Some implications of associated mycoflora during hydrated storage of recalcitrant seeds of *Avicennia marina* (Forssk.) Vierh.

by

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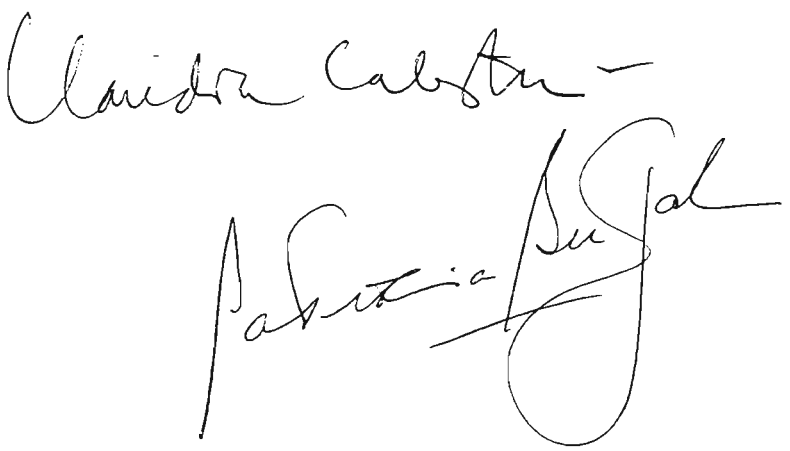
Preface

The experimental work described in this thesis was carried out in the School of Life and Environmental Sciences of the University of Kwazulu-Natal, Durban, South Africa, under the supervision of Profs Patricia Berjak and Norman W. Pammenter (School of Life and Environmental Sciences), Michelle McLean (Medical School) and Dr. Neil Koorbanally (School of Pure and Applied Chemistry).

These studies represent original work by the author, and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.



Dedication

I dedicated this thesis to my husband for his love and understanding and to my parents for everything that they have done for me.

Acknowledgements

To Patricia Berjak for giving me a chance back in 1993, for challenging and inspiring me, to Norman Pammenter and Michelle McLean for their support and encouragement and to Neil Koorbanally for his guidance through the plant metabolic pathways. I am truly grateful.

I am also indebted to other colleagues who helped me at various stages during the course of this work. To Dr. Peter Cheplogoi from the School of Pure and Applied Chemistry for his help and cooperation in the work on the elucidation of the structures of the antifungal compounds. To Dr. Vesselina Merhar for her assistance and cooperation in the detection and quantification of the PR-proteins. To Dr. James Wesley-Smith, Mrs. Priscilla Maartens and the late Dr. Fiona Graham for training and assistance in electron microscopy and dark room technology. To all members of the Plant Cell Biology Research Group for their help in seed collecting.

Abstract

Three questions are considered in the context of the possible effects of seed-associated mycoflora, typified by *Fusarium moniliforme*, during hydrated storage of recalcitrant seeds of the tropical species, *Avicennia marina*. These are: 1) whether fungal infection reduces storage lifespan; 2) whether seeds become more susceptible to fungal attack during storage and whether they posses defence mechanisms that might suppress fungal proliferation in hydrated storage (production of antifungal compounds and β-1,3-glucanase (EC 3.2.1.39) and chitinase (EC 3.2.1.14)] and 3) whether it is possible to discriminate ultrastructurally between inherent deteriorative changes and those that are fungally-induced.

1) The data indicate unequivocally that if fungal activity is curtailed, then the hydrated storage lifespan of *A. marina* seeds can be considerably extended.

2) When inoculated immediately with *F. moniliforme*, newly harvested seeds were extremely susceptible to the adverse effects of the fungus, while seeds that had been wet-stored for 4 days showed a considerably heightened resilience to the effects of the fungus prior to inoculation. The enhanced resilience, although declining, persisted in seeds stored hydrated for up to 10 days prior to inoculation, being lost after 12 days. This finding was supported by significant increase in β-1,3-glucanase and chitinase and in antifungal compound production during 10 days of wet storage. After 14 days of wet-storage, seeds become more susceptible to the effects of fungus than those in the newly harvested condition.

3) The resilience of seeds that had been stored in the short-term was associated with ultrastructural changes indicative of enhanced metabolic activity associated with the onset of germination (e.g. increase in vacuolation, well-developed mitochondria and endomembrane system [ER and Golgi bodies]). However, with sustained stress associated with wet-storage

conditions, the seeds became increasingly badly affected by the fungus, showing some ultrastructural fungally-induced abnormalities (e.g. nuclear lobing, presence of lipid bodies and prevalence of Golgi bodies that had many associated vesicles) and a decrease in β-1,3-glucanase and chitinase activity.

It is suggested that the decreased susceptibility of *A. marina* seeds during short-term storage relies on the ability to create an antifungal environment prior to infection (through synthesis and accumulation of pre-formed and induced antifungal compounds and antifungal enzymes), which would also be an effective strategy during germination in the natural environment.

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Chapter 1

Understanding recalcitrant seeds and their storage: An overview

1.1 General considerations

Presently, world plant genetic resources are being eroded through loss of old cultivars and replacement of primitive land races by genetically uniform cultivars. Furthermore, non-domesticated plants are severely threatened by loss of natural areas to agriculture, industrialisation and urbanisation, together with phenomenon such as desertification and climate change. Thus, there is an urgent demand for conservation of genetic resources via *ex situ* germplasm preservation, the most straightforward approach to which is seed storage.

- **Orthodox seeds**

The most common storage practices use the principle of extension of seed lifespan by storage at low moisture content and low temperatures (Justice and Bass, 1978). The seeds of a great many species can be stored successfully by maintenance under these conditions. Such seeds, termed orthodox, mostly undergo a period of maturation drying upon the parent plant as the final phase of their pre-shedding development, during which the seed moisture content falls to a low level, attaining equilibrium with the relative humidity of the atmosphere (Roberts, 1973). The International Board for Plant Genetic Resources (predecessor of The International Plant Genetic Resources Institute, IPGRI) recommended that for the purpose of conservation, orthodox seeds should be stored at a moisture content of approximately 5% (wet mass basis, wmb) at –20 ºC (IBPGR, 1976). In addition, to avoid accumulation of genetic damage, it is further suggested that plants should be generated when seed viability has declined by five percent. As long as their quality is good at the outset orthodox seeds can be stored for many decades following these guidelines. Storage lifespan of such seeds can, in fact, be predicted by application of the viability equation, an improved version of which was presented by Ellis and Roberts (1980).

- **Recalcitrant seeds**

Recalcitrant seeds are shed at relatively high water contents, undergoing little or no maturation drying upon the parent plant. In order to store this type of seed, moisture content must be maintained at, or only slightly below, that of the newly shed state (Berjak *et al.*, 1989). Nevertheless, viability retention in such hydrated storage generally ranges from only days to months, depending on the species (Roberts and King, 1980). Recalcitrant seeds may also be sensitive to low temperatures, being damaged by chilling injury at temperatures of 10–15 °C or lower, depending on the species (King and Roberts, 1980). Temperate species such as *Aesculus hippocastanum* (Dickie *et. al.*, 1991), *Acer pseudoplatanus* (Tompsett and Pritchard, 1993) and *Quercus robur* (Finch-Savage, 1992) are chilling tolerant, although they never become desiccation tolerant despite losing a portion of tissue water during their development. Seeds of some temperate species (exemplified by *Q. robur*, are recorded as surviving in hydrated storage for more than one year (Tylkowski, 1977). The recalcitrant seeds of the tropical species *Avicennia marina*, lose no water during their development and are very sensitive to dehydration prior to, and after they are shed (Farrant *et al.*, 1993a). Because recalcitrant seeds are desiccation and, in some cases, chilling sensitive and they also have quite unpredictable long-term storage behaviour, their maintenance is very difficult.

- **Intermediate seeds**

Another seed category has been identified, being described as intermediate. Coffee (*Coffea arabica* L. and *Coffea canephora*) (Ellis *et al.*, 1990, 1991a; Hong and Ellis, 1992, 1995), some *Citrus* species (Hong and Ellis, 1995), papaya (*Carica papaya*) (Ellis *et al.*, 1991b) and oil palm (*Elaeis guineensis*) (Ellis *et al.*, 1991c) fall into this seed category. Although such seeds have a degree of tolerance to dehydration to low water content (7 - 12%, wmb), some of them are also chilling sensitive, especially those of tropical origin that lose viability in the air dry state at temperatures below 10 °C (Ellis *et al.*, 1990; Ellis *et al.*, 1991a, b, c; Hong and Ellis, 1996).

These three seed categories are, however, open-ended, as it is often difficult to assign a species unequivocally to a particular seed group. A critical survey of the literature has concluded that there are 8376 orthodox or probably orthodox seeds, 526 recalcitrant or probably recalcitrant seeds and 145 intermediate or probably intermediate seeds (Tweddle *et al.*, 2003).

In the recalcitrant group alone, there are significant differences in the post-harvest responses of seeds of individual species (Farrant *et al.*, 1988; Berjak *et al.*, 1989). Among these seeds there is a varying degree of desiccation sensitivity, some species being intolerant to small levels of water loss and others tolerant to a more significant degree of dehydration. An additional complication that prevents categorical generalisations from being made, is that the degree of dehydration tolerated by recalcitrant seeds of individual species, depends on the rate of water loss (Pammenter *et al.*, 1998), and possibly also of the temperature at which this occurs.

1.2 Seed recalcitrance

Recalcitrant seeds are metabolic when they are shed and, depending on the developmental stage at which this occurs and the species, some seeds could go through the final stages of the development after shedding while other begin the germination immediately (Berjak *et al.*, 1989). In contrast to orthodox seeds that require water uptake to germinate (Côme and Corbineau, 1989), the ongoing metabolism of recalcitrant seeds will proceed at the water content at which they are shed.

The metabolic changes that have been identified in stored recalcitrant seeds have been linked to the processes of ongoing development and germination (Pammenter *et al.*, 1984; Berjak *et al.* 1989; Côme and Corbineau, 1989). Recalcitrant seeds do not pass through a developmental stage that induces any

significant degree of desiccation tolerance (Berjak *et al.*, 1984) in contrast to orthodox seeds that do acquire desiccation tolerance, loosing this only during the process of germination. After shedding, recalcitrant seeds have been likened to imbibed germinating orthodox seeds that have become susceptible to desiccation (Berjak *et al.*, 1984).

The number of plants known to produce recalcitrant seeds is increasing, as is the interest in regional indigenous biodiversity (Tweddle *et al.*, 2003). Recalcitrant seeds are produced by important food plants such as mango (*Magifera indica)* (Lizada, 1991; Girija and Srinivasan, 2000), cocoa (*Theobroma cacao*) (Li and Sun, 1999; Liang and Sun, 2002), mandarin (*Citrus reticulata*) (Khan *et al.* 2002) and avocado (*Persea* Americana) (Raja *et al.*, 2001). They are also known to be produced by commercial species such as rubber (*Hevea brasiliensis*) (Berjak *et al.*, 1989) and by medicinal plants such as the mangosteens (*Garcinia* spp.) (Normah *et al.*, 1997).

Although there are a few temperate recalcitrant species (horse chestnut – *Aesculus hippocastanum* [Bonner, 1990], English oak – *Quercus robur* [Finch-Savage *et al.*, 1996], Durant oak – *Q. durandii,* cherrybark oak – *Q. pagoda* [Connor and Sowa, 2002], sycamore – *Acer pseudoplatanus* [Bonner, 1990], trifoliate orange – *Poncirus trifoliata* [Purohit and Doijode, 1998] and tea – *Camellia sinensis* [Berjak *et al.*, 1993]), the ecological distribution of the majority of plants producing recalcitrant seeds is in tropical/subtropical aquatic or marshy regions or in humid rain forests. These environments generally do not have low temperature constraints and are conducive to rapid seedling establishment (Roberts and King, 1980).

If desiccation sensitivity is taken as the feature underlying seed recalcitrance, it appears that the category may be loosely sub-divided into highly, moderately and minimally recalcitrant (Farrant *et al.*, 1988; Berjak *et al.*, 1989). These sub-

categories were equated with apparently decreasing desiccation sensitivity by those authors, based on a survey of published information.

Tropical/sub-tropical forests or wetland areas provide conditions ideal for those plants that produce highly recalcitrant seeds, such as jackfruit [*Artocarpus heterophyllus*] (Wesley-Smith *et al.,* 2001), various mangroves (Farrant *et al.,* 1989; Farnsworth, 2000), Brazil nut [*Bertholletia excelsa*] (Cunha *et al.*, 1996) and hardwoods such as *Trichilia* spp. (Maghembe and Msanga, 1988; Choinsky, 1990). *Avicennia marina* is a mangrove species that produces recalcitrant seeds that are very sensitive to desiccation (Farrant *et al.,* 1993a). Immediately after shedding, seeds of *A. marina* begin the germination process which, in the absence of additional water, continues to a point short of radicle emergence. If additional water is provided, these seeds exhibit root protrusion within two to three days (Calistru *et al.,* 2000). When placed in storage under conditions that do not permit water loss, these highly recalcitrant seeds will nevertheless become debilitated, as additional water is required to sustain ongoing germination. Under such conditions, viability is lost generally within two weeks (Berjak *et al.*, 1984; Pammenter *et al.*, 1984; Farrant *et al.,* 1985; 1986; Calistru *et al.*, 2000).

Moderately recalcitrant seeds are produced by species in environments where water for seedling establishment is commonly available throughout the year (Farrant *et. al.*, 1988). Rubber (*Hevea brasiliensis*) produces seeds that Farrant *et al.* (1988) considered being moderately recalcitrant. The seeds appear to tolerate a certain degree of water loss in the short-term, but it remains to be ascertained how long they would survive in a 'sub-imbibed' condition (Eggers *et al.* [2003] suggested that sub-imbibed storage does not confer any benefit over *Trichilia emetica* seeds stored in fully hydrated state, but may in fact be deleterious to seed survival during storage). However, when placed in storage, if the relative humidity is kept elevated, moderately recalcitrant seeds should

remain viable for several weeks without provision of water, as their germination is slow.

In contrast to the highly and moderately recalcitrant sub-categories, minimally recalcitrant seeds appear to be able to tolerate a greater proportion of water loss. These species are distributed in subtropical and in some cases temperate regions, having in common conditions that are not continuously favourable for seedling establishment (Farrant *et al.*, 1988). As a consequence of their environment, these seeds might also tolerate lowered temperatures but not as low as O^0C (Roberts and King, 1980). Species of the gymnosperm, *Araucaria,* produce recalcitrant seeds (Tompsett, 1984; Galleschi *et al*, 2002) that are able to lose a significant proportion of the seed water content - up to 21% - without loss of viability (*A. hunsteinii*; Tompsett, 1982). Another representative species of this seed category is *Quercus alba* that is tolerant to storage at low temperatures, with germination occurring after eight months (Clatterbuck and Bonner, 1985). *Quercus rubra* has seeds in which the initial germinative events proceed very slowly, but may also require additional water to complete germination (Pritchard, 1991). *Podocarpus henkelii* also germinates slowly, after at least six months of cold storage (Dodd *et al.*, 1989). However, studies that systematically relate seed water content and storage conditions to lifespan are generally lacking.

