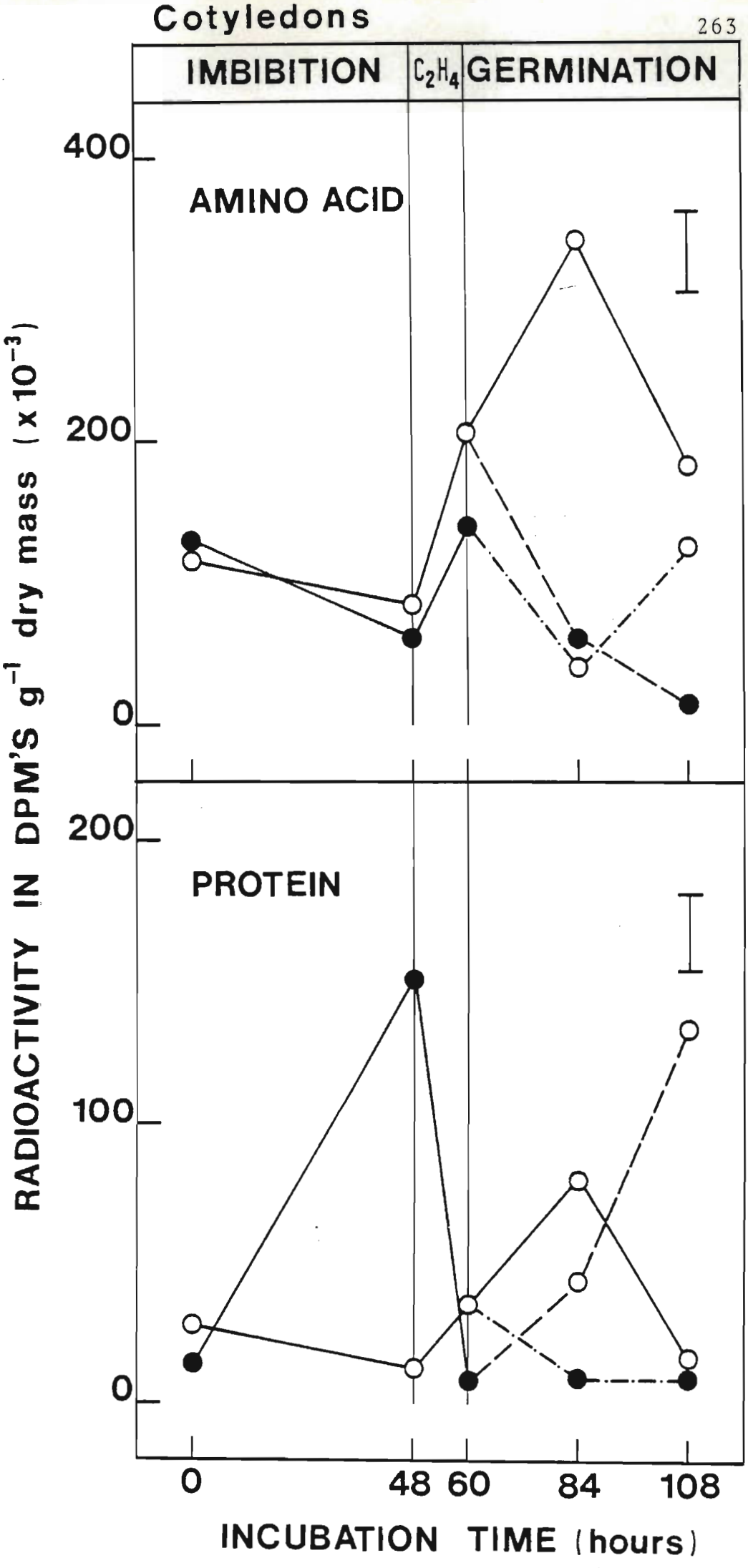

Cotyledons
IMBIBITION
C_2H_4
GERMINATION
AMINO ACID
PROTEIN
400
200
0
200
100
0
RADIOACTIVITY IN DPM'S g^{-1} dry mass ($x10^{-3}$)
0
48
60
84
108
INCUBATION TIME (hours)

FIGURE 3.25 The effect of cycloheximide on the incorporation of ^{14}C-leucine into the amino acid and protein fractions of endosperm tissue from ethylene treated seeds. Cycloheximide treatments were applied either throughout the experimental period (——), after gasing only (---), or up until the end of the gas treatment (-·-·). Non-cycloheximide treated tissue is represented by the closed symbols. The bars represent the maximum 95 per cent confidence limits.