1.3 Putative mechanisms conferring desiccation tolerance

During pre-shedding seed development, orthodox seeds undergo histodifferentiation, cell expansion with reserve deposition, followed by maturation drying. The events that occur during histodifferentiation are similar for both orthodox and recalcitrant seed categories (Farrant *et al.*, 1992a; 1993a). Recalcitrant seeds, with the exception of *A. marina*, demonstrate a decrease in water content towards the end of their development, but this

appears to be a consequence of dry mass accumulating faster than water (Hong and Ellis, 1990; Farrant *et al.*, 1992a; Tompsett and Pritchard, 1993; Finch-Savage and Blake, 1994; Fu *et al.*, 1994). Probably because the major reserves of *A. marina* are soluble sugars rather then insoluble materials, the water content of these seeds remains unchanged during development, with dry mass being accumulated at the same rate as water (Farrant *et al.*, 1992a).

Following histodifferentiation and through the entire developmental sequence, embryo cells of *A. marina* are highly vacuolated, lack lipids and storage proteins and have only small quantities of starch (Farrant *et al.*, 1992a; b). This is, however, not a consistent feature among recalcitrant seeds, as the embryo cells of some other species examined exhibited a lower degree of vacuolation and the accumulation of insoluble reserves (Farrant *et al.*, 1989). In order to tolerate desiccation, plant cells must withstand the mechanical stresses that are associated with volume reduction (Iljin, 1957). The ability to withstand mechanical stress could be attained either by the filling of large vacuoles with insoluble reserves or by sub-division of large fluid-filled vacuoles into smaller ones (Farant *et al.*, 1997). It has been suggested that in recalcitrant seeds, the presence of large vacuoles could be a major factor precluding desiccation tolerance, and interestingly, the development of extensive vacuolation in imbibed, germinating orthodox seeds, is associated with the loss of desiccation tolerance (Bewley and Black, 1978; Kermode, 1990; Berjak *et al*, 1993).

A study on two recalcitrant species, *A. marina* and *Aesculus hippocastanum*, and one orthodox seed type, *Phaseolus vulgaris* (prior to maturation drying), revealed that the vacuolar volume of all mature seed types was directly correlated, and the insoluble reserve accumulation inversely correlated, with the degree of desiccation sensitivity (Farrant *et al.,* 1997). In *A.marina* embryonic axes, when the water content was reduced below 0.5 g g^{-1} (dry mass basis, dmb), the meristematic root primordia lost viability while the vacuoles accounted for 60 to 90% of the cell volume. In addition, there was no significant

level of insoluble reserve accumulation, storage reserves being mainly in the form of sugars. In the case of the temperate species *A. hippocastanum*, the embryonic axes retained viability when dried to a water content of 0.42 - 0.25 g g^{-1}, fluid-filled vacuoles constituted only a small fraction of the total cell volume, and considerable accumulation of insoluble reserves had occurred. In contrast, the orthodox seeds of *P. vulgaris* tolerated water contents as low as 0.08 g g^{-1} and exhibited reserve-rich axis cells that were virtually unvacuolated.

The cytoskeleton could also play an important role in seed desiccation tolerance (Pammenter and Berjak, 1999; Mycock *et al.*, 2000). The inability of the cytoskeleton to re-assemble following dehydration could well be related to physiological and structural abnormality in the cells of desiccation sensitive seed tissues that have been injuriously dehydrated (Pammenter and Berjak, 1999). During dehydration, the cytoskeleton dissociates (Mycock *et al.*, 2000) and upon rehydration, in the case of desiccation tolerant seeds, must re-assemble in an orderly manner. When the status of the cytoskeleton in hydrated and dehydrated embryonic axes of recalcitrant *Quercus robur* seeds was investigated by fluorescence microscopy, it was observed that the microfilaments were distributed throughout the cytomatrix in the hydrated state. In contrast, on re-hydration that followed dehydration beyond a certain limit, the capability of the microfilaments to reassemble was lost (Mycock *et al.,* 2000). Progressive loss of the microfilaments ability to reassemble during re-hydration was also observed in *Trichilia dregeana* seeds (Gumede *et al.,* 2003).

Dehydration affects the conformation of not only the cytomatrical elements but also the integrity of the nucleoskeleton and the genetic material. According to Pammenter and Berjak (1999), maintenance of nuclear integrity during dehydration and in the dry state, as well as its re-establishment on re-hydration is a requisite for desiccation tolerance. In desiccation tolerant tissues, DNA replication ceases and, during dehydration, the chromatin becomes highly condensed (Sargent *et al.,* 1981), with replication resuming upon rehydration,

as the chromatin re-disperses. From work on resurrection plants, it has been suggested that this reversible chromatin compaction is associated with desiccation tolerance, while re-dispersion is specific to the desiccation sensitive state (Hallam and Luff, 1980). During dehydration, while in desiccation tolerant tissue the DNA is stable, it becomes highly degraded in desiccation sensitive tissues (Osborne and Boubriak, 1994; Osborne *et al.,* 2000).

Farrant *et al.* (1997) observed that at maturity, prior to shedding, highly recalcitrant *A. marina* seeds had a great number of well-developed mitochondria per unit cell volume, while in *P. vulgaris* seeds (prior to maturation drying), there were fewer mitochondria, which were all substantially de-differentiated. In these orthodox seeds, the observed organelle de-differentiation suggests that the membranes are sensitive to dehydration, and hence their surface area is minimised prior to water loss. In the recalcitrant seed situation, the retention of well-developed, differentiated organelles is thought to contribute significantly to the desiccation sensitivity of this seed type (Pammenter and Berjak, 1999).

Full metabolism occurs in plant/seed cells at water contents of 0.6 - 0.9 g g^{-1} or more (Vertucci and Roos, 1990, 1993; Vertucci and Farrant, 1995). According to those authors, particular metabolic activities become impaired or cease, as the water content declines through a series of well-defined levels. During dehydration, in the water content range of 0.45 - 0.25 g g^{-1} (intermediate water contents), the metabolism of seeds is suggested to be potentially unregulated, when free-radical-mediated damaging reactions could be prevalent (Leprince *et al.,*1990b; Hendry, 1993, Vertucci and Farrant, 1995). However, during controlled maturation drying of orthodox seeds, various protective mechanisms would also be operational, and again, when the seeds are imbibed the same would occur (Côme and Corbineau, 1996; Pammenter and Berjak, 1999). Additionally, mitochondrial de-differentiation and the decline in respiration prior to maturation drying of *Phaseolus vulgaris* seeds, suggest that by reducing the

surface area of mitochondria and the availability of respiratory substrates, desiccation tolerant seeds are able to pass through this critical water content range with minimal damage (Farrant *et al.*, 1997).

In contrast, in metabolically active, mature recalcitrant seeds, lethal damage has been shown to occur during slow dehydration when tissues reach the water content range of 1.0 - 0.7 g g^{-1}. As a consequence, it has been suggested that these desiccation sensitive seeds cannot pass through vulnerable water content ranges safely, as they do not possess, or do not express, the necessary mechanisms to curtail metabolism and protect against damaging reactants (e.g. free radicals) (Farrant. *et al.*, 1985; Pammenter *et al.*, 1991; Côme and Corbineau, 1996).

Seed development in many species is accompanied by an accumulation of late embryogenesis abundant proteins (LEAs). The synthesis of such proteins has been associated with the acquisition of desiccation tolerance prior to maturation drying in orthodox seeds (Kermode, 1990, 1997; Blackman *et al.*, 1991; Bradford and Chandler, 1992). Dehydrins, a sub-set of LEAs, have been identified in germinated *Medicago truncatula* seeds (Buitink *et al.*, 2003) recalcitrant seeds of *Quercus robur* (Finch-Savage and Blake, 1994), *Aesculus hippocastanum, Araucaria angustifolia, Camellia sinensis and Castanea sativa* (Farrant *et al.*, 1996), and *Acer saccharinum, Zizania palustris and Z. latifolia (*Gee *et al.*, 1994). The highly recalcitrant seeds of *Avicennia marina* do not produce LEAs, a deficiency that could contribute to their desiccation sensitivity (Farrant *et al.*, 1992b). Similarly, it was found that the seeds of some other tropical wetland species did not produce dehydrin-type proteins (Farrant *et al.*, 1996). However, although seeds of some temperate recalcitrant or intermediate species do produce dehydrin-like proteins, they remain desiccation sensitive (Bradford and Chandler, 1992). This implies that the expression of LEAs alone does not confer the ability to become desiccation tolerant but, on the basis of the continuum of recalcitrance (Farrant *et al.*, 1988), the production of

dehydrins associated with other protection processes, could perhaps determine the degree of desiccation sensitivity (Blackman *et al.*, 1992). LEAs (and possibly other proteins) could, however, act in synergy with soluble oligosaccharides to confer desiccation tolerance in orthodox seeds (Fu *et al.*, 1995; Walters *et al.*, 1997). It has been suggested that they play an important role in protecting macromolecules and cellular structures from deletrious effects of water removal by providing a water shell to the molecules and also by stabilising them in the dry state (Hoekstra *et al.*, 2001).

The increased level of oligosaccharides (especially raffinose and sucrose) has been linked to the onset of desiccation tolerance in orthodox seeds (Koster and Leopold, 1988; Leprince *et al*, 1990a; Black *et al.*, 1999). It has been suggested that oligosaccarides could be involved in desiccation tolerance in seeds by one of two mechanisms. One is that of "water replacement", where, during dehydration, the water that is associated with the membrane polar lipid surfaces, has been suggested to be substituted by the hydroxyl groups of oligosaccarides, thus maintaining the separation of the individual lipid molecules (Clegg, 1984; Crowe *et al*, 1992). The other mechanism, "vitrification", occurs in the case of the loss of water when sucrose and some oligosaccarides or galactosyl cyclitols interact to form aqueous, high-viscosity glasses (Koster and Leopold, 1988; Leopold *et al.* 1994; Obendorf, 1997). It has been suggested that glasses maintain seed viability in the dry state, through the possible reduction of the damaging effects of deranged metabolism and/or by protecting the macromolecules against denaturation (Leopold *et al.*, 1994). Some recalcitrant seeds do produce soluble sugars, but these may be located in cell compartments such as vacuoles where they would be unavailable to protect the cytoplasmic membranes (Dodd *et al.*, 1989; Farrant *et al.*, 1993b; Finch-Savage and Blake, 1994; Vertucci and Farrant, 1995). Whether or not vitrification occurs, Buitink (2000) has unequivocally linked intracellular viscosity with acquisition and maintenance of desiccation tolerance. However, when recalcitrant seeds are dehydrated under ambient conditions,

they lose viability at water contents that are much higher than those which are a prerequisite for glass formation (Farrant *et al.*, 1985; Hong and Ellis, 1990; Pammenter *et al.*, 1991; Leopold *et al.*, 1994), or even for the development of any effective level of intracellular viscosity.

When dehydration occurs, there are changes occurring at the intracellular level that have been suggested to include impaired intracellular transport, macromolecular conformational changes and abnormal (excessive or inadequate) accumulation of a variety of components that could disturb intracellular metabolism, leading to free radical generation (Smith and Berjak, 1995). In addition, the disruption of the anti-oxidant processes and impairment of the production of enzymatic free radical scavengers could also result in the uncontrolled generation and accumulation of free radicals (Senaratna and McKersie, 1986; Smith and Berjak, 1995). Under normal conditions, free radicals are components of intracellular metabolism, but their involvement is strictly controlled (Hendry, 1993). It has been suggested that the first wave of free radical generation could occur in the intermediate water content range of 0.45 - 0.25g g^{-1} (Vertucci and Farrant, 1995). During dehydration of desiccation sensitive maize, Leprince *et al.* (1990b) observed an accumulation of an organic radical and a considerable increase in lipid peroxidation, and although the free radical scavengers were not depleted, the activity of superoxide dismutase (SOD) was significantly affected. Lipid peroxidation and accumulation of stable free radicals have also been reported in desiccation stressed recalcitrant seeds such as *Quercus robur, Castanea sativa* and *Aesculus hippocastanum* (Finch-Savage *et al.*, 1994). Similarly, in the highly recalcitrant seeds of *Shorea robusta*, membrane damage was accompanied by the generation of a free radical, the superoxide anion (Chaitanya and Naithani, 1994), while in the aquatic recalcitrant seeds of *Zizania palustris*, dehydration was accompanied by significant hydroperoxide formation (Ntuli *et al.,* 1997). Free radical generation as a result of uncoordinated metabolism is considered to be one of the most significant injurious factors during the slow drying of

recalcitrant seeds that are still at relatively high water contents (Smith and Berjak, 1995). The hydrated cells of wet-stored recalcitrant seeds have been suggested to accumulate free radicals, the deleterious effects of which could be aggravated as water stress develops in wet storage (because the requirement for additional water cannot be met) (Pammenter *et al.,* 1994). However, Greggains *et al.* (2000) found no consisted evidence to show that metabolism-induced free radical activity was a significant contributing factor to deterioration of wet-stored recalcitrant seeds.

Other mechanisms that could also contribute to desiccation tolerance have been suggested. One of the consequences of deterioration in seeds is that lipid bodies in the cells coalesce (Smith and Berjak, 1995). This coalescence has been observed in both deteriorating orthodox seeds (Smith and Berjak, 1995) and in oil-rich recalcitrant seeds (Leprince *et al.*, 1998). Oleosins have been implicated in preventing lipid body coalescence in the desiccation tolerant seeds (Leprince *et al.*, 1997; Pammenter and Berjak, 1999). While no oleosins could be demonstrated in the highly desiccation sensitive seeds of *Theobroma cacao*, the temperate recalcitrant *Quercus rubra* and the intermediate seeds of *Azadirachia indica* demonstrated a very low oleosin:oil body ratio (Leprince *et al.,* 1998). Although the dehydration of *T. cacao* seeds did not negatively affect the oil bodies, on rehydration, the stability of these bodies was compromised. Leprince *et al.* (1998) have suggested that oleosins could play a significant role in stabilising the oil bodies of seeds in which these bodies are sizeable.

The movement of certain endogenous amphipathic molecules from the cytomatrix into membranes upon water loss could help maintaining the integrity of these membranes in the dry state in desiccation tolerant organisms (Hoekstra *et al.*, 1991, 1992; Golovina and Hoekstra, 1997). It has been demonstrated that upon water loss, some endogenous amphipathic molecules migrate into the membranes of desiccation tolerant pollen and embryos of seeds and are partitioned in the lipid bilayer (Hoekstra *et al.*, 1997). This

partitioning has been suggested to contribute towards maintenance of membrane integrity in the dry state in desiccation tolerant seeds, as it lowers the water content at which the membrane lipids undergo a change from the liquid crystalline to the gel phase. The phase changes of the membranes are reversible, restoring them to normal function upon rehydration when the amphipathic molecules re-partition into the cytomatrix (Hoekstra *et al.*, 1992, 1997). Although studies have not been conducted on recalcitrant seeds, these migratory endogenous amphipathic molecules could be non-functional or absent. Therefore, dehydration could result in non-reversible membrane phase changes or, as in the case of *Camellia sinensis* seeds, a semi-reversibility in which the membrane lipids return to their crystalline phase but the proteins remain irreversibly damaged (Sowa *et al.,* 1991; Vertucci and Farrant, 1995). In the case of recalcitrant embryos of *Aesculus* and *Castanea*, however, signs of phase separation were never revealed, even after re-hydration, when the distribution of membrane proteins was homogenous and integrity was maintained for some time (Hoekstra *et al.,* 2003).

It is likely that further mechanisms and processes implicated in the acquisition and maintenance of desiccation tolerance will be identified. However, even at present, there is significant evidence from those characterised, to indicate their absence or incomplete expression in desiccation sensitive seed tissues. Furthermore, as Pammenter and Berjak (1999) pointed out, it is the interactions among the mechanisms and processes that must determine the presence and degree of desiccation tolerance.