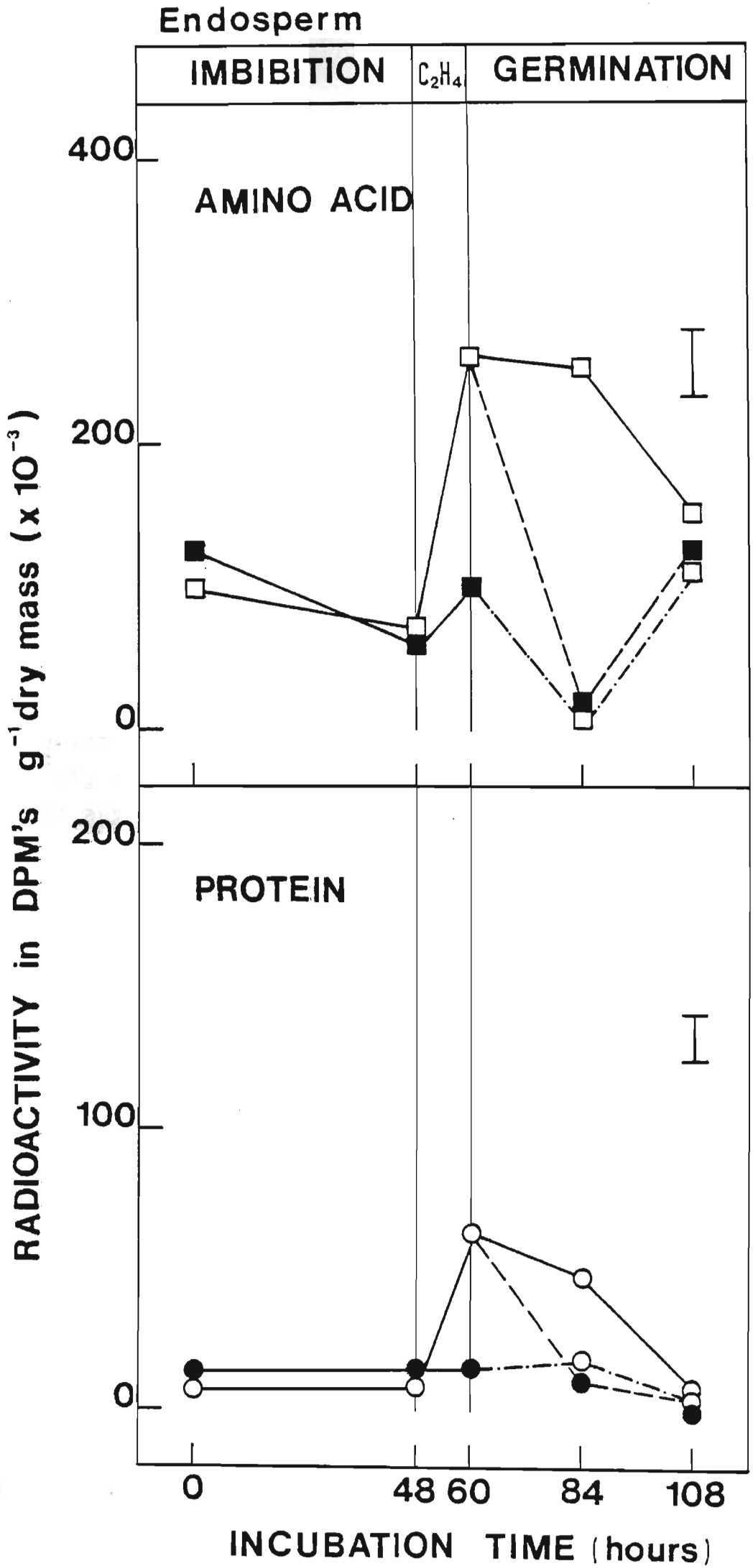
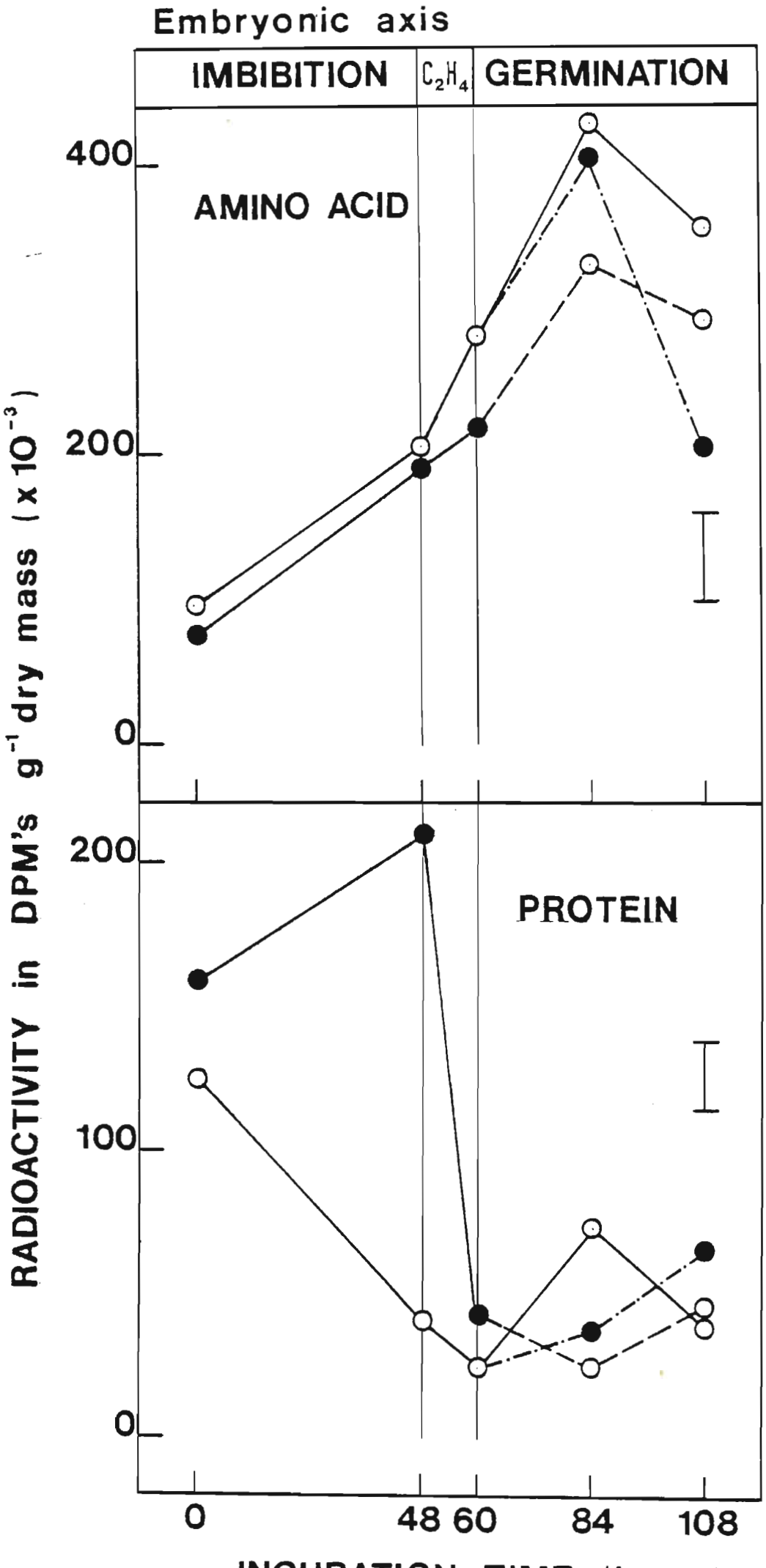

Endosperm
IMBIBITION
C_2H_4
GERMINATION
400
AMINO ACID
200
0
200
PROTEIN
100
0
RADIOACTIVITY in DPM's g^{-1} dry mass ($\times 10^{-3}$)
0
48
60
84
108
INCUBATION TIME (hours)

FIGURE 3.26 The effect of actinomycin D on the incorpo-
of ^{14}C-leucine into the amino acid and pro-
tein fractions of embryonic axes from ethylene
treated seeds. Actinomycin D treatments
(open symbols) were applied either throughout
the experimental period (——), after gasing only
(– – –), or up until the end of the gas treatment
(–·–·–). Non-actinomycin D treated axes are
represented by the closed symbols. The bars
represent the maximum 95 per cent confidence
limits.


Embryonic axis
IMBIBITION
C_2H_4
GERMINATION
AMINO ACID
PROTEIN
400
200
0
200
100
0
RADIOACTIVITY in DPM's g^{-1} dry mass (x 10^{-3})
0
48
60
84
108
INCUBATION TIME (hours)

served in the cotyledonary and endosperm tissues from the same seeds (Figures 3.27 and 3.28). Thus, protein synthesis was only reduced by actinomycin D in tissues which had been imbibed for 48 hours. In addition, post-ethylene protein synthesis was higher in those tissues from seeds which experienced the longest delay in germination.

In general, these results support the view that protein synthesis can take place in freshly imbibed seed tissues and that this probably results from preformed components. Subsequent increases in protein synthesis appeared to be dependent on the synthesis of new RNA fractions. The effects of cycloheximide were very marked but this compound failed to prevent protein synthesis completely. Actinomycin D, on the other hand, had little effect on the protein synthesic activities in 'dry' seeds, but markedly suppressed subsequent protein synthesis. This strongly suggested that initial protein synthesis is initiated by preformed components, whereas new fractions of RNA are required to support subsequent increases in this aspect of metabolism. In spite of this effect, seeds treated with actinomycin D still germinated, indicating that either the synthesis of new RNA was not essential for germination, or that the concentration of this antibiotic was insufficient to have its full effect. One of the most notable features of these data, is the fact that ethylene treatments did not necessarily stimulate general protein synthesis. On the contrary, protein synthesis appeared

FIGURE 3.27 The effect of actinomycin D on the incorporation of ^{14}C-leucine into the amino acid and protein fractions of cotyledon tissue from ethylene treated seeds. Actinomycin D treatments (open symbols) were applied either throughout the experimental period (——), after gasing only (– – –) or up until the end of the gas treatment (—·—·—). Non-actinomycin D treated cotyledon tissue is represented by the closed symbols. The bars represent the maximum 95 per cent confidence limits.

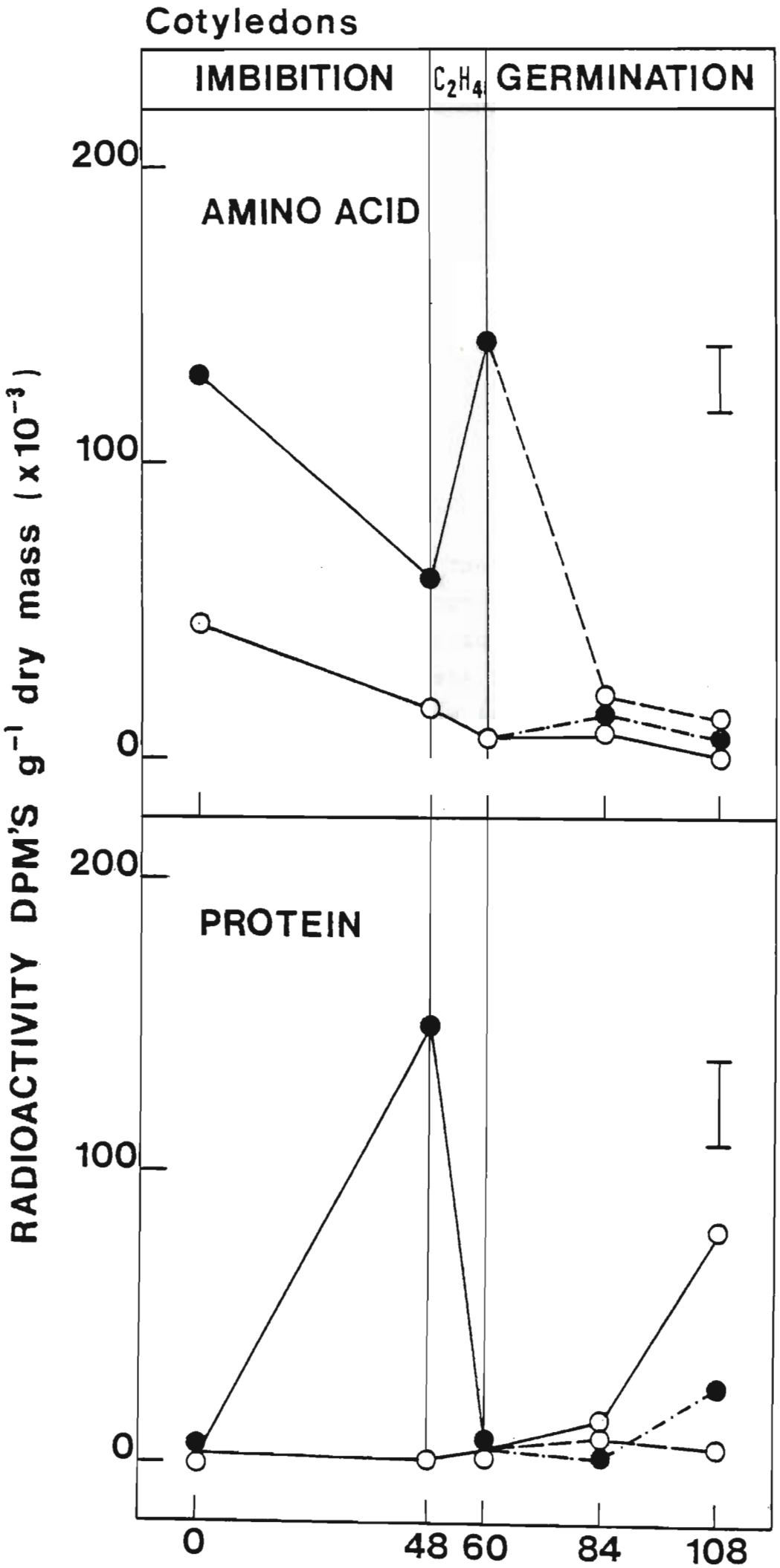
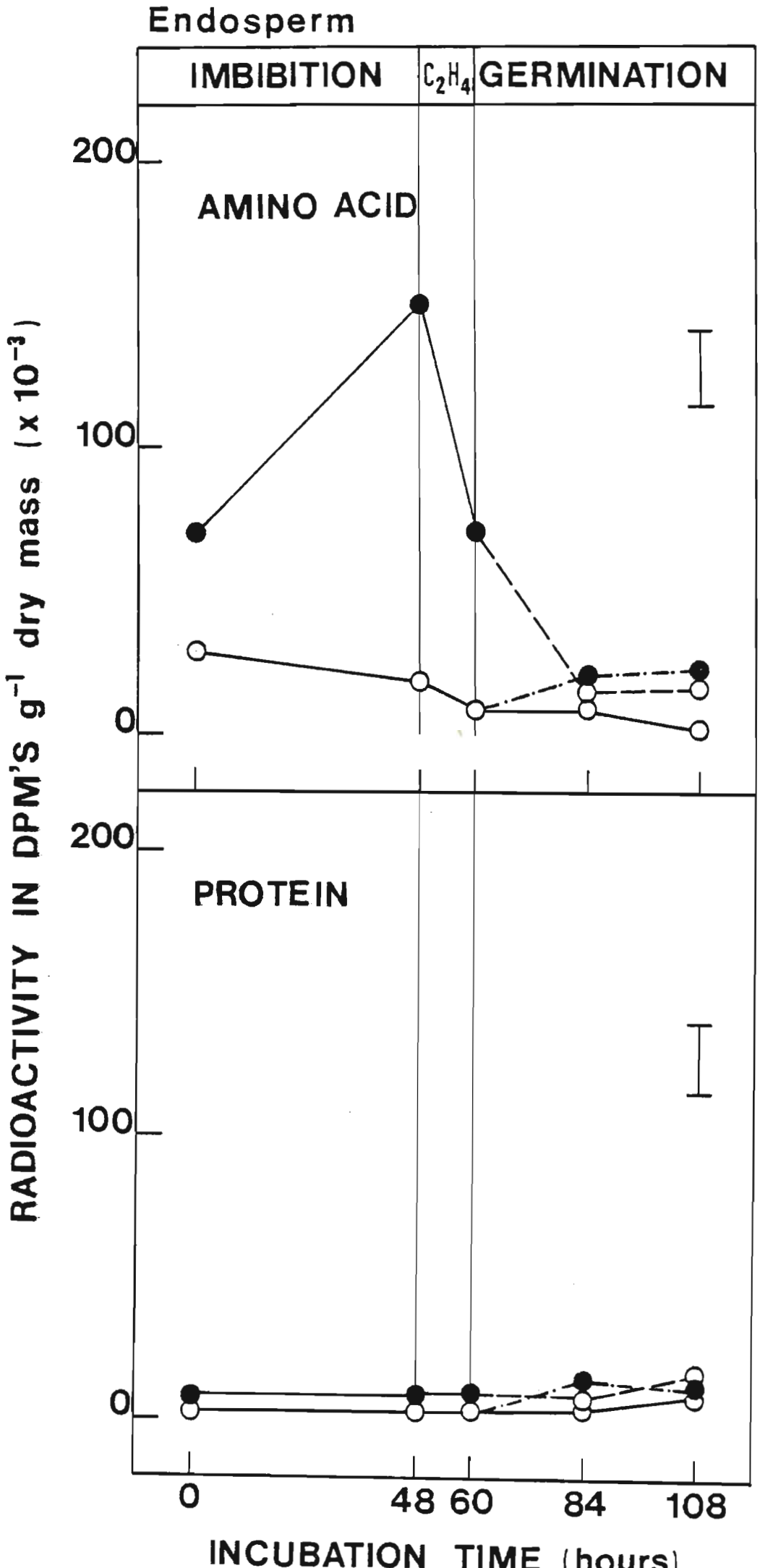

Cotyledons
IMBIBITION
C_2H_4
GERMINATION
AMINO ACID
PROTEIN
200
100
0
RADIOACTIVITY DPM'S g^{-1} dry mass ($\times 10^{-3}$)
0
48
60
84
108

FIGURE 3.28 The effect of actinomycin D on the incorporation of ^{14}C-leucine into the amino acid and protein fractions of endosperm tissue from ethylene treated seeds. Actinomycin D treatments (open symbols) were applied either throughout the experimental period (——), after gasing only (- - -), or up until the end of the gas treatment (—·—·—). Non-actinomycin D treated endosperm tissue is represented by the closed symbols. The bars represent the maximum 95 per cent confidence limits


Endosperm
IMBIBITION
C_2H_4
GERMINATION
AMINO ACID
PROTEIN
200
100
0
200
100
0
RADIOACTIVITY IN DPM'S g^{-1} dry mass ($\times 10^{-3}$)
0
48
60
84
108
INCUBATION TIME (hours)

to be marginally higher in those instances where the seeds either did not germinate, or experienced the greatest delay in germination. These findings are somewhat anomalous in the sense that germination and dormancy breaking are usually associated with increases in protein synthesis (DE KLERK and LINSKENS, 1979). These data also show that the increase in extractable protein noted as a result of ethylene treatments (Figure 3.15), was not due to an increase in protein synthesis. However, it is possible that ethylene may affect qualitative rather than quantitative changes in proteins. DE KLERK and LINSKENS (1979) found that dormancy correlated with a particular protein band in the cotyledons of 48 hour imbibed *Agrostemma githago* seeds. In addition, they reported that one particular protein disappeared as a result of after-ripening treatments. Thus, in the same way, ethylene may affect specific proteins, (possibly enzymes) in *R. rautanenii* seeds. On the other hand, ethylene treatments consistently caused a marked increase in the uptake of the labelled amino acid. This suggested that ethylene may have its immediate effect by increasing the passive permeability of membranes and this is made more feasible by the fact that it is a fat-soluble molecule which would tend to concentrate in lipid phases (LESHEM, 1973). ABELES (1973) suggested that, in this way, ethylene could control the movement of certain compounds across membranes.