1.4 Storage of recalcitrant seeds

Recalcitrant seeds are metabolically active when harvested or shed and they are able to maintain this status for varying periods of time depending on the species and storage conditions, if the water loss is kept minimal (Berjak *et al.*, 1989; Poulsen and Eriksen, 1992; Chien and Lin 1997; Farrant *et al.*, 1997). The longevity of recalcitrant seeds in storage is, to some extent, dependent on their provenance: as a general rule, species originating from warmer tropical regions have a shorter storage life than those from subtropical and temperate areas (King and Roberts, 1980; Farrant *et al.*, 1989; Fu *et al.*, 1990; Tompsett, 1992; Pammenter *et al.*, 1994; Motete *et al.*, 1997). Storage of germplasm of many of these species is becoming an increasing necessity to ensure conservation of biodiversity and to facilitate propagation of possibly threatened species. Economically important crop species that produce recalcitrant seeds such as mango (*Mangifera indica*), tea (*Camellia sinensis*), cocoa (*Theobroma cacao*), and rubber (*Hevea brasiliensis*) must be stored, even for the short-term, in order to facilitate their transport between centres of cultivation (Chin, 1980; Berjak *et. al.*, 1989).

In order to maintain recalcitrant seeds at their shedding water content, the most practical way of storing them is under conditions of high relative humidity (King and Roberts, 1980; Pammenter *et al.*, 1984; Farrant *et al.*, 1988; Berjak *et al.*, 1989). However, in hydrated storage, depending on the species, within a short time germinative metabolism may be initiated, or the progression of developmental stages that lead to the onset of germination will take place (Berjak *et al.*, 1989). Germinative metabolism has been found to negatively influence the longevity of the seeds in hydrated storage. During radicle extension, the seeds lose vigour and eventually viability, as they are not supplied with additional water that is necessary to support this active growth stage (Farrant *et al.*, 1986).

Studies conducted on unrelated recalcitrant species showed that when seeds were removed from storage and planted out, they germinated in a shorter time than did newly shed seeds (Farrant *et al.,* 1989). This is consistent with the suggestion that germination of recalcitrant seeds begins upon shedding in a variety of species (Berjak *et al.*, 1984; Pammenter *et al.*, 1984). This is also supported by ultrastructural and biochemical evidence, showing that increased sub-cellular organisation and metabolic activity of the embryonic axes cells occurs during short-term storage (Farrant *et al.*, 1985, 1989; Berjak *et al.*, 1993).

It has been observed that the storage lifespan in the hydrated condition varies from two to three weeks for some tropical species to two to three years for temperate recalcitrant seeds that are chilling tolerant and that were stored at low temperatures (King and Roberts, 1980; Farrant *et al.*, 1989; Tompsett, 1992). Fungal contamination that is associated with most recalcitrant seeds can lead to deterioration especially if the seeds are stored at high temperatures and relative humidiy (RH). Even in the cases where mycoflora has been reduced, recalcitrant seeds eventually lose viability in storage (personal observations). Since it is virtually impossible to obtain fungal-free seeds in the tropics and sub-tropics (Mycock and Berjak, 1992), understanding the underlying mechanisms of the rapid loss of viability of recalcitrant seeds during storage is complicated by the effects of the mycoflora.

Several hypotheses have been proposed for the decline in recalcitrant seed viability, one of which cites development of an imposed water stress as germination is initiated in storage, with a concomitant increase in fungal and bacterial activity within the seed tissues (Berjak, 1996, 2000). Pammenter *et al.* (1994) postulated that unless additional water is provided, the tissues of recalcitrant seeds may suffer a mild, but prolonged water stress, interfering with normal regulatory biochemical reactions. As one consequence, free radicals, which would normally be quenched in fully hydrated tissues could accumulate,

resulting in peroxidation of lipids and proteins with subsequent membrane damage (Leprince *et al.*, 1990b; Hendry *et al.*, 1992; Greggains *et al.*, 1997). In hydrated stored seeds of *Avicennia marina*, the decline in seed viability was associated with an increasing electron paramagnetic resonance (EPR) signal ascribed to a stable free radical (Greggains *et al.*, 1997) which lead to the proposal of the involvement of oxidative mechanisms. Pammenter *et al.* (1994) have suggested that such free radical damage, and the inability to repair the resultant damage, might be a major contributing factor to the loss of viability of recalcitrant seeds in storage. However, this water stress hypothesis, although not disputed, has not yet been substantiated (Motete *et al.*, 1997; Greggains *et al,*, 2000).

In the humid storage environment seed deterioration that is accompanied by leakage of solutes as a consequence of the loss of membrane integrity, provides ideal conditions for fungal proliferation. Furthermore, any manipulation of recalcitrant seeds such as lowering the water content to prevent ongoing germination could also impose a stress on the seeds, which could exacerbate the possible deleterious effects of the seed-associated microflora, in particular fungi (Berjak, 1996; Drew *et al.*, 2000). If the fungi are located on the external seed surfaces only, then the application of fungicides can be affective. For example, The longevity of *Hevea brasiliensis* seeds has been prolonged from three to 12 months through seed coating with 0.3% Benlate® (Chin, 1988).

In contrast to orthodox seeds, recalcitrant seeds cannot be stored at low water contents. This implies that even partial drying is not an appropriate approach to extending storage life of recalcitrant seeds, although it has been observed that in some species a certain level of drying is necessary in order to prevent germination in storage (Fu *et al.*, 1990). However, Drew *et al.* (2000) have shown that the storage lifespan of recalcitrant *Trichilia dregeana* seeds is deleteriously affected if water content is reduced.

If the cause of the loss of viability in hydrated stored recalcitrant seeds is the ongoing metabolic activity, then the reduction of the metabolic rate could be a practical method of prolonging storage lifespan. This approach has been effective in chilling tolerant species that have been stored at lowered temperatures (Pritchard *et al*, 1995). However, this approach cannot be utilised for chilling sensitive seeds, which include those of *Avicennia marina* (Lewis, 2002).

In an attempt to prolong seed longevity in storage, manipilations have involved the removal of the pericarp of freshly harvested *A. marina* seeds and coating the exposed tissues with a crude alginate gel (Pammenter *et al.*, 1997; Motete *et al.*, 1997). It was suggested by those authors that the more than four-fold extended seed viability - up to 70 days - may have been a result of the slowing of the post-shedding development, and, more importantly, it appeared that the alginate coating reduced the rate at which the root primodia lost water and suffered desiccation damage. However, no measurable change in a range of metabolic parameters could be discerned (Motete *et al.*, 1997; Pammenter *et al.*, 1997). From their observations those authors have suggested that the improved storage was due to the alginate gel that inhibited fungal proliferation.

Although hydrated storage or even successful manipulation by alginate encapsulation could help towards extending storage lifespan of recalcitrant seeds, they are not suitable for purposes of germplasm conservation, as the stored seeds will sooner or later lose viability in storage.

Another approach used to prolong recalcitrant seed storage has been to manipulate the storage atmosphere. Treatment of *Litchi chinensis* and *Dimocarpus logan* seeds with nitrous oxide, an anaesthetic which reduces respiration rates, increased storage lifespan (Sowa *et al.*, 1991). However, a high-oxygen storage atmosphere is still essential to maintain recalcitrant seed

viability in storage (Willan, 1985), presumably because their active metabolism cannot be curtailed indefinitely.

The quality of light during seed storage could also play a role in increasing seed longevity, as it could affect precocious germination. Studies have demonstrated that darkness inhibits germination of seeds of some forest species (Teketay, 1998), this being a possible adaptation to suppress germination when the seeds are buried deep in the soil or shaded by vegetation canopy. Although the role of phytochrome and ultraviolet radiation in germination has been well-studied (Singh and Amriphale, 1992; Kim *et al.*, 1998; Leinonen and Dechantal, 1998), it appears that the actual effect differs amongst species, and even between seedlots of the same species (Singh and Amriphale, 1992). Hence the effect of light/dark on stored seeds should be determined empirically.

What presently appears to be the only feasible method for long-term storage of recalcitrant germplasm is *in vitro* cryopreservation (Wesley-Smith *et al.*, 1992). However, production of normal seedlings from cryostored seeds is almost impossible as recalcitrant seeds cannot be stored at sub-zero temperatures that are typical of even conventional freezers. This is largely because intact recalcitrant seeds are too large and too wet to survive conventional freezing. As a consequence, the explants affording successful cryopreservation are excised embryonic axes (Wesley-Smith *et al.,* 1992; Kioko *et al,* 1998). In order to attain successful cryostorage, several parameters have to be taken into consideration including the freezing rate and thawing conditions. In addition, it is essential that the preliminary rapid dehydration of embryonic axes (flash drying) should be optimised to ensure only minimal water removal and that the cryogen is chosen appropriately to avoid biological injury and also to conduct heat rapidly away from the axes (Wesley-Smith *et al.,* 1992; Wesley-Smith, *et al.,* 1999).

Despite cryopreservation of recalcitrant germplasm being a complicated process, success has been achieved for several temperate and tropical species

(Pritchard and Predergast, 1986; Kioko *et al.*, 1999; 2000; Berjak *et al.*, 1999). One of the complications encountered with excised axes to be cryostored, is the incidence of intraseminal fungi, necessitating procedures to remove or kill them. Thus, as for recalcitrant seeds in hydrated storage, the seed-associated mycoflora remain a significant problem.

1.5 Effects of associated mycoflora on stored recalcitrant seeds

Although mycofloral infection of orthodox seeds has been relatively extensively studied, to date there have been few data published on the implication of microflora, especially fungi, in the curtailed storage life of recalcitrant seeds. In order to understand the implication of fungal infection on stored hydrated recalcitrant seeds, a brief review of fungal contamination of orthodox seeds is necessary.

1.5.1 Aspects of associated mycoflora of orthodox seeds

The major causes of loss in quality and quantity of stored seeds are rodents, insects and seed-associated pathogens that include viruses, bacteria, fungi and nematodes. Fungi, which are adapted to survive in air, soil, water and seeds, and in or on living or dead organic matter, cause most plant diseases, with more than 8000 fungal plant pathogens having being described (Agarwal and Sinclair, 1987).

Work over the years has established that more than 150 species of fungi are associated with stored seeds, most having a major influence on seed condition and storability of grain. According to Christensen and Kaufmann (1969), the major changes caused by fungi in stored seeds are decrease in germinability, discolouration of parts or all of the seed, heating, various chemical changes,

loss in weight and accumulation of mycotoxins that may be harmful to humans or domestic animals.

- **Classification of fungi**

Seeds are seldom entirely free of microbial contamination, and the seed-associated fungi may be on the external surface, be located internally, or both. Individual species comprising the mycoflora have traditionally been assigned to one of two major categories: storage fungi or field fungi. Field fungi gain access to the growing plants and their incidence declines with orthodox seed maturation, i.e. as the seeds become dehydrated. In contrast, the storage fungi proliferate once the seeds have been harvested and stored. However, studies have suggested that this classification may not be as clear cut as originally reported, as storage fungi, mostly species of *Aspergillus* and *Penicillum* have been isolated from developing seeds, and field fungi, in particular *Fusarium* species, frequently persist during seed storage, suggesting that *Fusarium* may be a facultative storage fungus (McLean and Berjak, 1987; Mycock and Berjak, 1992; Mycock *et al.*, 1992).

The storage group of fungi, comprises genera dominated by *Aspergillus* and *Penicillium*. Storage fungi are metabolically active in seeds stored at 70% RH or higher, under which conditions grains will equilibrate to moisture contents of 13% or more (Christensen and Kaufmann, 1969, 1974; Roberts, 1972). *Aspergillus* species have been isolated from seeds of many species, including maize (Payne, 1992), cotton (Khalid *et al.,* 2001) and different types of nuts (Diener, 1989; Doster and Michailides, 1994; Waliyar *et al.,* 1994). In addition, the occurrence of these fungal species has also been reported in seed-derived spices (black, white and red pepper (Ito *et al.,* 1994). Many storage fungi produce mycotoxins that contaminate seeds, resulting in poor seed quality or seed losses. In particular, the significant incidence of *A. flavus* and *A. parasiticus* are a cause of concern in many parts of the world as they produce aflatoxins (Groopman *et al.*, 1988; Newberne, 1993),

The field fungi are pathogens that infect the seed prior to harvest. This group comprises species of the genera *Alternaria, Cladosporium, Curvularia, Epicoccum, Fusarium and Verticillium*. Their activity requires high relative humidity (in excess of 95%) and moisture content of above 25% at least for the cereal grains. These fungi have a deleterious impact on seeds of cultivated plants around the world: illustrative examples include wheat, rice, cotton and groundnut (in Asia) (Rahman and Mia, 1998; Naeem *et al.,* 2001; Khalid *et.al.,* 2001; Ammara *et al.,* 2001; Bhattacharya and Raha, 2002), soybean and melon (in Nigeria) (Bankole *et al.,* 1999; Umechuruba and Nwachukwu, 2002) and maize (in North and South America and in Europe) (Pittet *et al.,*1992; Nelson *et al.*, 1993; Bullerman and Tsai, 1994; Rice and Ross, 1994). Fusaria are plant pathogens that can be active from seed germination, through harvest, and in the post-harvest period (Bottalico *et al.,* 1989). The detrimental effects of *Fusarium* infection continue to be documented from various plants (Bottalico *et al.*, 1989; Somani and Indira, 2000; Dillep and Tribhuwan, 2001; Dharam and Mahashwari, 2002; Liu *et al.*, 2002; Costa *et al.*, 2003). More than 24 toxigenic species of *Fusarium* produce not only trichothecenes, but also metabolites such as fusarins and gibberellic acid (Savard and Miller, 1992).

- **Mechanisms of fungal infection**

Seeds can be colonised by micro-organisms from the newly-fertilised stage, in the nascent and developing fruit, to maturation and ripening. The infection process can occur at any time during the preharvest period, at harvest, during seed drying and in storage in orthodox seed-types. Furthermore, any seed in the soil is susceptible to attack by both external and internal pathogens (Agarwal and Sinclair, 1987).

In the field, there are various factors that facilitate fungal infection. For example, insects and nematodes may damage the seeds, causing lesions of the testa and underlying tissues. The leakage of solutes from the seed tissues, which occurs during germination, can stimulate germination of dormant microbial

structures within the soil. Under favourable conditions of temperatures, pH, water and nutrient availability, any fungal structure will germinate and is likely to infect the seeds in its close proximity (Lillehoj *et al.*, 1987; Cotty, 1989).

During invasion of developing seeds, micro-organisms require a variety of conditions (for fungal spores to germinate), the first of which is the presence of sufficient free water. During seed development, the water present in the underlying tissues determines the availability of water to fungi. Additionally, with seed maturation, the nutrient availability may also change.

Fungi can enter plants and seeds through natural openings and through wounds made by various abiotic and biotic agents. In this regard, *A. flavus* has been shown to invade wounds and infect seedling tissues (Mycock *et al.,* 1990). Some fungi use mechanical pressure or enzymic action, or both, to penetrate directly into plant and seed tissues. The invasive process is facilitated by the production of extracellular secretions, which include lytic enzymes. Some of these enzymes have the ability to degrade the cellulose and polygalacturonic acid of the plant cell wall (Raper and Fennel, 1965; McLean *et al.,* 1986). In the growing plant, chlorophyll and proteins are lost as the concentration of degradative enzymes increases, and nutrients are also lost from the cells, as membranes lose their integrity. All these changes can be reflected in the rate and extent of development and persistence of the seed-associated mycoflora (Lacey *et al.,* 1991).