Another aspect of metabolism which was examined, was the possible involvement of the pentose phosphate pathway in

the process of dormancy breaking. In experiments carried out using compounds known to stimulate the pentose phosphate pathway, no germination was obtained. This lack of response indicated that it was unlikely that ethylene released dormancy by directly stimulating alternate respiration. In this respect, *R. rautanenii* seeds are similar to those of *Spergula arvensis* (JONES and HALL, 1981) and more recently, SATOH and ESASHI (1980) produced overwhelming evidence in favour of the view that this pathway is not involved in the breaking of *X. pensylvanicum* seed dormancy. This was in spite of the fact that the germination of these latter seeds could be stimulated by nitrates, nitrites and cyanide. Thus, present evidence suggests that ethylene is not involved in this particular aspect of metabolism.

The earlier finding that applied gibberellins could stimulate manketti seed germination, indicated that endogenous gibberellins may be important in the phenomenon of dormancy breaking in this species. Thus, two inhibitors of gibberellin synthesis, CCC and phosphon D, were applied to seeds in an effort to determine whether ethylene stimulated this particular aspect of gibberellin metabolism. The results presented in Figure 3.29 showed that these compounds, irrespective of when they were applied, had no effect on the germination of manketti seeds. The results presented in Table 3.8, on the other hand, indicated that gibberellin synthesis was essential for early seedling growth. Significant differences in the dry matter content

Figure 3.29 The germination response of manketti seeds treated with inhibitors of gibberellic acid biosynthesis. CCC and phosphon D were applied either throughout the experimental period (■) or during the periods of imbibition (●) or gasing (⬣) only. Incubation was at 30°C. Control seeds (▲) were gased and incubated in the normal manner.

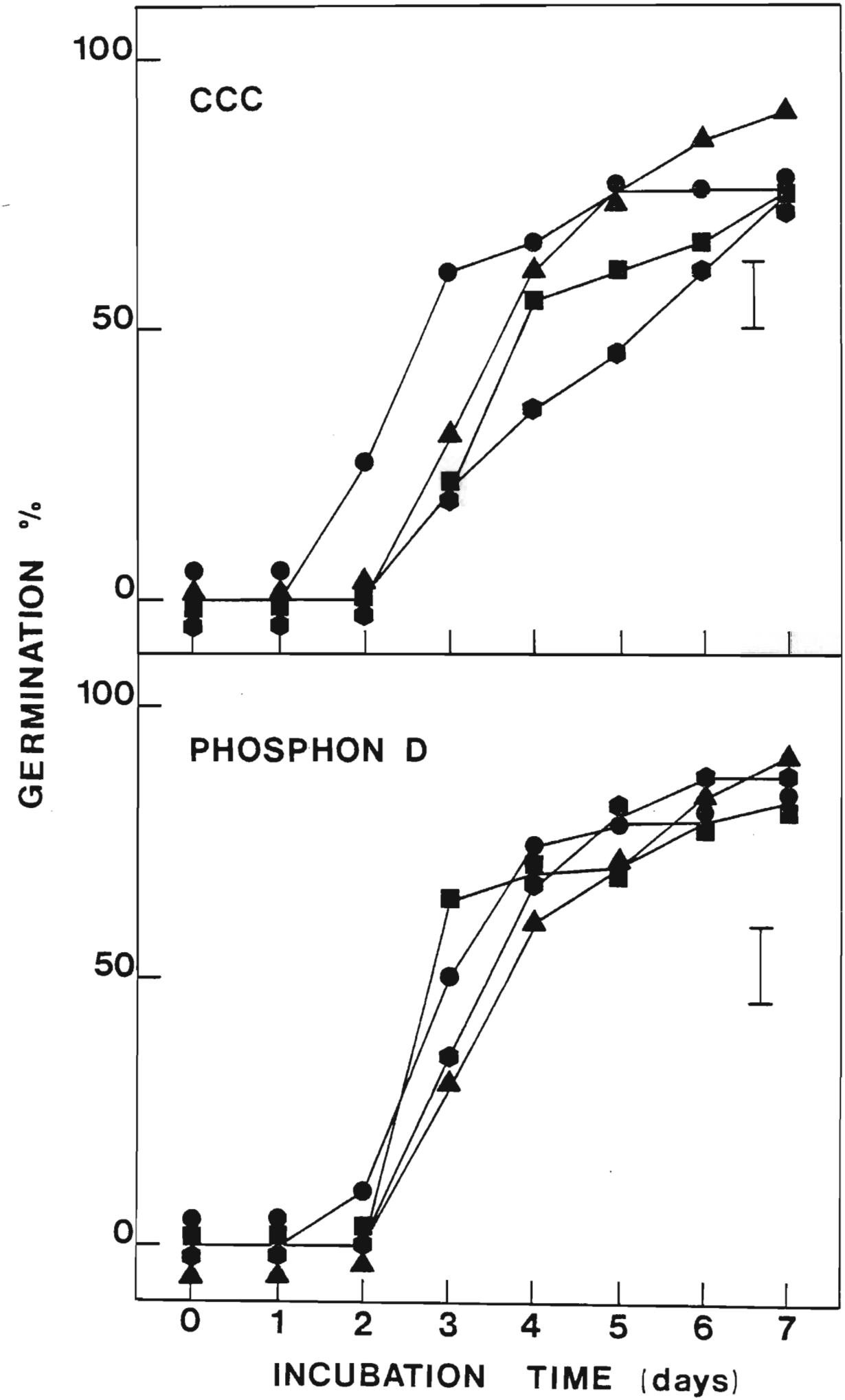

100
CCC
50
0
100
PHOSPHON D
50
0
GERMINATION %
0
1
2
3
4
5
6
7
INCUBATION TIME (days)

Table 3.8 Effect of phosphon D and CCC, applied at various times, on the growth of ethylene treated *R. rautanenii* seedlings. Dry mass determinations were after seven days incubation. Figures in parentheses represent the confidence limits where p = 0,05.

Period of Treatment	Dry Mass (grammes per seed)		
	Roots	Shoots	Endosperm
Control (dist. H_2O)	0,02 (± 0,006)	0,09 (± 0,025)	0,79 (± 0,02)
Phosphon D:			
During imbibition	0,03 (± 0)	0,14 (± 0,02)	0,76 (± 0,2)
During gasing	0,02 (± 0,01)	0,07 (± 0,03)	0,79 (± 0,14)
Throughout	0,02 (± 0,002)	0,10 (± 0,03)	0,73 (± 0,07)
CCC:			
During imbibition	0,03 (± 0,007)	0,13 (± 0,06)	0,71 (± 0,05)
During gasing	0,01 (± 0,008)	0,07 (± 0,01)	0,82 (± 0,1)
Throughout	0,02 (± 0,001)	0,15 (± 0,09)	0,77 (± 0,26)

were noted between seeds kept in the inhibitors and those removed from them after the ethylene treatments. This is in agreement with the generally accepted role of gibberellins which is that they are required for normal development of the seedlings.