Infection may occur during flowering, and the systemic transmission of pathogens from one generation to the next via the seeds has been reported for some species of fungi (Mycock *et al.*, 1992). Studies have shown that a succession of fungal species was manifested prior to harvest and during seed storage, arguing for the pre-harvest presence of the inoculum of the different species (McLean and Berjak, 1987; Mycock and Berjak, 1992; Mycock *et al.,* 1988; 1992). During storage, it appears that the seed tissues that are utilised,

and the rate at which they are degraded by storage fungi, is determined by seed moisture content, temperature and by the extracellular enzyme capabilities of each fungal species (Christensen and Kaufmann 1969, 1974; McLean and Berjak, 1986; Mycock *et al.*, 1992). Apart from increasing seed moisture content through the release of metabolic water, it appears that the metabolism of each specific component member of the fungal succession could also release substrates that are suitable for its successor. Irrespective of the type of fungal succession, the contamination produces a decline in orthodox seed vigour and viability.

1.5.2 Aspects of fungal contamination on recalcitrant seeds

As for orthodox seeds, fungal contamination of recalcitrant seeds could occur at any developmental or handling stage. In addition, recalcitrant seeds could be internally infected by mycoflora through systemic transmission via the parent plant; such a process has been demonstrated for the developing orthodox seeds of maize (Mycock *et al.,* 1992). Alternatively, infection could occur through the stigma-style continuum during flowering (Marsh and Payne, 1984). When fungal contamination occurs by systemic transmission, the mycelium is likely to become established deep in the seed tissues, making the eradication process almost impossible (Berjak, 1996). Post-harvest recalcitrant seeds also remain very sensitive to fungal contamination and proliferation as they are shed at high water contents, offering an immediately available moisture source and substrate for opportunistic fungi.

There is no doubt that at harvest, recalcitrant and intermediate seeds (i.e. the non-orthodox seed category) harbour a spectrum of fungal species as well as bacteria (Table 1.1). Species of *Alternaria, Cladosporium* and *Fusarium* as well as non-xerotolerant *Aspergillus* and *Penicillium* have been isolated from the surface and the internal tissues of seven unrelated recalcitrant species from a

variety of provenances in South Africa, from sub-tropical estuaries to warm-temperate montane areas (Mycock and Berjak, 1990). *Hevea brasiliensis* seeds of Malaysian provenance harboured 23 fungal species (Singh and Singh, 1990). The prevalence of fungal infection may well be related to the fact that most of the seeds that were tested in those studies had a tropical/sub-tropical provenance, thus possibly being more prone to fungal infection. However, it was found that seeds of *Podocarpus henkelii*, an African gymnospermous temperate species, also harboured fungi, as did those of *Camellia sinensis*. In addition, a spectrum of fungi has been found to be associated with seeds of species of cool temperate origin such as *Quercus robur* (Murray, 1974). The fungus that commonly infects the acorns after shedding, *Sclerotinia batschiana* (Kehr and Schroeder, 1996), was also found in the seeds of *Castanea* species (Delatour, 1978).

The opinion has been expressed that the spectrum of fungal species could differ with seed provenance, where geographical areas are widely separated (Sutherland *et al.*, 2003). It appears that the recalcitrant seeds that have been tested in Southern Africa (Mycock and Berjak, 1990) harboured an essentially different spectrum of fungi from those originating in the Asia-Pacific area (Pongapanich, 1990). There are however, some exceptions to this apparently limited distribution: for example, *Phomopsis* spp. have been isolated from oak seeds (Kehr and Schroeder, 1996), *Trichilia dregeana* (Sutherland *et al*, 2003), neem seeds, *Azadirachta indica* (Pongapanich, 1990), and *Hevea brasiliensis* (Singh and Singh, 1990). Although this fungal genus is common to seeds from all these trees, it appears that the species were different, as *P. hevea* was isolated only from seeds of the rubber tree, *H. brasiliensis*, while *P. azadirachtae* contaminated only neem seeds (Table 1.1).

Reference to Table 1.1 reveals that fungi such as *Cladosporium, Mucor, Phoma, Phomopsis, Alternaria* and *Botryodiplodia* are the most frequent contaminants of recalcitrant seeds from a wide range of provenances. While

Fusarium species have been isolated from a variety of seeds, appearing to become the dominant fungal contaminant during storage (Mycock and Berjak, 1990; Calistru *et al.*, 2000), only few representatives of species of *Aspergillus* and *Penicillium* are apparently associated with non-orthodox/recalcitrant seeds (Mittal and Sharma, 1982; Pongapanich, 1990; Singh and Singh, 1990), none of which was categorised as a storage fungus (Sutherland *et al.*, 2003).

This apparent absence of the storage species from the fungal spectrum of recalcitrant seeds is not surprising. The xerotolerant species of *Aspergillus* and *Penicillium* predominate in air-dried orthodox seeds during storage, when the low water activity and the osmotic conditions became unfavourable to the field fungi. However, in intermediate seeds that are dehydrated to relatively low water contents, xerotolerant fungi could become active if their propagules are present intra-seminally or in the storage containers. In contrast, the recalcitrant seeds offer ideal conditions and substrates for the field fungi, such as *Fusarium*, as these pathogens need high seed water content for their ongoing activity.

All non-orthodox seeds tested to date have been shown to harbour a variety of fungal species as well as bacteria. Furthermore, fungal inoculum was found in fresh seeds even when newly hand-harvested (Mycock and Berjak, 1990; Calistru *et al.*, 2000). Despite this, no evidence has been recorded of visible proliferation of fungi externally or internally, in freshly-harvested seeds or during the early periods of hydrated storage. In the case of *Avicennia marina*, no fungal proliferation was observed when newly-harvested seeds were planted out for germination (personal observations). It is only when longer storage periods are involved that fungal proliferation becomes evident either during wet storage or when the seeds are planted out. This interesting phenomenon may be explained by the operation of defence mechanisms that could exist in mature, fully hydrated recalcitrant seeds, particularly considering that

Table 1.1. Microbial contaminants of a variety of non-orthodox seeds (Murray, 1974; Delatour *et al.*, 1980; Mittal and Sharma, 1982; Mycock and Berjak, 1990; Pongpanich, 1990; Singh and Singh, 1990; Abdelmonem and Rasmy, 1996; Kehr and Schoeder, 1996; Calistru *et al.*, 2000; Sutherland *et al.*, 2003)

Contaminants	Non-orthodox species											
	Avicennia marina	*Azadirachta indica*	*Camellia sinensis*	*Castanospermum australe*	*Hevea brasiliensis*	*Landolphia kirkii*	*Litchi chinensis*	*Podocarpus kenkelii*	*Quercus* spp.	*Scadoxus membranaceus*	*Shorea robusta*	*Trichillia dregeana*
Alternaria spp.	Present				Present	Present						Present
A.. alternata									Present		Present	Present
A. tenuis												
Acremonium spp..					Present							
Aspergillus spp.	Present					Present	Present			Present		
A. flavus		Present			Present						Present	
A. niger	Present	Present			Present						Present	
A. glaucus											Present	
Aureobasidium Pullulans									Present			
A. apocryptum									Present			
Botryodiplodia sp.		Present			Present						Present	
B. theobromae					Present							
Botrytis cinerea									Present			
Calcanosiphon sp.					Present							
Colonostachys cylindrospora					Present							
Chaetomium sp.		Present									Present	
Chrysonilia sitophila												Present
Ciboria batschiana									Present			
Cladosporium spp.			Present			Present					Present	Present
C. cladosporioides									Present		Present	
C. herbarum									Present			
C. sphaerospermum									Present			
Colleotrichum sp.												Present
C. gloeosporioides					Present							Present
Colonostachys cylindrospora					Present							
Codinea simplex									Present			
Coniothyrium sp.									Present			
Corynespora sp.											Present	
Cylindrocladium sp.					Present							
Curvularia spp.	Present											
C. eragragrostidia											Present	
C. lunata											Present	Present
C. verruculosa		Present										
Diaporthe eres									Present			
Discula umbrinella									Present			
Epicoccum nigrum									Present			
Fusarium sp./spp.	Present	Present	Present	Present			Present	Present	Present	Present	Present	
F. equiseti												Present
F. moniliforme	Present											
F. oxysporum					Present				Present			Present
F. semitectum	Present				Present							Present
F. subglutinans												Present
F. solani					Present							Present
Gliocladium sp.					Present							
G. roseum												Present
Geotrichum sp.					Present							
Metarhizium anisopliae												Present
Mucor sp.		Present			Present						Present	
Mucorales spp.									Present			
Myrothecium sp.											Present	
Nigrospora oryzae												Present
Pecaelomyses sp.					Present							
Penicillium sp./spp.	Present	Present	Present	Present	Present	Present		Present	Present		Present	
P. albicans											Present	
P. aurrantiogriseum												Present
P. canadense											Present	
P. frequentans											Present	
P. olsoni												Present
P. oxalicum												Present
Pestalotiopsis maculaans												Present
Pezicula cinnamonea									Present			
Phoma spp.		Present			Present				Present			Present
Phompsis sp./spp.					Present				Present			Present
P. glandicola									Present			
P. quercella									Present			
Rhizoctonia solani									Present			
Rhizopus sp.								Present				Present
R. nigricans											Present	
R. oryzae											Present	
Sphaeropsis sp.												Present
Sporormiella sp.					Present							
Syncephalastrum sp.											Present	
Stagnospora sp.					Present							
Ulocladium atrum									Present			
U. chartarum									Present			
Bacteria	Present					Present		Present	Present	Present		

physiologically these seeds could be considered as seedlings in many instances (Berjak, 1996).

1.6 Aspects of plant defence mechanisms

It is widely recognised that in their natural environment, plants encounter a very wide range of potential pathogens including bacteria, fungi, viruses and nematodes. However, the majority of plants withstand pathogen attack, and successful colonisation of the plant is the exception rather than the rule, in plant-pathogen interactions.

1.6.1 Plant pathogens

Some fungi that infect plants are unspecialised micro-organisms described as necrotrophic, and could damage the host through production of toxins and/or enzymes. In addition, it has been found that such pathogens are equipped with avoidance or inactivation mechanisms, which counteract defence mechanisms of the plant (Lamb *et al.* 1989). On the other hand, specialised micro-organisms are biotrophic and they do not activate host defences nonspecifically. These pathogens have mechanisms that, in the early stages of the infection, do not damage the host plant. For those specialised pathogens that have no avoidance mechanisms, the plant defences, if induced, are usually effective (Lamb *et al.*, 1989).

In plant disease terms, a compatible plant-pathogen interaction (host susceptible and pathogen virulent) is one where the pathogen can elude the defence mechanisms of the host, which is consequently followed by manifestation of the disease. In contrast, in an incompatible interaction (host

resistant and/or pathogen non-virulent), the plant is able to recognise the pathogen and rapidly express appropriate defence reactions.

The fact that a plant may be an incompatible host for potential pathogens can be explained in several ways. First plant may not actually support the requirements of the pathogen and thus does not represent a potential substrate for microbial growth. Secondly, the host could also have preformed barriers that are of a structural nature, and/or it could possess preformed antimicrobial compounds. The plant could also react to the infection by activating an endogenous complex defence reaction (Hammond-Kosack and Jones, 1996).

Approximately 1600 bacterial species can induce plant diseases, some being Gram-positive such as *Streptomyces* and *Clavibacter (Coryobacterium)* or Gram-negative as *Agrobacterium, Erwinia, Pseudomonas* and *Xanthomonas* (Walton, 1997). While nematodes cause plant diseases and some 1000 viruses infect the higher plants, more than 8 000 fungal species are potential or active plant pathogens. Some of these diverse pathogens exist generally as saprophytes, but also can opportunistically infect plants. Other species are obligate pathogens, growing only in the host plant and being highly specialised (Walton, 1997). The following sections review the plant-fungal interaction.

1.6.2 Mechanisms of fungal pathogenicity

Pathogens possess a range of biochemical specialisations that facilitate infection of a plant. Such specialisations comprise those traits that differentiate a pathogenic organism from its closely related, non-pathogenic species. A pathogen can be distinguished by its ability to colonise and survive inside a seed, to adhere to a plant leaf, to penetrate the plant cuticle, to remain dormant

during crop rotations, to germinate in response to plant signals and/or to degrade the plant antimicrobial compounds (Walton, 1997).

- **Penetration and spread**

One of the most important characteristics of invasion is the ability of the pathogen to penetrate and spread inside its host. As the cuticle and the epidermal cell walls represent an effective barrier, pathogens must have the ability to penetrate these plant barriers, generally through secretion of a cocktail of hydrolytic enzymes including cutinases, cellulases, pectinases and proteases (Prusky, 1997). Although the majority of fungi enter the host plant through natural openings and wounds, others have developed specialised mechanisms of penetration such as appressoria. The spores of *Colletotrichum gloesporoioides,* for example, adhere to the surface of a fruit, germinate and produce appressoria for penetration. These structures are able to pass through the waxy fruit cuticle. As the appressoria reach the host cell walls, the pathogen becomes quiescent until the fruit ripens after harvest (Prusky *et al.,* 1997).

- **Enzymes produced by fungi**

Studies have shown that plant pathogens produce one or more enzymes that are capable of degrading the polymers of plant cell walls. At least 20 different cell wall-degrading enzymes have being described (Walton, 1997). The oat root-infecting *Gaeumannomyces graminis* requires the saponin-detoxifying enzyme, avenacinase, during the infection process (Bowyer *et al.*, 1995; Osbourn *et al.*, 1995a). *Botrytis cinerea, Fusarium moniliforme, Helminthosporium oryzae* and *Chaetomium globosum* have demonstrated the ability to release extracellular pectinolytic enzymes that elicit defence reactions in carrots (Amin *et al.,* 1986), while *Gaeumannomyces graminis* var. *tritici* has been shown to produce cell wall-degrading enzymes in wheat roots (Dori *et al.,* 1995). Similarly, it was found that the phytoalexin-degrading enzymes of

Nectria haematococca, a pathogen of pea and chickpea, also contribute to its pathogenicity (Enkerli *et al.,* 1998). These are but a few examples of the spectrum of enzymic mechanisms involved in fungal pathogenesis of plants.

- **Toxins produced by fungi**

There are a variety of phytotoxic compounds that are elaborated by plant pathogens, but for most, their involvement in the pathogenesis has not been established. The compounds of diverse structures that are known as elicitors will be discussed later. The fungal genera, *Bipolaris* and *Alternaria,* are known for producing host-selective toxins (Walton, 1997). The host-specific toxins produced by *Alternaria* have been considered to be virulence factors that allow the normally weakly pathogenic *Alternaria* to inflict severe damage on plants that are sensitive to the particular toxin produced (Panopoulos *et al.*, 1984; Kohmoto *et al.,* 1987). *Bipolaris sorokiniama* has been reported to produce a range of phytotoxins, but prehelminthosporol, a precursor of helminthosporol, constitutes the main compound involved in the phytotoxigenicity of this fungus (Olbe *et al.,* 1995). Host-specific toxins are also characteristic in fungal species such as *Helminthosporium, Periconia* and *Phyllosticta*, pathogens that cause diseases in oats, sorghum, maize and sugarcane (Scheffer, 1976; Daly, 1987). Neovasipyrones and neovasifuranones are two toxins that are produced by *Neocosmospora vasinfecta*, a pathogen that causes root- and fruit-rot and seedling damping off in the Malvaceae, Leguminosae and Cucurbitaceae (Furumoto *et al.*, 1995).

- **Detoxification and degradation**

In some fungi, the ability for phytoalexin (low molecular weight antimicrobial compounds produced by plants [Hammerschmidt, 1999]) detoxification is related to virulence (Kuc, 1995). *Fusarium solani* f. sp. *phaseoli* is able to detoxify kievitone and phaseollin simultaneously (Smith *et al.,* 1981; Turbek *et al*, 1992). This fungus, that causes serious foot and root disease of bean, can also metabolise other anti-fungal compounds such as phaseollin and

phaseollinisoflavan (Wietor-Orlandi and Smith, 1985). In alfalfa, pathogens such as *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *medicaginis* have been shown to be able to detoxify the phytoalexins, mediscarpin and maackiain, produced by the plant (Soby *et al.,* 1996). Studies have also demonstrated that fungi such as *Gaeumannomyces graminis, Drechslera* spp., *Fusarium* spp. and *Erysiphe graminis* are able to metabolise avenalumins (Bratfaleanu and Steinhauer, 1994). In the case of the phytoalexin wyerone, that is produced by faba beans (*Vicia faba*), the two fungal pathogens, *Botrytis cinerea* and *B. fabae*, can convert this phytoalexin to a less inhibitory hydroxyester, wyrol (Gorge and Werner, 1991). Similarly, the pathogenic fungus *Sclerotinia sclerotiorum* has demonstrated its ability to adapt, and survive exposure to glyceollin, a soybean phytoalexin (Marciano *et al*, 1991), while *Leptosphaeria maculans*, a contaminant of cruciferous crops, is also capable of metabolising the phytoalexin, brassinin (Soledade *et al.*, 1993).

- **Suppressors of plant defence responses**

Fungl suppressors are determinants for pathogenicity without evident phytotoxicity. They are produced by the pathogens at the site of infection and participate in suppression of the general plant resistance and in induction of local susceptibility in hosts. These fungal suppressors are not toxic to the host plants (Shiraishi *et al.*, 1997). The chemical nature of these suppressors has been determined and is represented by complex compounds including water-soluble glucan, phosphoglucan, glycopeptide, glycoproteins or peptides (Shiraishi *et al.*, 1997). Several plant pathogenic fungi of the genus *Mycosphaerella* secrete substances of low molecular weight into the spore germination fluid, that appears to suppress the elicitation of the defence reaction of host plants (Oku *et al.*, 1987). It has also been demonstrated that *Phytophthora megasperma* f. sp. *glycinea*, a soybean pathogen, produces a glucan with suppressor activity, in addition to the plant cell wall degradation elicitors (Yoshikawa and Sugimoto, 1993). Similarly, *P. infestans* is suggested

to produce water-soluble glucans that prevent the hypersensitive reaction and phytoalexin accumulation in the incompatible interaction (Oku *et al.*, 1987).

- **Non-degradative mechanisms**

Non-degradative tolerance mechanisms could also be important in enabling opportunistic fungi to invade plants. Some of these pathogenic fungi may have innate resistance to the defence substances of the host. Resistance to a wide range of antifungal compounds has been often associated with fungal ATP-binding cassette (ABC) membrane transport proteins. Through this strategy, the phytopathogenic fungi may avoid plant defence compounds and prevent their intracellular concentration from building up to toxic levels (Osbourn, 1999).

1.6.3 Mechanisms of plant resistance

The natural defence mechanisms of plants are known to be effective in preventing or limiting pathogen colonisation and hence disease (Dangl and Jones, 2001). Plant resistance is a complex system, with passive and active constitutive and inducible elements.

1.6.3.1 Constitutive resistance

The cuticle is considered to be a first line of constitutive defence against pathogens. Constitutive resistance factors, comprising preformed structures and compounds, are morphological and chemical entities that are present in the plants prior to any pathogenic attack. Plants produce a variety of constitutive secondary metabolites that have antifungal properties. These preformed substances could inhibit either the germinating pathogen spores that have produced structures entering plant tissues through wounds or natural openings,

or the pathogens that have penetrated directly through the cuticle and cell walls (Osbourn, 1996).

While some of the preformed compounds exist in plants in their biologically active form, others are present as inactive precursors and are activated only by the pathogenic infection (Walton, 1997). It is believed that the preformed compounds are generally present in the outer layers of plant tissues and that these compounds are compartmentalised predominantly in vacuoles in healthy plants (Prusky and Keen, 1993). The biological activity of the constitutive substances has been suggested to depend on the extent of fungal damage and the amount of compound released (Prusky, 1997).

In unripe mango fruits, a mixture of antifungal compounds was found to be fungitoxic, and to be associated with resistance to *Alternaria alternata* (Droby *et al.,* 1987). In avocado, the resistance of unripe fruits to *Colletotrichum gloesporioides* infection was achieved by keeping appressoria quiescent through preformed antifungal compounds present in the exocarp (Prusky *et al.*, 1983; Prusky *et al.*, 1990; Kobiler *et al*, 1993; Prusky *et al.*, 1997). Resistance of celery stalks to *Botrytis* may be the result of a variety of active preformed compounds (Afek *et al.*, 1995). As another example, although citrus plants may be heavily infected with *Penicillium digitatum and P. italicum* the fruits produced are resistant to these pathogens during growth. This is achieved by the release of a preformed compound, citral, which decreases in older fruits, allowing decay to occur rapidly (Ben-Yehoshua *et al*, 1995; Rodov *et al.*, 1995; Ben-Yehoshua *et al.,* 1997).

Preformed phenols have been reported to play a significant role in the inhibition of *Botrytis cinerea* in strawberries, where resistance in unripe fruit could be explained by the fact that the extracellular fungal hydrolases are bound by proanthocyanidins, thus becoming inactive (Prusky, 1997). In addition, for

grapes, the concentration of proanthocyanidins is also influential in resistance to *B. cinerea* (Prusky, 1997).

Other studies have demonstrated the possible involvement of preformed proteins in plant resistance. The plant cell walls have proteins that could inhibit fungal polygalacturonases (PG). There is a significant evidence of the presence and action of PG-inhibiting proteins in unrelated plants such as apples (Yao *et al.*, 1995) and tomato (Stotz *et al.*, 1994).

1.6.3.2 Induced resistance

This type of resistance refers to defence mechanisms that are triggered only by the presence of a pathogen. The plant reaction to pathogenic infection is represented by a range of events starting with the perception of a foreign signal by the plant cell, followed by the intracellular transduction of this recognition signal and the synthesis and transport of strategic defence molecules (Fig. 1.1). The interplay between the host plant and the pathogen is reflected in a substantial and continuing exchange of information, resulting in activation of complex responses that determine the outcome of the interaction (Lamb *et al.*, 1983).

As mentioned earlier, (see Mechanisms of Pathogenicity), in order to facilitate a successful infection, the pathogen is equipped with a variety of devices and mechanisms such as attachment to the plant surface, formation of fungal penetration structures, toxin production and degradation of the plant cuticle and cell wall.

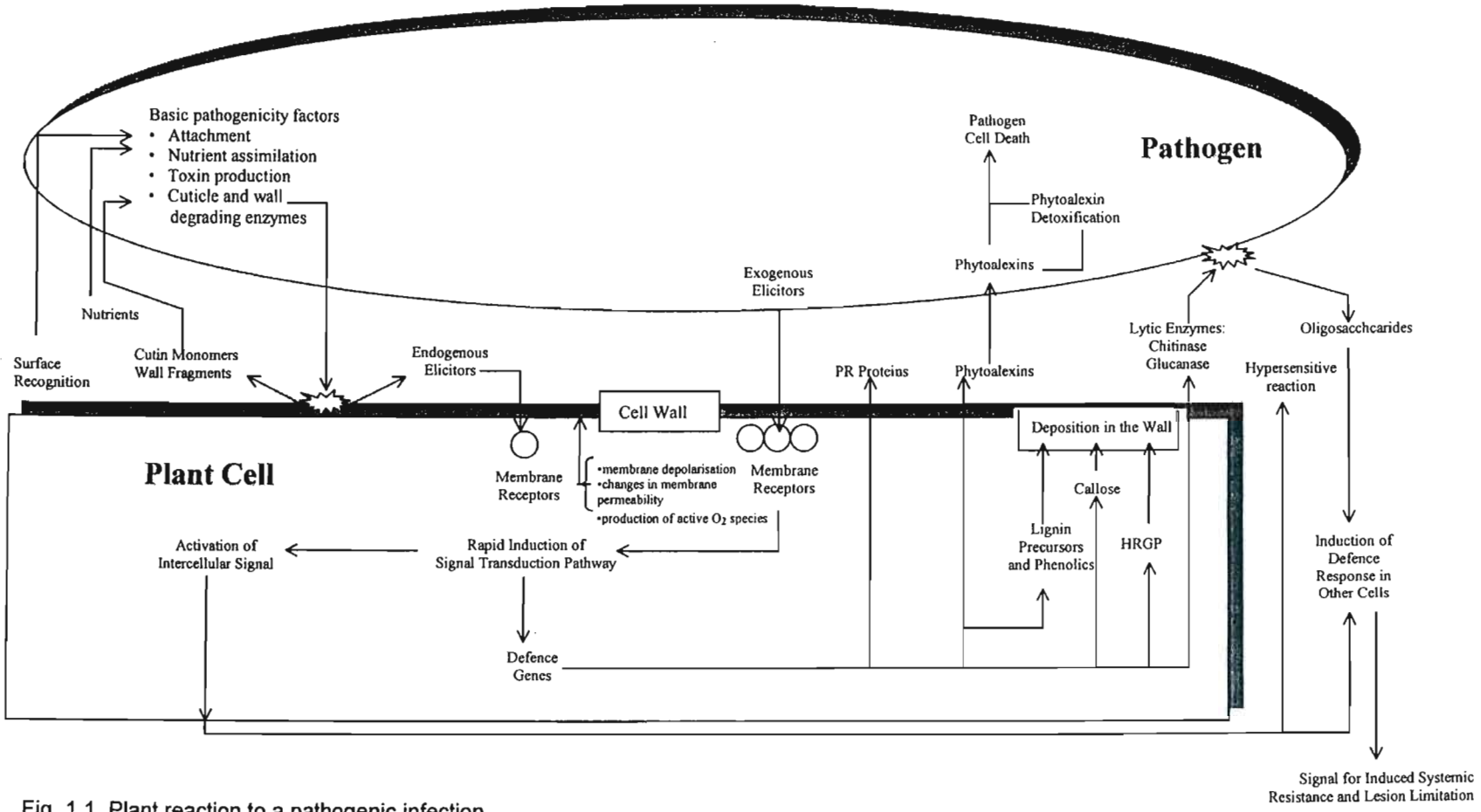


Fig. 1.1. Plant reaction to a pathogenic infection.
The text deals with items approximately in the order displayed from left to right. Adapted from Lamb *et al.*, 1989.

Numerous studies have highlighted that the early events of host infection include membrane depolarisation, changes in membrane permeability, increase of intracellular Ca^{2+} concentrations and production of active oxygen species (Apostol *et al.,* 1989; Lamb and Dixon, 1997; Ebel and Mithofer, 1998). Within minutes of contact with the pathogen, its presence is detected chemically and transmitted to the informational level in the plant. It is believed that these chemicals are then recognised as signal compounds by appropriate perception systems in the plasmalemma (Benhamou, 1996). While biotic elicitors are represented by macromolecules that originate either from the host plant (endogenous elicitors) or from the plant pathogens (exogenous elicitors), heavy metals, chloroform and detergents represent abiotic elicitors of defence responses (Darvill and Albersheim, 1984; Rouxel *et al.,* 1991). These various substances, representing a diversity of chemical structures, have been shown to have the ability to activate a spectrum of defence reactions in plants (Yoshikawa and Sugimoto, 1993). Fungal elicitors can affect the host defence mechanisms by altering pathways and synthesis of defence compounds (Yuan *et al.*, 2002).

- **Elicitor production and recognition**

Four classes of oligosaccharide elicitors have been identified: oligoglucans, oligochitins, oligochitosans of fungal origin and oligogalacturonides of plant origin (West, 1981; Nothnagel *et al.*, 1983; Keen and Yoshikawa, 1983; Davis *et al.,* 1984; Ranansky *et al.*, 1996; Umemoto *et al.*, 1997; Ebel, 1998). In addition, a number of proteinaceous elicitors have also been isolated, some of these being glycoproteins that have been identified in fungal genera such as *Colletotrichum* (Chawla *et al.*, 1992; Coleman *et al.*, 1992), *Puccinia* (Kogel *et al.*, 1991) and *Phytophtora* (Nurenberger *et al.,* 1994). Recent investigations have demonstrated that plants also detect peptides and proteins such as elicitins as factors that are thought to elicit defence reactions (Ebel and Mithofer, 1998).

Elicitor production is rapidly followed by the recognition of the signaling molecule by a receptor that is located in plasma membranes (Nishi, 1994). Although knowledge of the molecular structure of such receptors is limited, glucan-elicitor-binding-receptors on the plasmalemma of soybean have been identified (Benhamou, 1996). In addition, a high-affinity receptor of a *Phytophtora-sojae*-derived oligopeptide has recently been identified in parsley plasma membranes (Nurenberger, 1999). It is believed that these receptors have a dual function: first, that of perception of an extracellular signal produced by elicitors and secondly, that of initiation of an intracellular signal transduction cascade (Nurenberger, 1999).

- **Signal transduction**

The interaction of elicitor substances with membrane receptors is accompanied by a complex response that will trigger events, ultimately leading to defence responses. Cytoskeleton disorganisation (Benhamou, 1996) and production of reactive oxygen intermediates such as the superoxide anion and hydrogen peroxide have been reported (Wojtaszek, 1997b). It has been suggested that the activation of the oxidative burst is a product of the most rapid signalling events in the plant-pathogen interaction. The oxidative burst stands temporarily between early events such as the stimulation of ion fluxes across the plasmalemma and later changes in gene expression, being, in fact, a central component of integrated signalling expression (Bolwell and Wojtaszek, 1997b; Jabs *et al.*, 1997). The oxidative burst could lead to the cross-linking of the cell wall proteins, rendering the plant cell walls more resistant to attack by the enzymes produced by fungi (Ebel and Mithofer, 1998).

Irrespective of the oxidative burst, the response to elicitors includes a rapid change in permeability of the plasma membrane to calcium ions, protons, chloride and potassium ions (Benhamou, 1996). Changes in the ion fluxes (Cl^- and K^+ efflux and Ca^{2+} influx) appear to be early inducible responses that occur within minutes after elicitor application (Zimmermann *et al.*, 1997; Nurenberger,

1999) and have also been correlated with the activation of defence responses. In supporting this, Ca^{2+} has been demonstrated to be involved in phytoalexins elicitation in soybean, potatoes and carrots (Nishi, 1994).

Other molecules such as ethylene, jasmonic acid (JA) and salicyclic acid (SA) have been implicated in the signal transduction pathway, leading to plant disease resistance (Benhamou, 1996).

Ethylene regulates many aspects of plant growth, development and senescence and has been shown to elicit defence responses in melon, soybean, tomato and rice (Lyon *et al.,* 1995). Studies have recorded an increase in ethylene synthesis in advance of release of the resistance substances, leading to the suggestion that ethylene could either be an elicitor-mediated symptom or a second messenger transducing the signal to the nucleus (Mauch *et al.,* 1984).