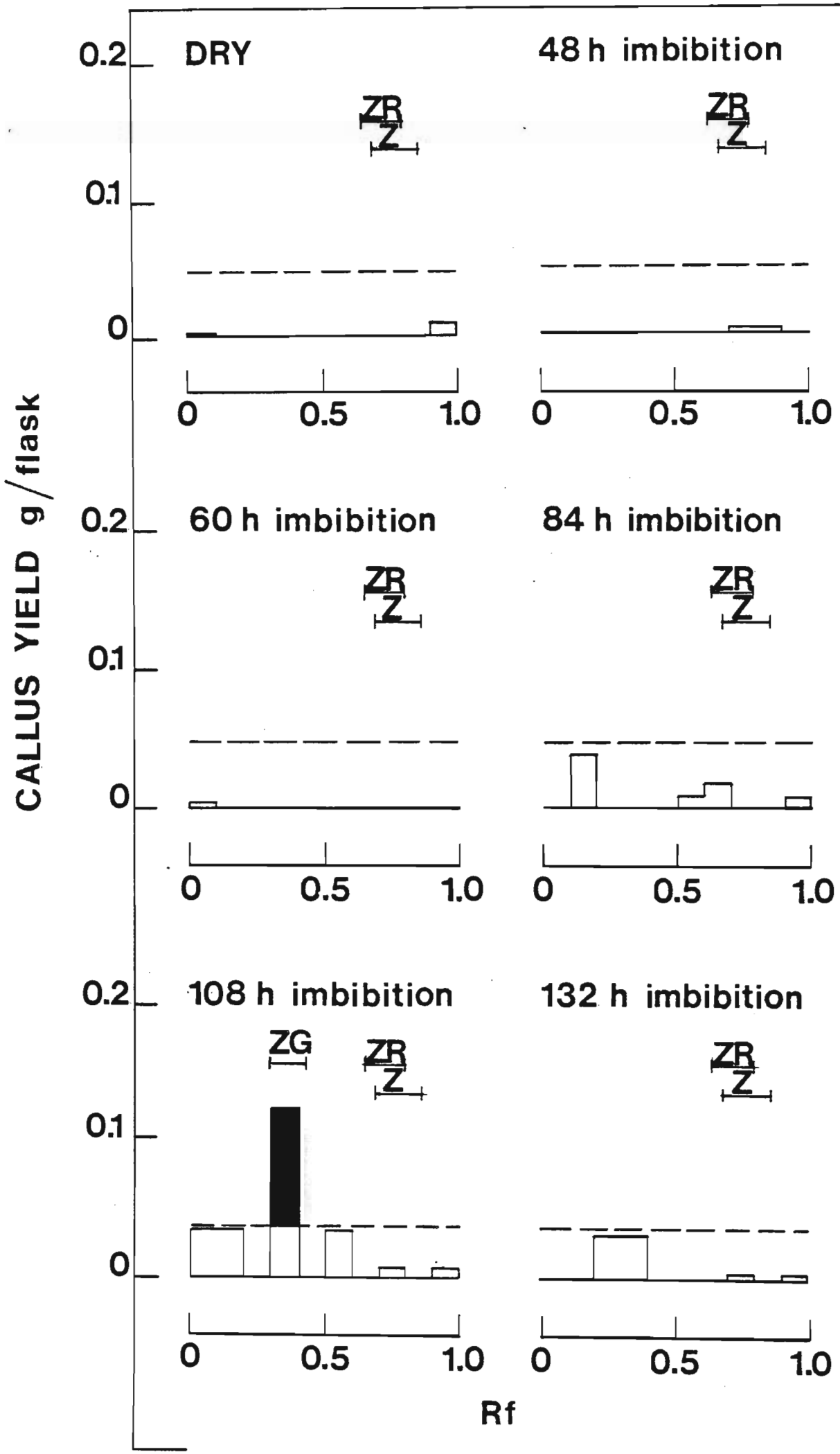
From these data it seemed anomalous that gibberellin synthesis was not involved in dormancy breaking, although applied gibberellins could actually stimulate germination. However, the possibility exists that ethylene may stimulate a qualitative change in gibberellins where biologically inactive forms of the hormone are metabolized to the more ac-

tive forms. Inhibitors of gibberellin synthesis would clearly have no effect on this aspect of metabolism and germination would thus proceed in the presence of such compounds. Applied gibberellins, on the other hand, would simply satisfy the requirement for biologically active gibberellins, thus making the presence of ethylene unnecessary. This aspect should be examined in more detail in future studies on *R. rautanenii* seeds.

Present evidence indicates that, in conjunction with gibberellins, cytokinins also play an active role in germination (WEBB, VAN STADEN and WAREING, 1973). In terms of KHAN'S model for dormancy and germination, this would be related to the 'permissive' role cytokinins have in controlling the effects of gibberellins (KHAN, 1971). THOMAS (1977) suggested that cytokinins could achieve this control over gibberellins by regulating their movement across membranes. Thus, in *R. rautanenii* seeds, dormancy may be due to the absence of a threshold cytokinin concentration which would be required to stimulate radicle elongation. The effect of ethylene could then be to alter this condition by stimulating cytokinin synthesis or interconversion.

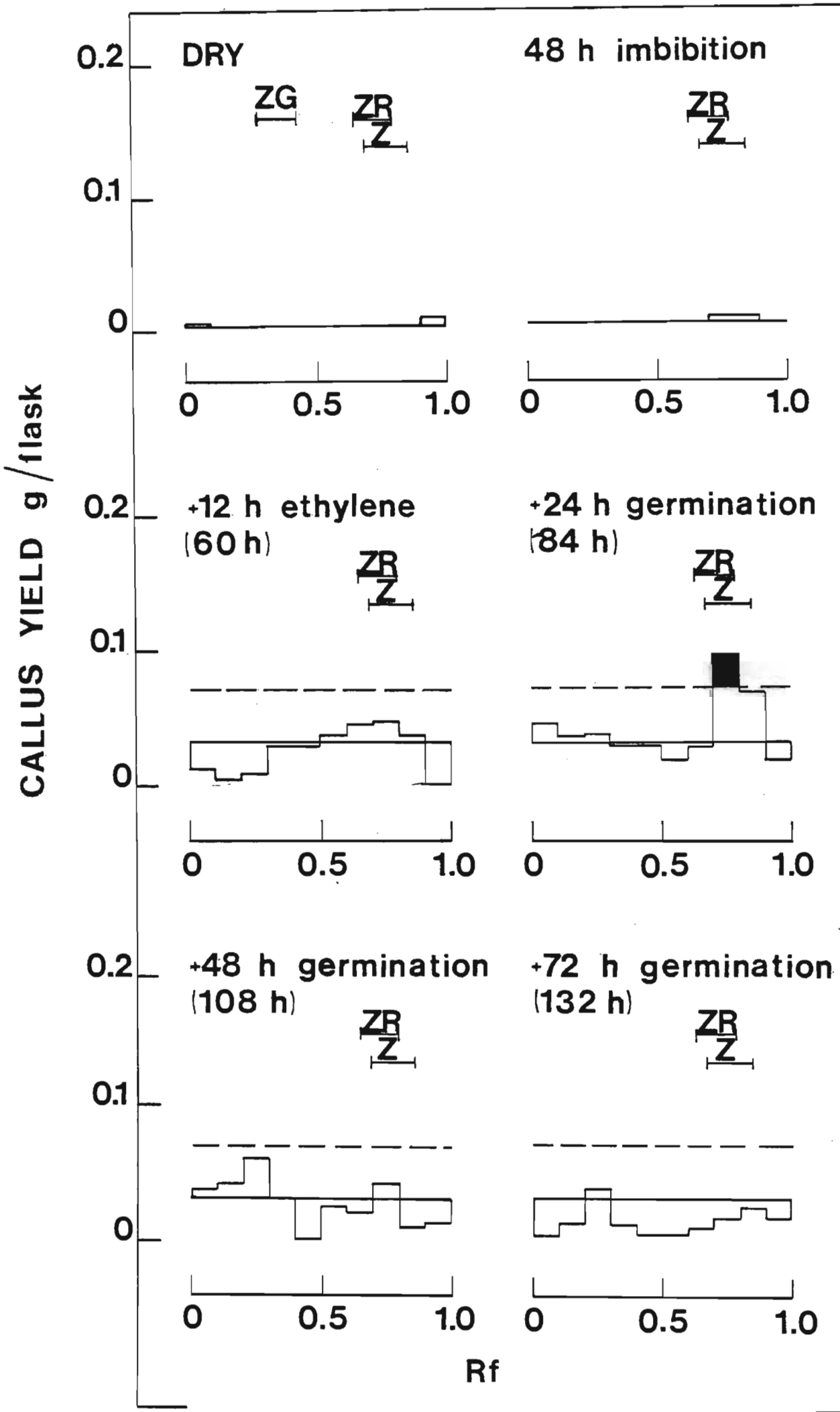
The results presented in Figure 3.30 show that, unlike *Zea mays*, (SMITH and VAN STADEN, 1978) and *Leucadendron daphnoides* (BROWN and VAN STADEN, 1975), dry embryonic axes of *R. rautanenii* seeds contained no detectable levels of cytokinins. Furthermore, no changes were observed as a