In addition to ethylene, JA may also play a role in the signal for elicitor activation of plant defences, as has been demonstrated in tomato plants and *Arabidopsis* (Reymond and Farmer, 1998). However, there are some uncertainties related to the specificity of the JA signals in relation to the effects induced by exogenous treatments and whether endogenous JA is essential for the activation of the defence mechanisms (Benhamou, 1996).

The involvement of SA, as an endogenous signal that is capable of inducing the release of plant defence compounds has been supported by numerous studies of a spectrum of plant species against a variety of pathogens (Godiard *et al.*, 1994; Druner *et al.*, 1997; Murphy *et al.*, 1999; Pieterse and van Loon, 1999). Although it has been established that SA has a significant role in plant resistance, the possibility that it could also be a primary signal that travels from the infection site to distal cells of the contaminated tissues, remains controversial (Benhamou, 1996). It has been hypothesised that local infection

could lead to a production of a compound that in turn, requires the presence of SA in the distant tissues, in order to influence the expression of the systemic resistance (Klessing and Malamy, 1994).

- **Expression of plant defence reactions**

The complex set of structural and biochemical mechanisms that is activated upon perception of the transduced signal is an adaptation to protect the plant from the infecting pathogen.

- **The hypersensitive reaction (HR)**

The hypersensitive reaction, characterised by localised and rapid cell death, represents one of the defence responses of plants to fungi. During the HR, recognition of the invading pathogen triggers the activation of a cell death pathway that results in the formation of a zone of necrosis around the infection site. Although certain tissue damage occurs, the affected plant is protected by limiting the spread of pathogenic infection (Mittler and Lam, 1995; Mittler *et al.*, 1996). The HR is also associated with induction of multiple defence mechanisms such as strenghtening of cell walls, accumulation of phytoalexins, salicyclic acid and pathogenesis-related proteins (PR-proteins) (Greenberg *et al.*, 1994). In addition, the HR has also been associated with increased plant resistance to subsequent infection by a variety of pathogens (Greenberg *et al.*, 1994).

- **Reinforcement of the plant cell wall**

The reinforcement of the mechanical properties of the cell walls of the infected plant has been shown to occur concomitantly with deposition of freshly produced compounds such as callose, lignin and phenolics as well as hydroxyproline-rich glycoproteins (HRGPs). Since it has been observed that upon fungal penetration, callose accumulates in significant quantities, it has been suggested that the presence of this compound delays pathogen spread

while allowing the plant to activate further defence responses (Benhamou, 1996).

A further permeability barrier against pathogen infection is the enrichment of the plant cell wall with phenolic or lignin-like polymers. The formation of lignin as a defence mechanism against fungal pathogens has long been recognised (Bennett and Wallsgrove, 1994). Furthermore, studies have demonstrated that the phenolic compounds are associated with the resistance of plants such as yam (*Dioscorea alata*) (Plumbley and Sweetmore, 1994) and carnation (Niemann and Steijl, 1994) to *Colletotrichum gloesporioides* and *Fusarium oxysporum* f.sp. *dianthi,* respectively.

Plants cell walls contain a number of structural glycoproteins that are important in strengthening the entire structure (Walton, 1997). Although cell wall HRGPs accumulate during normal plant development, being associated with cell maturation after the phase of elongation growth, studies have indicated that the increase in HRGPs may be linked to plant resistance (Lamb *et al.*, 1989; Benhamou, 1996).

- **Phytoalexins**

When plants encounter pathogens, they may also undergo biochemical changes such as accumulation of phytoalexins and synthesis of pathogenesis-related proteins (PR-proteins). The term, phytoalexin, was used first time to describe a chemical molecule produced in response to threatened infection by *Phytophthora infestans* (Müller, 1958). Based on the subsequent investigations on beans, phytoalexins have been defined as antibiotics that inhibit the growth of pathogens (Ersek and Kiraly, 1986). The definition of the phytoalexins was later modified to antifungal compounds produced *de novo* by plants when infected by fungi or other micro-organisms, or when treated with abiotic agents (Dakora and Philips, 1996). Phytoalexins have been defined as low molecular weight antimicrobial compounds that are both synthesised by, and accumulate

in, plants after the exposure to microorganisms (Ebel, 1986; Hammerschmidt, 1999).

The accumulation, distribution, enzymology and molecular biology of phytoalexin synthesis following pathogen attack have been researched. The phytoalexins are typically synthesised in a highly localised region around the site of infection and are either absent from, or present only in negligible amounts in, healthy tissues (Keen, 1986; Whitehead and Threlfall, 1992). Quantification of the phytoalexin concentrations in these localised areas of infection has shown sufficient levels of the compounds necessary to inhibit pathogen growth (Walton, 1997).

Studies have demonstrated that certain levels of the relevant biosynthetic enzymes regulate the accumulation of phytoalexins (Kuc, 1995). An increase in phytoalexin biosynthetic enzymes, themselves, is a result of the increased expression of the genes encoding the enzymes. If elicitors stimulate phytoalexin synthesis, *de novo* transcription of the gene encoding phytoalexin biosynthetic enzymes begins within minutes (Kuc, 1995) in the cells surrounding the site of infection (Walton, 1997).

Compounds with phytoalexin activity demonstrate the range and structural complexity typical of higher plant natural products, belonging to a variety of different, but frequently related, chemical classes: phytoalexins include benzofuran, chromone, coumarin, diterpene, flavonoid, furanoacetylene, isoflavonoids, polyacetylene, sesquiterpene, stilbene and triterpene (Smith, 1996). This diversity of chemical structures makes it difficult to adopt a single definition of a phytoalexin based purely on chemical aspects. However, all phytoalexins can be classified as secondary products of plant metabolism with some compounds being related, both by chemical structure and pathway of biosynthesis, to other constitutive secondary metabolites in the same or related species (Smith, 1996).

More than two hundred phytoalexins have been characterised from approximately 30 plant families, with around 125 being isolated from the Leguminosae alone (Kuc, 1995). Most phytoalexins have been described from dicotyledonous families including Convolvulaceae, Euphorbiaceae, Leguminosae, Malvaceae, Solanaceae and Umbellifereae, but they have also been characterised from monocotyledons including rice, barley, maize and onion (Smith, 1996). In general, phytoalexins have been isolated from roots, stems, leaves and fruits though not always from the same plant (Table 1.2). It has been observed that the different members of the same plant family produce phytoalexins that belong to no more than three structural classes that are characteristic of that family. For example, in the Solanaceae, capsidol, rishitin and gossypol (sesquiterpenes) dominate, while Leguminosae accumulate predominantly medicarpin and sativan (isoflavonoid derivates).

- **Pathogenesis-related proteins (PR-proteins)**

In a plant-pathogen interaction, the complex of defence responses, including the inducement of pathogenesis-related proteins (PR), counteracts the possible damage that could occur. These PRs are defined as proteins coded by the plant but induced specifically in pathological (infection by bacteria, fungi and viruses) or related situations (application of chemicals that mimic the effect of pathogens or induce similar stress) (van Loon and van Strien, 1999). The presence of PRs has been observed in some organs including roots, limited parts of seedlings and in cultured cells of many plant species belonging to various families, suggesting that they are involved in general adaptation process to biotic stress (van Loon, 1997).

The pathogenesis-related proteins were originally divided into five groups on the basis of findings of serological and sequence analysis (Bowles, 1990; Stintzi *et al.*, 1993). Subsequently, another nine groups of proteins induced by pathogens have been included (Ebner and Breiteneder, 2002). Many known plant-derived food allergens are homologous to PRs, such as chitinase (PR-3

family) from avocado, banana and chestnut; thaumatin-like proteins (PR-5) from cherry and apple; proteins homolougus to the major birch pollen allergen, Bet v 1 (PR-10), from vegetables and fruits; and lipid transfer proteins (PR-14) from cereals (Ebner and Breiteneder, 2002).

Although proteins that are part of the first five groups (PR-1 to PR-5) are known to have antifungal activity both *in vivo* and *in vitro*, with the exception of PR-2 (β-1,3-glucanase) and PR-3 (chitinase), the molecular activity of the other PR proteins is not yet known (Kitajima and Sato, 1999).

The accumulation of PRs as a stress response has been detected in cultivated plants such as tomato, tobacco and potato (Kladnitskaya *et al.*, 1996; Veronese *et al.*, 1999). The presence of pathogenesis-related proteins was observed in maize (Serna *et.al.,* 2001), wheat (Anguelova *et al.*, 1999), barley (Roberts and Selitrennikoff, 1986; Tamas *et al.*, 1997, 1998), sorghum (Seetharaman *et al.*, 1997; Waniska *et al.,* 2001), some Australian native plants (Last and Llewellyn, 1997), cotton (Chung *et al.*, 1997), pumpkin (Vassiliou *et al.,* 1998), yam (Rompf and Kahl, 1999), sugar beet (Nielsen *et al.,* 1996), radish (Schaaper *et al.*, 2001). The seeds of Asteraceae, Hippocastanaceae and Saxifragaceae have demonstrated production of PRs (Osbourn *et al.*, 1995b).

Table 1.2. Phytoalexins isolated from a variety of plant tissues.

Plant tissue	Pathogen	Phytoalexin	Reference
Rutaceae			
Citrus fruit (*Citrus* spp.) (lemon, orange, grapefruit, lime)	*Phytophthora citrophthora* *Penicillium digitatum*	6,7-dimethoxycoumarin Scoparone Scorpoletin	Sulistyowati *et al.*, 1990 Ben-Yehoshua *et al.*, 1997
Leguminosae			
Chickpea (*Cicer arietinum* L.) roots	*Fusarium oxysporum* f.sp. *ciceri*	Medicarpin Maackiain	Stevenson *et al.*, 1997
Soybean (*Glycine max* L. Merr.) leaves, hypocotyls, seeds and shoots	*Phytophtora megasperma* *Aspergillus flavus* *Rhizoctonia solani* *Sclerotinia sclerotiorum*	Glyceollins PAI, PAII, PAIII, PAIV	Morris *et al.*, 1991; Song and Karr, 1993; Zvyagintseva *et al.*, 1997; Hammerschmidt 1999
Pigeon pea (*Cajanus cajan*) plants	*Fusarium udum*	Hydroxygenistein Genistein Cajanin Cajanol	Marley and Hillocks, 1993
Groundnuts	*Aspergillus flavus* *A. parasiticus*	Stilbene phytoalexins	Strange and Subba Rao, 1994
French bean (*Phaseolus vulgaris*) pod, hypocotyl	*Phytophthora infestans* *Rhizoctonia solani* *Penicillium expansum*	Phaseolin Coumestrol	Muller, 1958; Rathmell and Bendall, 1971
Malvaceae			
Cotton (*Gossypium barbadense*) roots and stems	*Verticillium dahliae*	Desoxyhemigossypol	Mace *et al.*, 1993; Mace and Stipanovic, 1995
Cocoa (*Theobroma cacao* L.) stems	*Verticillium dahliae* Kleb.	3,4 dihydroxyacetophenone 4-hydroxyacetophenone	Resende *et al.*, 1996
Umbelliferae			
Parsnip (*Pastinaca* sativa) roots	*Fusarium sporotrichioides*	Furanocoumarin	Desjardins *et al.*, 1989
Poaceae			
Sorghum (*Sorghum bicolor* L. Moench	*Collelotrichum graminicola*	3-Deoxyanthocyanidin	Hipskind *et al.*, 1990
Rice (*Oryza sativa*) plants	Variety of fungi	Diterpenoids Flavones	Tamogami *et al.*, 1993; Koga *et al.*, 1997; Kodama *et al.*, 1992
Brassicaceae			
Oilseed rape (*Brassica* spp.) seeds and leaves	*Leptosphaeria maculans*	Sirobrassinin Cyclobrassinin sulphoxide Brassilexin Cyclobrassinin	Rouxel *et al.*, 1989; Storck and Sacristan, 1995
Caryophyllaceae			
Carnation (*Dianthus caryophyllus*) stem	*Fusarium oxysporum* f.sp. *dianthi*	Dianthalexin Hydroxidianthramide B Methoxydianthramide R & B Methoxydianthramide S	Baayen and Schrama, 1990; Niemann *et al.*, 1990
Vitaceae			
Grape (*Vitis vinifera*) berries	*Rhizopus stolonifer*	Resveratrol Pterostilbene	Sarig *et al.*, 1997
Solanaceae			
Potato (*Solanum tuberosum* L.) tubers	*Phytophthora infestans* (Mont.)	Rishitin Lubimin	Mucharroma *et al.*, 1995
Brinjal (*Solanum melongena* L.) fruits	Variety of fungi	Sesquiterpenoid phytoalexins	Giri *et al.*, 1996
Alliaceae			
Onion (*Allium cepa* L.)	Necrotrophic fungi	Tsibulin 1d Tsibulin 2d	Dimitriev *et al.*, 1990
Pinaceae			
Pinus spp.	*Fomes annosus* *Peridermium pini*	Pinosylvin Monomethyl ether	Harborne, 1999
Cupressaceae			
Italian Cypress (Cupressus sempervirens L.) bark	*Diplodia pinea*	Glucosides	Madar *et al.*, 1995a; 1995b

1.7 The present study

The only way to store intact recalcitrant seeds is through short- and medium term hydrated storage (Chin, 1980; King and Roberts, 1980; Berjak *et al.*, 1989; Berjak and Pammenter, 2001). The high RH and relatively high temperatures of hydrated storage of recalcitrant seeds are also conducive to the proliferation of associated mycoflora (Berjak 1996; Sutherland *et al.*, 2003). Fungal proliferation associated with wet-stored recalcitrant seeds has always been accepted as being an inevitable event. However, despite considerable research that has been conducted on aspects of recalcitrant seed post-shedding behaviour there are no in-depth studies of mycoflora on recalcitrant seeds.

The current study investigates one of the most characterised species, *Avicennia marina*, that produce seeds that are highly recalcitrant and have been found to rapidly deteriorate in hydrated storage (Farrant *et al.,* 1985; 1986). The study was undertaken to provide an understanding of the role of associated mycoflora in curtailing the lifespan of hydrated stored recalcitrant seeds of *A. marina* and to investigate possible defence mechanisms of these recalcitrant seeds againt *Fusarium moniliforme*.

In order to ascertain whether it is possible to prolong hydrated storage time of *A. marina* seeds by limiting the associated fungi, studies were conducted to investigate the effects of seed-associated mycoflora in 1) seeds with which fungal contamination was inherent and 2) seeds with which fungal contamination was experimentally manipulated. The untreated (non-manipulated) seeds (1) were placed in wet storage without any attempts to minimise/alleviate fungal status and analysed as outlined below. In experimental manipulation (2), responses of seeds that were deliberately disinfected [fungal contamination kept minimal] were compared with those of seeds that were deliberately inoculated and with those of disinfected seeds

which were later fungally-inoculated. All seeds were assessed for fungal contamination and germination performance.

Microscopical assessment (SEM and TEM) was used in an attempt to discriminate between fungal-induced deterioration changes and those inherent degenerative changes that could result from prolonged wet-storage.

Associated fungi were found to have a significant role in limiting recalcitrant seed hydrated storage lifespan although seeds were differentially susceptible depending on the storage period. It was then considered important to understand the observed post-harvest seed resilience to fungal attack and the increased seed susceptibility to the deleterious fungal effects with increase storage time, in particular. In order to explain this observed resilience, seeds were investigated for some defence mechanisms and were studied in the context of the production of antifungal compounds and antifungal enzymes (β-1,3-glucanase and chitinase).

The implications of associated mycoflora in the loss of viability of recalcitrant seeds are discussed.

Chapter 2

The implication of mycoflora in curtailed storage lifespan of recalcitrant seeds of *Avicennia marina*[#]

1. **Investigation of inherent fungal infection of the stored seeds**
2. **Manipulation of the stored seeds in relation to fungal contamination**

[#] Parts of this chapter were incorporated into a paper published in: *Seed Science Research* **10**, 341-353 (2000).

2a. INTRODUCTION

Recalcitrant seeds are shed at relatively high water contents, undergoing little or no maturation drying upon the parent plant. Such seeds are damaged by subsequent dehydration (Roberts, 1973) and, in some instances, by low temperatures (King and Roberts, 1980). In order to store recalcitrant seeds, their water content must be maintained at, or only slightly below, that of the newly shed state (Berjak *et al.*, 1989). Nevertheless, viability retention in such hydrated storage ranges from only days to months, depending on the species. It has been reported that some tropical recalcitrant seeds are damaged by chilling injury at temperatures below 15 °C (Roberts, 1973; King and Roberts, 1980; Roberts and King, 1980) which would effectively preclude their low-temperature storage. Seeds of a few temperate species such as *Quercus robur* (Finch-Savage, 1992), *Acer pseudoplatanus* (Tompsett and Prichard, 1993) and *Aesculus hippocastanum* (Dickie *et al.*, 1991) do lose a proportion of tissue water during development, but never become truly desiccation tolerant. When maintained at 16 °C, seeds of *A. hippocastanum*, the dormancy of which is broken by low storage temperatures, have a lifespan of two to three years (Pritchard *et al.*, 1995). The seeds of other chilling tolerant, temperate species are also reported to have a two to three year lifespan at low temperature. In contrast, some tropical species survive in storage up to three weeks only (King and Roberts, 1980; Suszka and Tylowski, 1980; Farrant *et al.*, 1989; Fu *et al.*, 1990; Tompsett, 1992).

The conditions necessary to maintain the viability of stored recalcitrant seeds (high humidity and relatively high temperatures) are, however, also conducive to fungal and bacterial proliferation (Berjak, 1996; Sutherland *et al.*, 2003). Although mycofloral infection of orthodox seeds has been relatively extensively studied, to date, there have been few data published on the implication of microflora, especially of fungi, on the curtailed storage life of recalcitrant seeds.

Recalcitrant seeds, like their orthodox counterparts, generally harbour a range of fungi and bacteria even when they are newly harvested (Mycock and Berjak, 1990). While species of *Aspergillus* and *Penicillium* can be found in recalcitrant seeds, it is apparent that *Fusarium* species may emerge as the dominant fungal species during storage of recalcitrant seeds (Mittal and Sharma, 1982; Pongapanich, 1990; Dalbir-Singh and Singh, 1990; Singh and Singh, 1990; Sutherland *et al.*, 2003), as in the case of *Avicennia marina* seeds (Mycock and Berjak, 1990).

It is now known that recalcitrant seeds not only have a high water contents, but are metabolically active on shedding and that in many cases the development progresses, without discernible interruption, into germinative metabolism (Berjak *et al.*, 1989; Farrant *et al.*, 1993a; Pammenter *et al.*, 1994). However, when these seeds are wet-stored, they become progressively debilitated, eventually losing vigour and viability, the time period involved depending on the species (Pammenter *et al.*, 1994). This is also the time when the seeds-associated mycoflora often proliferates (Berjak, 1996). No research, however, has previously been undertaken in recalcitrant seeds to determine whether seed deterioration offers the mycoflora favourable growth conditions, or whether the fungi themselves cause, or at least accelerate, seed debilitation.

The present study is aimed towards the understanding of the complex relationships between associated mycoflora and wet-stored recalcitrant seeds of *Avicennia marina*. The investigation was undertaken to ascertain whether it is possible to prolong hydrated storage time of *Avicennia* seeds by limiting the associated fungi. A further question was whether the associated mycoflora triggers seed degeneration during wet storage, or if inherent deterioration of the seeds facilitates fungal proliferation and invasion. Newly harvested and stored seeds were presently studied in the context of seed-fungal interaction. The implications of associated mycoflora in the loss of viability of recalcitrant seeds are discussed.

2b. MATERIALS AND METHODS

2b.1 Seed collection

Seeds of *A. marina* used in this study were collected in March and/or April of each year from a population of trees in the Beachwood Mangroves Nature Reserve, Durban, South Africa. Mature seeds (that showed no signs of a aruptured or blemished pericarp and were easily detached from the mother plant) were carefully hand-picked and transferred in plastic bags to the laboratory.

2b.2 Explanatory note

The present study was conducted over a period of four years. The investigation was divided into two distinct studies: hydrated storage of non-manipulated *A. marina* seeds (Investigation of the natural fungal infection of the stored seeds: Experiment 1 - inherent infection) and wet storage of manipulated *A. marina* seeds (Manipulation of stored seeds in relation to fungal contamination: Experiment 2 - manipulation).

2b.3 Preparation of seeds for storage

2b.3.1 Experiment 1 – inherent infection

In this experiment, the seeds were wet stored with and without pericarps for a period of 21 days (Diagram 2.1). In the laboratory, freshly harvested seeds were divided into two batches: seeds that were stored with the pericarp (Fig. 2.1) and those that were stored without pericarp (artificially removed, Fig. 2.3). In the first situation, the pericarps became naturally detached during storage as this outer covering sloughs spontaneously under damp conditions. These were provided by the high RH of the storage conditions under which an individual seed usually

remained lying upon, or loosely surrounded by, the sloughed pericarp (Fig. 2.2). For the second seed category, pericarps were immediately removed by allowing spontaneous sloughing to proceed during soaking in water for 30 minutes (Fig. 2.3). In neither case were the seeds subjected to surface sterilisation.

Seeds were then arranged in one to two layers on plastic mesh grids. The grids were suspended at a height of 100-200 mm over saturated paper towel, within individual plastic buckets. The buckets were then sealed and maintained at an ambient temperature range of 25-28 °C.

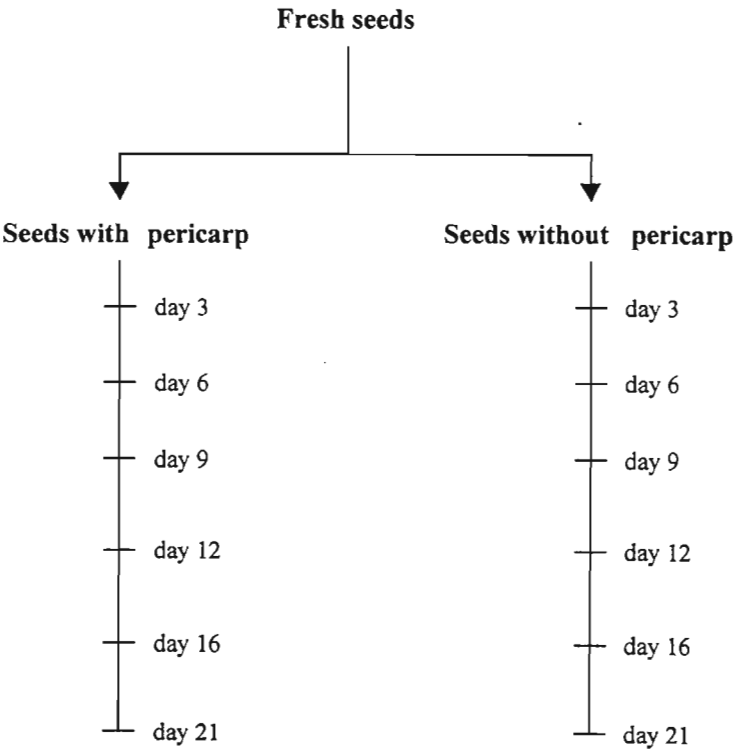


Diagram 2.1. Hydrated storage of *A. marina* seeds with or Without pericarp (Experiment 1 – inherent infection). The numbered days represent sampling intervals.

2b.3.2 Experiment 2 – manipulation

In this experiment, the seeds of *A. marina* were stored under hydrated conditions as described above, for 21 days (Diagram 2.2), but in all cases, the seeds were stored after pericarp removal. In the laboratory, pericarps were sloughed by soaking seeds in water for 30 minutes. All seeds were then surface sterilised in 1% sodium hypochlorite containing 0.02% Tween 20 (Sigma, USA) for 20 minutes. Following rinsing with sterile, distilled water (three changes), seeds were separated into two categories. One lot of seeds was maintained clean (naturally occurring fungal contamination kept minimal to zero) throughout the hydrated storage period, while the second lot was experimentally fungal inoculated prior to storage. From this point, these seeds will be referred to as *clean* and *infected seeds*.

In order to minimise microbial contamination, the clean seeds as well as the interior wall of the storage containers were sprayed at 3-day intervals with Previcur N® solution (2.5 ml l^{-1}) (AgrEvo, Pietermaritzburg, South Africa) throughout the 21 days of storage. As depicted in Diagram 2.2, after various periods of storage (4, 7, 10, 12 14, 16 and 21 days), samples of clean seeds of *A. marina* were removed from the initial storage containers and inoculated with *F. moniliforme* (as it is described below). These seeds were then re-stored in individual batches in separate, pre-sterilised containers for a further period of 12 days (referred to as *clean stored-infected seeds*).

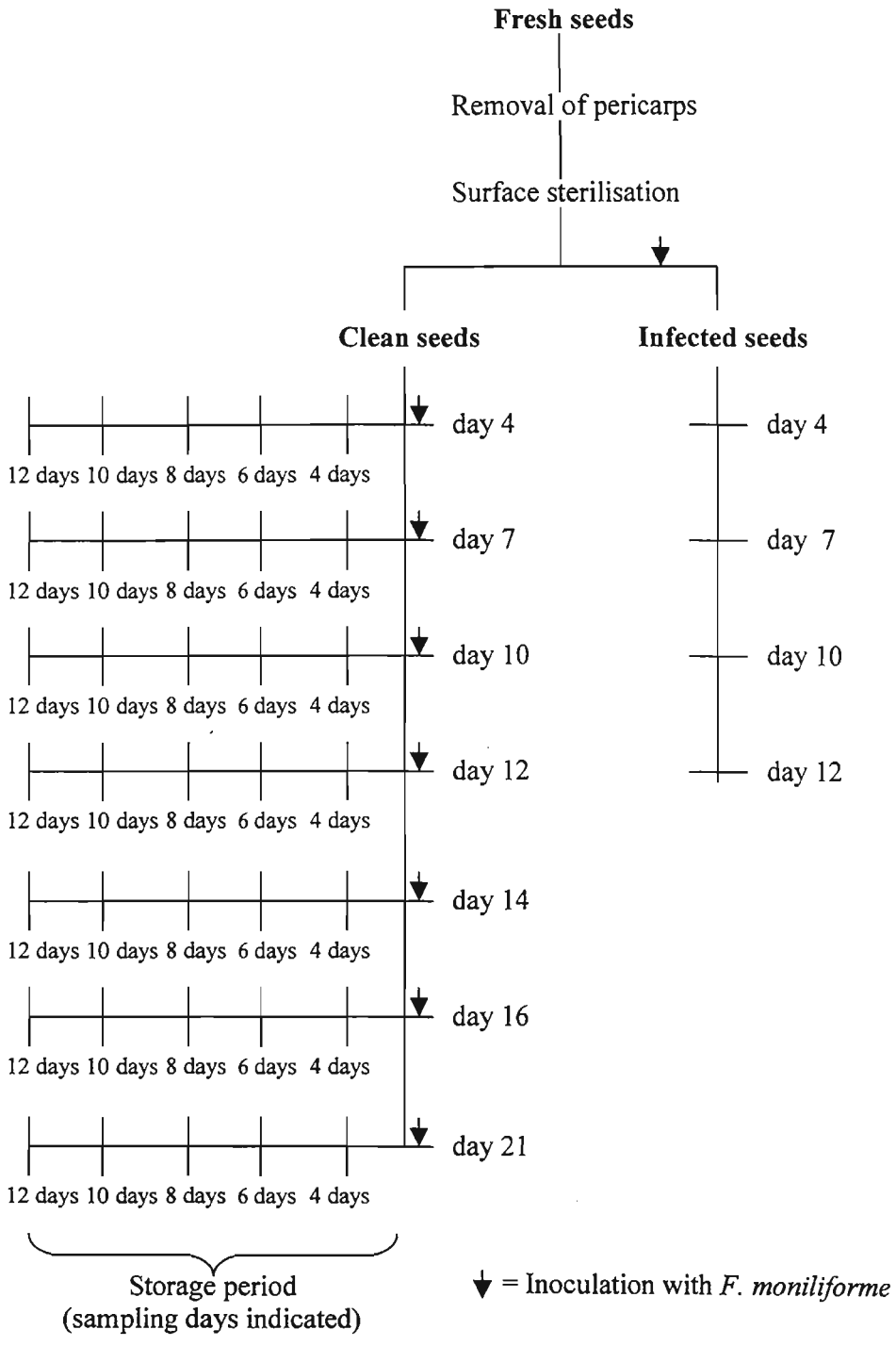


Diagram 2.2. Hydrated storage of *A. marina* seeds (Experiment 2 - manipulation).

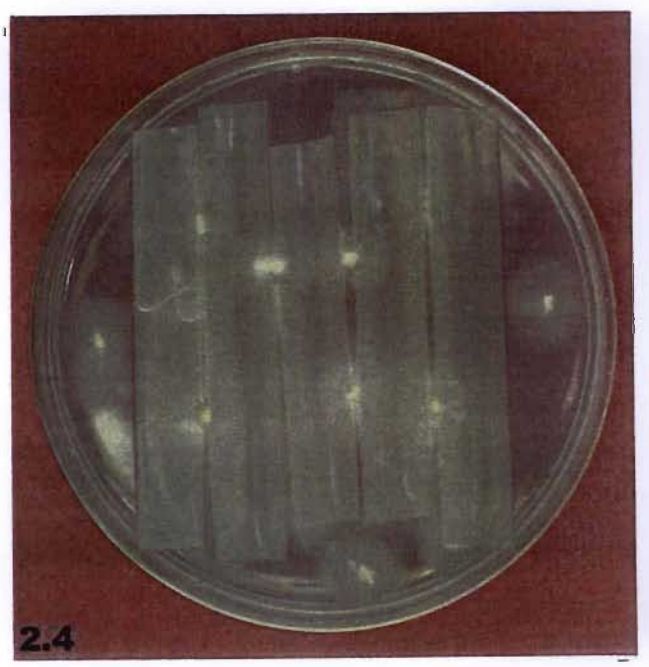
Figs. 2.1 - 2.3. **Seeds of *Avicennia marina*:** freshly harvested with pericarps (Fig. 2.1), spontaneous sloughing of pericarps (Fig. 2.2) and freshly harvested with pericarps removed (Fig. 2.3).