Figure 3.30 Soybean bioassay results for cytokinins extracted from 0,15 grammes of untreated dry and imbibed embryonic axes. Chromatograms were developed in *iso*-propanol:25% ammonium hydroxide:water (10:1:1 v/v). Z = zeatin, ZR = ribosyl-zeatin and ZG = glucosylzeatin. The broken line indicates the confidence limit at p = 0,01. The results are the means of two bioassays and 10 µg/ℓ of kinetin standard gave 0,07 g/glask callus growth.


DRY
48 h imbibition
60 h imbibition
84 h imbibition
108 h imbibition
132 h imbibition
ZR
Z
ZG
0.2
0.1
0
0
0.5
1.0
CALLUS YIELD g/flask
Rf

result of imbibition except after 108 hours. At this time, a transient peak in the more polar cytokinins occurred. This peak co-chromatographed with zeatin glucoside which is regarded as the biologically inactive storage form of the hormone (PARKER and LETHAM, 1973). On the basis of this, it is unlikely that this particular cytokinin played an active role in the metabolism of these dormant seeds. The small, transient peak in cytokinin levels observed 24 hours after the application of ethylene may be significant in terms of germination (Figure 3.31). This is based on the fact that this peak co-chromatographed with the biologically active cytokinin, zeatin. Thus, the appearance of this compound prior to any visible signs of germination may be linked to its suggested role as an initiator of radicle growth (PINFIELD and STOBBART, 1972). This is supported to some extent by the fact that the cytokinin makes its appearance at a time when the only visible signs of activity are located in the nucleus and coincides with the transient reappearance of glucose and sucrose. The subsequent disappearance of cytokinins in this tissue is consistent with the observations made in germinating *Leucadendron daphnoides* seeds (BROWN and VAN STADEN, 1973) in which case the authors suggested that the cytokinins are rapidly metabolized once growth is resumed. The impression gained from this preliminary investigation was that cytokinins are probably not directly involved in mediating the ethylene response and that the transient peak observed was more likely the result of germination rather than the cause of dormancy breaking. This

Figure 3.31 Soybean bioassay results for cytokinins extracted from 0,15 grammes of ethylene treated embryonic axes. Chromatograms were developed in *iso*-propanol:25% ammonium hydroxide:water (10:1:1 v/v). 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 10 µg/ℓ of kinetin standard gave 0,1 g/flask callus growth.


DRY
48 h imbibition
+12 h ethylene (60 h)
+24 h germination (84 h)
+48 h germination (108 h)
+72 h germination (132 h)
ZG
ZR
Z
0.2
0.1
0
0.5
1.0
CALLUS YIELD g/flask
Rf

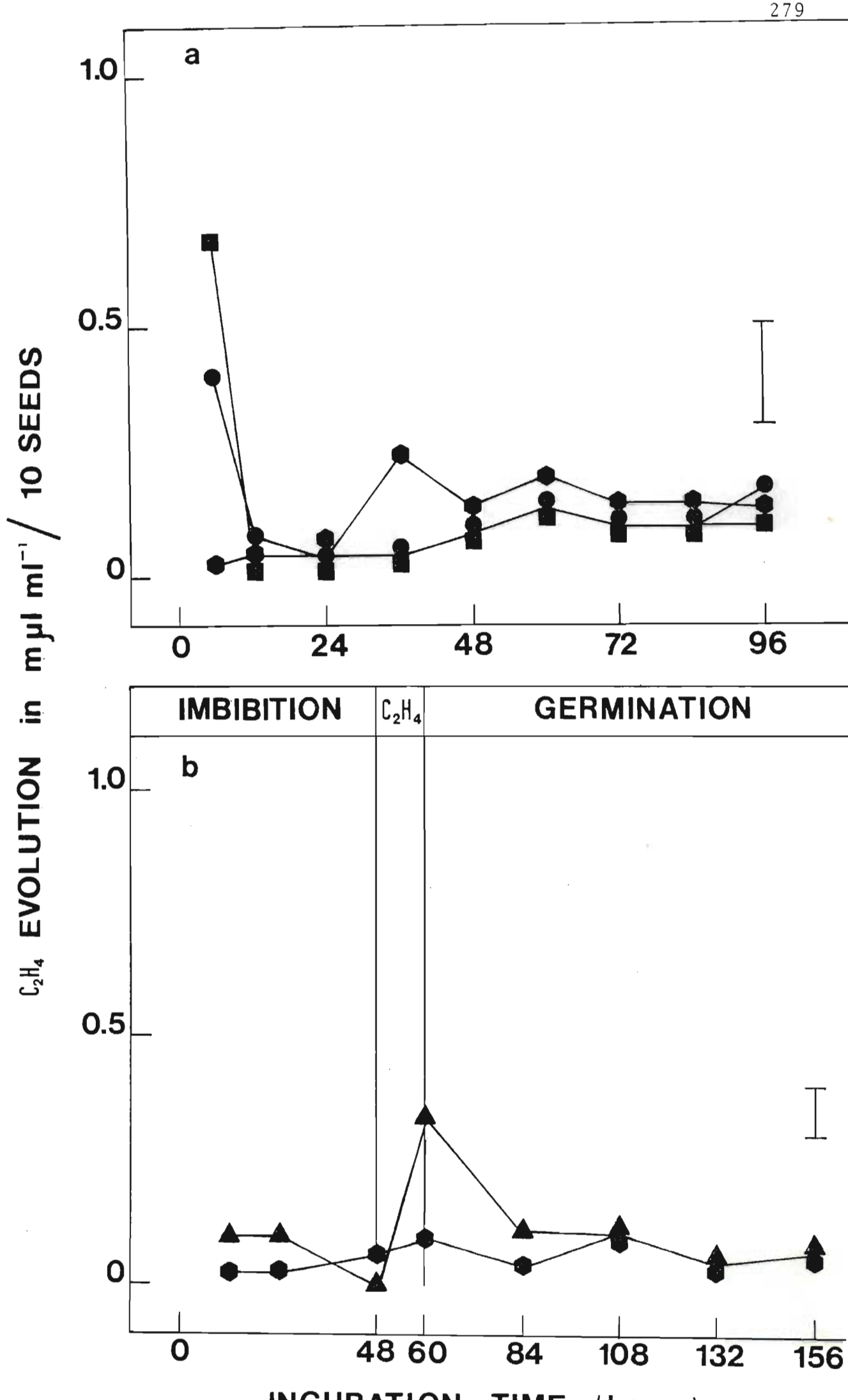
is supported by the fact that in previous studies, applied cytokinins could not overcome the dormant condition (Chapter 1). However, more detailed research is required, particularly with regard to the possible changes occurring in the cotyledon and endosperm tissues during dormancy breaking and germination.

In terms of KHAN'S model for seed dormancy, the results obtained suggested that it was unlikely that any inhibitors present in the embryonic axis were responsible for dormancy. This is based on two observations. Firstly, if inhibitors were involved, it would be expected that applied cytokinins would have had the effect of negating their influence. Similarly, ethylene treatments would have been expected to cause a marked increase in endogenous cytokinin levels. In the second instance, applied gibberellins would not have had any effect on germination, since the seeds would essentially require the additional presence of cytokinins before the gibberellins could operate. Studies on the actual levels of endogenous inhibitors are now required to examine this possibility.

As mentioned previously, applied ethylene, as well as various other dormancy breaking treatments, very often have their effect by stimulating the production of endogenous ethylene. An investigation was carried out to determine whether or not, in the case of *R. rautanenii* seeds, the effects of applied ethylene, ethrel and gibberellic acid were mediated in this manner. Chromatographic

analysis of samples taken at various times from control seeds treated with distilled water showed that, upon imbibition, manketti seeds produced a basal amount of ethylene. This ranged between 0,001 and 0,02 millimicrolitres per litre per seed (Figures 3.32a and b). However, Figure 3.32a shows that after the first 6 hours of incubation, ethrel and gibberellin treated seeds produced significantly more ethylene than the controls. This increased ethylene synthesis was not maintained and after 12 hours incubation, ethylene evolution by these treated seeds was the same as the controls. It thus appeared that the initial peaks observed were only transient phenomena. Gas treated seeds behaved in a similar manner and a transient increase in ethylene production was also observed immediately after the dormancy breaking treatment. These results suggested that a transient increase in the synthesis of ethylene could be associated with the early stages of dormancy breaking in *R. rautanenii* seeds. However, it is difficult to assess the significance of this finding since the increases in ethylene production were not sustained as was found to be the case in stimulated *Arachis hypogaea* (KETRING and MORGAN, 1971) and *Xanthium pensylvanicum* (KATOH and ESASHI, 1975a) seeds. It thus seemed more likely that in manketti seeds, ethylene production was a result of renewed embryo activity, rather than a cause of it. In a recent study on bean seeds, DE GREEF, DE PROFT, VEROUSTRAETE and FREDERICQ (1980) found that the onset of vigorous embryo growth coincided with a transient increase in ethylene release. However, unlike the situation in

FIGURE 3.32 Ethylene synthesis by (a) ethrel (■) and GA_3 (●) treated seeds and (b) seeds in which dormancy was broken by a 12 hour ethylene treatment (▲). Untreated control seeds are represented by ⬢. The bars represent the maximum 95 per cent confidence limits.


a
1.0
0.5
0
0
24
48
72
96
IMBIBITION
C_2H_4
GERMINATION
b
1.0
0.5
0
0
48
60
84
108
132
156
C_2H_4 EVOLUTION in mμl ml^{-1} / 10 SEEDS
INCUBATION TIME (hours)

Ricinus communis (SPENCER and OLSON, 1965), *R. rautanenii* seeds exhibited no peak in ethylene production during radicle emergence.

The view that endogenous ethylene production is unimportant in dormancy breaking in this species, was supported by the findings of an experiment using an ethylene-removing agent. A one Molar solution of potassium permanganate (SALTVEIT, 1980) was used to remove ambient ethylene from the air surrounding ethrel, gibberellin and gas treated seeds. This treatment had no effect on germination and 90 per cent of the seeds germinated within four days. A similar treatment applied to ethylene sensitive *Trifolium subterraneum* seeds resulted in a drastically reduced germination response (ESASHI and LEOPOLD, 1969). Thus, in *R. rautanenii* seeds, endogenously produced ethylene was most probably a by-product of metabolism.

These investigations into the possible involvement of other phytohormones in mediating the effects of applied ethylene provided some interesting information regarding the dormancy breaking process. Firstly, it appeared that although applied gibberellins had a marked effect on germination, the synthesis of this hormone was not affected by ethylene treatments. If endogenous gibberellins are involved in the breaking of manketti seed dormancy, it would most likely be through qualitative changes. In the second instance, it seemed that cytokinins were also not involved in the process of dormancy breaking. Their

transient appearance after the ethylene treatment was probably more related to the onset of germination rather than a cause of it. This is supported by the earlier finding that applied cytokinins had no effect on germination. However, this hormone was also present, albeit in a storage form, in dormant, imbibed tissues and more research is required to ascertain the reason for this. Finally, one of the most interesting findings to emerge from this series of experiments, was that dormancy breaking treatments only caused a transient increase in the basal ethylene production of these seeds. This is unlike *Arachis hypogaea* and *Xanthium pensylvanicum* seeds, in which dormancy breaking was closely related to a marked increase in the general ethylene biosynthesis. The transient increase in ethylene production noted in manketti seeds appeared to be unimportant in the process of dormancy breaking, since removal of the gas from the surrounding atmosphere had no effect. This suggested that ethylene had its effect in a somewhat different manner in *R. rautanenii* than it appears to have in other ethylene sensitive species. It would be interesting to note, in future studies, whether increased ethylene production in species such as *Xanthium pensylvanicum* and *Arachis hypogaea* could be correlated with increased levels of 1-aminocyclopropane-1-carboxylic acid (ACC). This would indicate whether ethylene was deliberately being synthesized or whether it was merely a metabolic by-product.

To summarize, the results obtained from the experiments

detailed in this chapter failed to indicate which aspect of metabolism was directly affected by ethylene. However, it is possible to state that reserve hydrolysis, respiration and protein and RNA synthesis could take place in the absence of any dormancy breaking treatments. These findings were supported by the fine structural studies which showed that numerous ultrastructural changes took place in imbibing, dormant tissues. Protein synthesis was found to be essential for the successful consummation of ethylene treatments, whereas inhibition of RNA synthesis and DNA replication did not prevent germination. More detailed studies are now required to determine whether qualitative changes in proteins could be associated with dormancy breaking. With regard to other aspects of metabolism, ethylene did not appear to directly affect the pentose phosphate pathway since compounds which are known to stimulate alternate respiration had no effect. The effects of ethylene on other phytohormones as well as its own endogenous production were also examined. The results indicated that the changes which took place were most likely the result of germination, rather than a cause of it. However, this study did not cover the possibility that ethylene initiates qualitative changes in gibberellins and this aspect must be thoroughly investigated in future research. One feature that was consistently related to ethylene treatments, was the increased uptake of the labelled amino acid, leucine. The rapidity of this response suggested that ethylene may have its effect by changing the permeability of membranes. In having this

effect, ethylene may also alter cell wall extensibility and BURG, ABELBAUM, EISINGER and KANG (1971) in fact stated that, in their opinion, the immediate effect of ethylene binding is the promotion of cell expansion. A change in wall plasticity would enable the embryonic axes of manketti seeds to expand and hence crack through the enclosing testa. Thus, in future studies on *Ricinodendron rautanenii* seeds, attention must be given to the possibility that ethylene overcomes dormancy by promoting cell expansion and thus embryo enlargement.

CONCLUSIONS

Numerous similarities and differences were found to exist between seeds of *Ricinodendron rautanenii* and those of many other ethylene sensitive species. Studies on their germination requirements showed that manketti seeds were unique since ethylene was the only naturally occurring stimulus that would relieve their dormant condition. (In other species, treatments such as changes in temperature also stimulated germination). In addition to this ethylene requirement, manketti seeds also exhibited a form of coat imposed dormancy. The tough, woody endocarp which encloses these seeds was found to physically restrict embryo enlargement even in the presence of ethylene. It was thus concluded that in order for ethylene to be effective under natural conditions, the endocarp would have to undergo considerable degradation.

Ricinodendron rautanenii seeds were found to have a remarkable sensitivity to ethylene. The threshold and saturating ethylene concentrations were found to lie below those most frequently recorded in many other species. Furthermore, dry seeds also exhibited the capacity to respond to ethylene. These features alone suggested that manketti seeds were the most sensitive to ethylene yet recorded. Added to this, was the fact that the seeds required only a very short period of exposure to the gas, indicating that the effects of this hormone were extremely rapid.

This remarkable sensitivity to ethylene was, however, only observed over a well defined temperature range and this appears to be a common feature amoung ethylene sensitive species. The optimum temperature range lay between 25 and 35°C and little or no germination was recorded at temperatures below 20°C. This may be due to the inability of ethylene to act at these low temperatures. Germination was reduced when temperatures exceeded the optimum range, indicating that some mechanism was present which may prevent germination at excessively high temperatures. It is possible that this feature may have evolved to prevent germination under conditions which are detrimental to seedling growth and which are linked to high temperatures. An autecological study is necessary to investigate this further. Manketti seeds differ from a number of other species in that they do not have a period of carbon dioxide sensitivity associated with their ethylene requirement nor do they require light in order to germinate. Nevertheless, both parameters would be necessary for the normal growth of the resultant seedlings.

Another unique feature of *R. rautanenii* seeds was their ability to retain the effects of the gaseous hormone even after a period of almost complete re-dehydration. This indicated that, not only were the seeds able to respond to extremely low ethylene concentrations, but that they could do so even after a considerable delay between the breaking of dormancy and the advent of reasonable germination conditions. Furthermore, the effects of ethylene were

clearly permanent since dehydration of treated seeds did not prevent germination. From these results it was concluded that, in the field, seeds which had received sufficient ethylene would retain the ability to germinate should immediate conditions not be suitable.

As already mentioned, dry manketti seeds appeared to have the ability to respond to ethylene. Additional experiments on this aspect showed that in fact, seeds which had imbibed for only three hours, germinated as well as those which had been imbibing for 48 hours. Thus, the ethylene receptor protein may be sufficiently hydrated in dry tissues to respond to ethylene or it may require very little water to enable it to do so. Whichever of these is correct, this ability of barely hydrated manketti seeds to perceive the dormancy breaking stimulus adds to the remarkable sensitivity of these seeds to ethylene.

The investigations carried out into which aspect of metabolism was affected by ethylene, showed that initial food reserve mobilization was probably not involved in the breaking of dormancy. By actually measuring the levels of lipids, sugars, starch and protein, it was found that some mobilization occurred prior to any dormancy breaking treatments, and this was supported by ultrastructural studies. However, *R. rautanenii* seeds were not unique in this respect, since both VILLIERS (1971) and ROST (1972) found that reserve hydrolysis took place in dormant tissues. The implication of these findings was that the enzymes

necessary for reserve mobilization were already present in the dry seeds, or were rapidly synthesized after the commencement of imbibition. Nevertheless, ethylene was not required to stimulate the activity of these hydrolytic enzymes.

Another interesting finding to emerge from this study, was that the food reserve levels in dormant seeds returned to their original values after an extended period of imbibition. This indicated that the reserves mobilized during the first 48 hours had been replaced suggesting that synthetase enzymes were also active in these tissues.

In spite of the disorganized appearance of the internal membranes of mitochondria visible in imbibed, dormant axes, dormancy did not result from an inability to produce energy rich compounds. This is based on the observation that carbon dioxide production (and hence, probably, respiration) occurred prior to ethylene treatments. In addition, the poor response obtained from seeds incubated under sterile conditions on nutrient media, suggested that dormancy was not due to an inability to metabolize certain substrates. The reason for this is that such media contain nutrients and energy sources in forms which are readily usable.

Protein synthesis also occurred in the absence of any dormancy breaking treatments. This activity was attributed to preformed components which were probably represented in the dry tissues as ribosomes. Subsequent protein syn-

thesis which relied on the synthesis of new RNA fractions also occurred in dormant embryonic axes and the inhibition of this particular aspect of metabolism did not prevent germination. Thus, it seemed that dormancy did not result from the inability of these seeds to synthesize proteins or new RNA.

Changes in fine structure and levels of food reserves which occurred as a result of the dormancy breaking ethylene treatments were typical of those normally described in the literature for other species (BEWLEY and BLACK, 1978; CHING, 1972). However, a 48 hour lag period existed between the application of the dormancy breaking treatment and the onset of germination. During this time, metabolic readjustments may have been taking place as suggested by changes observed in the nuclei of the cells. It was noted that this organelle expanded considerably and that the granular content of the associated nucleoli increased at the same time as nucleolar vacuoles disappeared. These observations suggested that ribosomal RNA synthesis had commenced. Thus, RNA fractions produced prior and subsequent to ethylene treatments may differ qualitatively, although not necessarily quantitatively. This aspect should be investigated in more detail.

Protein synthesis was generally lower in the embryonic axes from ethylene treated seeds during the 48 hour, post-ethylene lag period. It is not clear from this whether ethylene suppresses the production of certain inhibitory

compounds, but the results do show that ethylene treatments cannot be associated with a rise in protein synthetic abilities. However, this does not eliminate the possibility that qualitative changes in proteins may be involved in dormancy release. In these experiments, one notable effect of the dormancy breaking ethylene treatment was a marked rise in the uptake of the labelled amino acid, leucine. This phenomenon occurred consistently throughout the experiments on protein synthesis and was always observed in those seeds which would germinate. From this it was concluded that ethylene may relieve dormancy by altering membrane permeability. In this way, ethylene may affect germination by regulating the movement of certain compounds, one of which may be gibberellin. Alternatively, this result may reflect ethylene's effect on the plasma membrane, the result of which is an increase in cell expansion. By promoting cell expansion, ethylene would have a marked effect on the ability of the embryo to penetrate the testa. It may be significant in this respect that germination in this species was characterized by a cracking of the testa followed by radicle protrusion. Future studies on the effects of ethylene on *Ricinodendron rautanenii* seeds should investigate this in more detail.

Applied gibberellins were found to stimulate manketti seed germination. However, this was not brought about by an effect on endogenous gibberellin synthesis, since inhibitors of the gibberellin biosynthetic pathway did not prevent germination. Thus, if ethylene does influence gibberellin

metabolism in any way, it would most likely have been through stimulating the conversion of bound, inactive forms of gibberellin to the more biologically active ones. Alternatively, ethylene may, by its effect on membranes, influence the movement of gibberellins between compartments in a manner similar to that suggested for cytokinins (THOMAS, 1977). Finally, the possibility also exists that the effects of gibberellin were simply pharmacological and that at certain concentrations they simulate the effects of ethylene on membrane permeability. The possibility that gibberellins may effect cell membranes has, in fact, been investigated in a number of instances. PALEG, WOOD and SAWNHEY (1971) suggested that this hormone controlled barley endosperm hydrolysis by increasing the permeability of the membrane's surrounding enzyme containing organelles.

A preliminary investigation on the changes in cytokinins showed that this hormone was probably not directly involved in dormancy breaking or gibberellin metabolism.

The finding that applied ethylene, ethrel and gibberellins did not result in a continued enhancement of endogenous ethylene production, suggested that in *R. rautanenii* seeds, ethylene had a somewhat different mode of action from that in other species. The changes in ethylene production which occurred as a result of the various dormancy breaking treatments were transient and by all accounts appeared to be the result of renewed activity, rather than a cause of it. Nevertheless, it is interesting to note that in

those instances where applied ethylene does stimulate endogenous ethylene production, workers have failed to question the role of this ethylene. Future research on this aspect should thus concentrate on determining the metabolic 'target' of this endogenous ethylene.

Finally, another major conclusion that was drawn from this study, was that the endosperm played no role in the process of dormancy breaking. The reserves contained within these organs were only mobilized at a very late stage and therefore probably served to nourish seedling growth, rather than germination. This finding highlights the value of examining the various plant tissues separately. Many of the earlier studies on seed dormancy failed to do this, resulting in a situation where the data creates the wrong impression regarding the processes involved in dormancy breaking. Future studies on this aspect of physiology should continue to seek out a unifying concept for seed dormancy. The sophisticated techniques which have been developed with regard to molecular biology will no doubt assist in this quest.

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