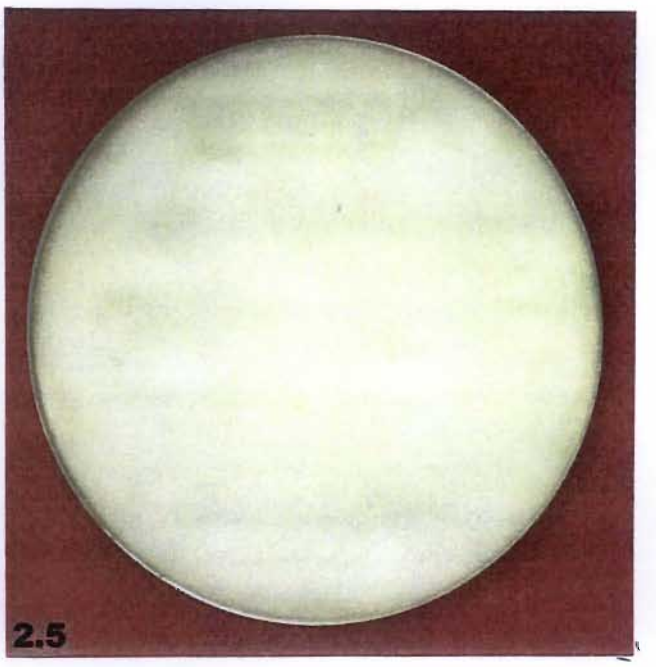
2b.3.2.1 Fungal inoculation of *A. marina* seeds

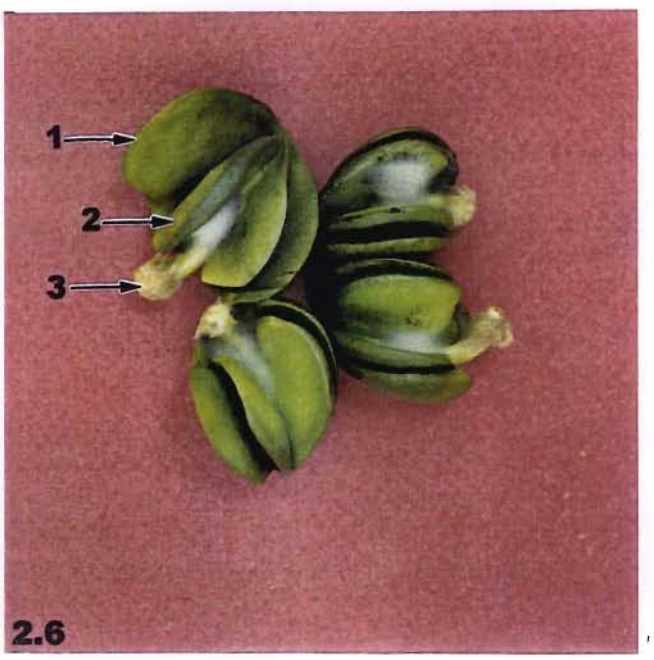
Avicennia marina seeds that were inoculated immediately after pericarp removal as well as those that were stored clean and subsequently inoculated, were manipulated using a technique adapted from Marciano *et al.* (1991) and described below.

Fusarium moniliforme (isolated from *A. marina* seeds and identified by the Plant Protection Research Institute, Pretoria, South Africa) was inoculated on plates of fresh potato dextrose agar (potato dextrose agar 25.0 g, bacteroagar 15.0 g, sodium chloride 40.0 g and distilled water 1000 ml [The Oxoid Manual, 1990]). The cultures were then incubated in the dark for six days at 25-26 °C.

Sterile dialysis tubing was cut into strips of 60-80 mm and placed onto fresh potato dextrose agar in Petri dishes (Fig. 2.4). Fungal mycelium was removed from the growing perimeter of the *F. moniliforme* colony and inoculated into the agar plates containing the dialysis tubes (Fig. 2.4). These inoculated Petri dishes were then incubated for a further period of ten days, in dark, at 25-26 °C. After this period (Fig. 2.5), under sterile conditions, each dialysis tube was removed from the plate and cut into segments 5 mm long. These fungally-contaminated segments of dialysis tubing were then used to inoculate the *A. marina* seeds: a segment was placed between the surfaces of the inner cotyledon of each seed, in the proximity of the embryonic axis (as depicted in Fig. 2.6). Inoculated seeds were then placed into hydrated storage for various periods (Diagram 2.2).







Figs. 2.4 - 2.6. **The seed inoculation process.** *Fusarium moniliforme* was inoculated on PDA Petri dishes that contained dialysis tubes (Fig. 2.4) and after 10 days of incubation (Fig. 2.5) was used to inoculate *A. marina* seeds as illustrated in Fig. 2.6.*

* 1, outer cotyledon; 2, inner cotyledon; 3, embryonic axis

2b.4 Sampling procedures

Both experiments were repeated over two years. In the case of the experiment 1 (inherent infection), the seeds stored with, or without, pericarps were randomly sampled fresh and after three, six, nine, 12, 16 and 21 days of hydrated storage (Diagram 2.1).

The seeds of the experiment 2 (manipulation) were randomly sampled fresh and for both seed categories, clean and infected, after 4, 7, 10 and 12 days. The clean seeds were also sampled after 14, 16 and 21 days of storage. As shown in Diagram 2.2, sub-samples of clean stored-infected seeds were collected at intervals of two days over a period of 12 days (with the exception of the seeds stored clean for four days). For easier description for the reader, each of sample has been allocated a code (Table 2.1).

Samples from both experiments were assessed for germination performance and fungal contamination. While samples from experiment 1 (inherent infection) were viewed using scanning electron microscopy (SEM), the samples and sub-samples of experiment 2 (manipulation) were assessed by transmission electron microscopy (TEM).

2b.5 Germination assessment

Random samples of 25 seeds each, per treatment were germinated in trays of moistened vermiculite at 25-26 °C, under 16 hours/8 hours photoperiod conditions. Germination was assessed at three-day intervals over a period of 12 days. The seeds were considered germinated when the root primodia of each seed had extended by at least 2 mm.

Table 2.1. The coding system used throughout the research

Treatment		Codes
Storage days prior to inoculation	**Storage days following inoculation**	
0d	0	Fresh
	4	4d infected
	7	7d infected
	10	10d infected
	12	12d infected
4d	0	4d clean
	4	4d-4
	6	4d-6
	8	4d-8
	10	4d-10
	12	4d-12
7d	0	7d clean
	4	7d-4
	6	7d-6
	8	7d-8
	10	7d-10
	12	7d-12
10d	0	10d clean
	4	10d-4
	6	10d-6
	8	10d-8
	10	10d-10
	12	10d-12
12d	0	12d clean
	4	12d-4
	6	12d-6
	8	12d-8
	10	12d-10
	12	12d-12
14d	0	14d clean
	4	14d-4
	6	14d-6
	8	14d-8
	10	14d-10
	12	14d-12
16d	0	16d clean
	4	16d-4
	6	16d-6
	8	16d-8
	10	16d-10
	12	16d-12
21d	0	21d clean
	4	21d-4
	6	21d-6
	8	21d-8
	10	21d-10
	12	21d-12

2b.6 Fungal contamination

Twenty-five seeds were randomly selected from each treatment. Fungal contamination was monitored for the pericarp (if present), cotyledons and embryonic axis of each seed. In order to assess surface contaminants, portions (5 x 5 mm) of pericarp, cotyledon and embryonic axis were dissected out, and, under sterile conditions, placed onto potato dextrose agar (PDA) plates. For internal contaminants, the seed tissues (with the exception of pericarp and embryonic axes of the seeds of experiment 2) were aseptically excised (all outer surfaces were dissected away) and surface sterilised in 1% sodium hypochlorite containing 0.02% Tween 20, for 10 minutes. The tissue segments were plated onto PDA. The Petri dishes were incubated in dark, at 25 ± 1 °C and monitored at three - day intervals for contamination (data presented after 12 days of incubation). All fungal and bacterial samples were identified by the Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa.

2b.7 Microscopical studies

As mentioned above, the seeds of *A. marina* from experiment 1 were viewed with SEM. In contrast, the samples and sub-samples from experiment 2 were monitored by TEM.

2b.7.1 Scanning electron microscopy (SEM)

Five replicate samples were taken from each seed treatment. Plant material was dissected from each seed, each sample being excised from the same region of all seed tissues. The 5 x 5 mm portions of pericarp (where present) and cotyledon (the edge of the outer cotyledon) and the root primordium region of the embryonic axis were processed according to the protocol of Mycock and Berjak (1991):

- Plant material was fixed for a minimum of 24 hours in 2.5% phosphate-buffered glutaraldehyde (pH 7.2).
- After fixation, the samples were gently washed three times for five minutes each in 0.5 M phosphate buffer.
- The material was post-fixed for one hour in 1.5% aqueous osmium tetroxide.
- Specimens were washed three times for five minutes each, in 0.5 M phosphate buffer.
- Samples were then dehydrated through an alcohol series (25%, 50%, 75% and 100% ethanol), with 10-30 minutes immersion, at each concentration.
- The plant material was transferred from absolute alcohol to a critical point drier (Hitachi C.P.D.).
- The dried samples were mounted on stubs and coated with a mixture of gold/palladium in a Polaron sputter coater.
- Viewing and photography on Kodak film were carried out using an Hitachi SEM 520.

2b.7.2 Transmission electron microscopy (TEM)

Five seeds were randomly selected from each treatment. Segments (1 mm x 4 mm) of the cotyledons were excised from the same region of all of the sampled seeds (the edge of the outer cotyledon). Three root primodia were excised from each of the five seeds.

- Plant material was fixed in 2.5% glutaraldehyde buffered at pH 7.2 with 0.1 M phosphate buffer containing 1% caffeine, for a minimum of 24 hours
- Samples were then post-fixed in 0.5% aqueous osmium tetroxide for one hour.
- Samples where then rinsed with three changes of distilled water.
- Dehydration in a graded acetone series (30-100%) followed.

- Material was then infiltrated in 50:50 acetone:epoxy resin for four hours.
- Overnight infiltration in pure resin (Spurr, 1969) followed.
- The material was then embedded in fresh, pure resin, which was polymerised at 80 °C for 8 hours.

The embedded plant material was sectioned using an LKB ultramicrotome. Ultra-thin sections were cut for TEM analysis. The sections were post-stained for 10 minutes with saturated aqueous lead citrate (Reynolds, 1963), and viewed and photographed on Kodak film, using a Jeol 1010 transmission electron microscope.

A qualitative analysis was carried out at a descriptive level of the micrographs, with particular attention being focused on metabolic status indicated by the ultastructure (e.g. the development of the endomembrane system) and any possible abnormalities (e.g. abnormal vacuolation, aberrations of membranes and possible presence of fungal structures) indicative of cell damage.

2c. RESULTS

As described in the Materials and Methods Section of the present chapter, the results of the two experiments (Experiment 1 – inherent infection and Experiment 2 – manipulation) will be discussed separately.

2c.1 An investigation of natural infection of the stored seeds: Experiment 1 – inherent infection

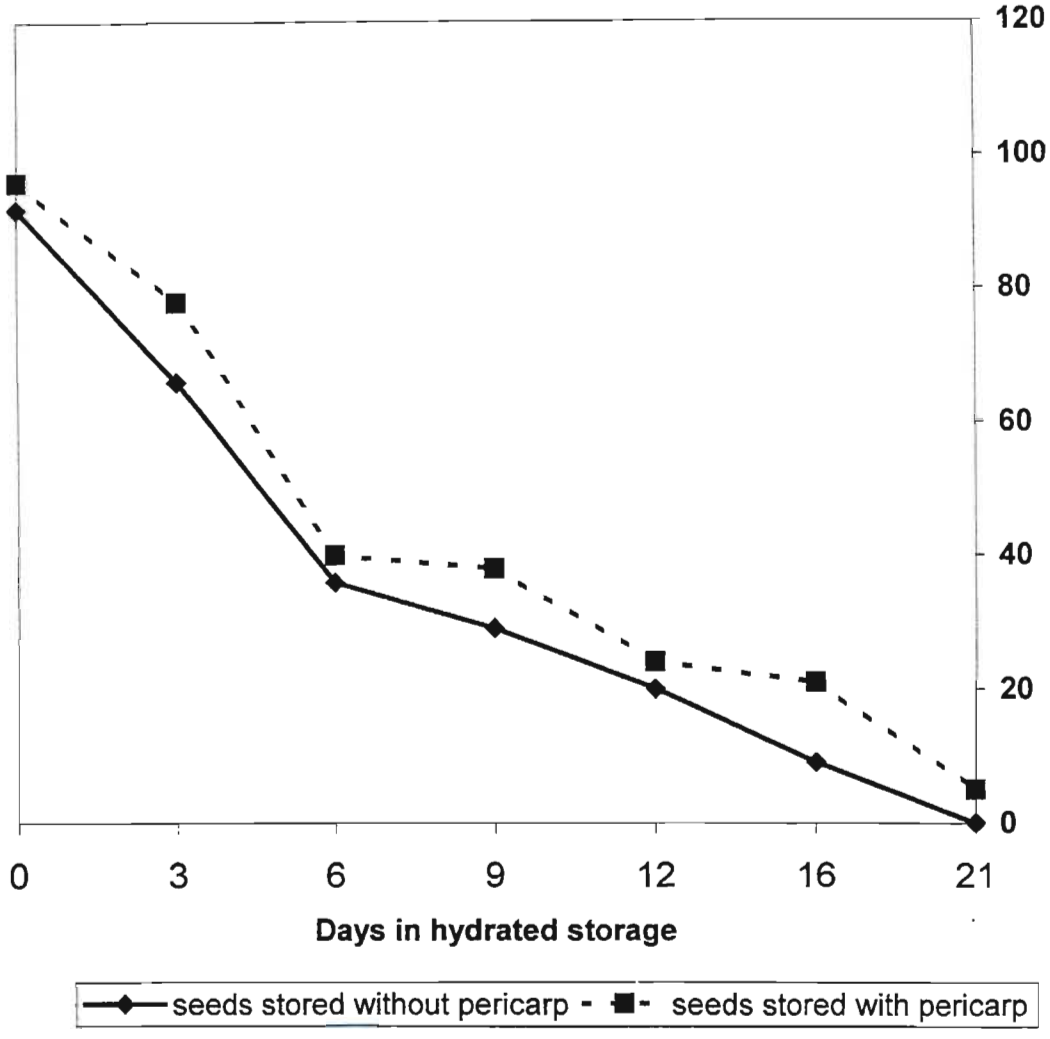
The results of this experiment are presented in terms of the germination assessment, the fungal contamination and the scanning electron microscopical study.

2c.1.1 Germination assessment

The assessment of germinability of *A. marina* seeds stored with and without pericarps is depicted in Figure 2.7. Germinability of *A. marina* seeds stored for three days, declined from 96% to 78% for seeds stored with pericarp, and from 92% to 66% for those in which the pericarp had been removed prior to storage. Following six days of storage, the germinability decreased further to 40% for the seeds stored with pericarp and to 36% for those *A. marina* seeds stored without pericarp. After nine days of hydrated storage, the seeds stored with pericarp exhibited a germinability of 38%, while those stored without pericarps had a germination percentage of 29%. *Avicennia marina* seeds stored with pericarp for 12 days demonstrated a decreased germinability of 24%, while the seeds stored without coats for the same period had a germination of 20%. It was observed that further into the hydrated storage, at day 16, the seeds stored with coats exhibited only 5% germinability and 0% after three weeks of storage. A similar pattern for seeds stored without pericarps was observed by 16 days of storage, where germinability had decreased to 21% and to a further low 5% after 21 days of hydrated storage. From these data, it was apparent that

removal of the pericarp had no effect on retention of seed viability during hydrated storage.

Fig. 2.7. Germination of hydrated seeds of *Avicennia marina* stored with or without pericarps



2c.1.2 Fungal contamination

During experiment 1, no attempts were made to identify the associated seed bacteria, or fungi to species level. In contrast, experiment 2 presents an in-depth identification of the microorganisms that were isolated from the *A. marina* seeds.

Newly harvested seeds of *A. marina* harboured fungi of several species, as well as bacteria. A mixed microflora was found externally on all seed tissues, and as internal contaminants (Table 2.2). All seed parts were contaminated with a minimum of three fungal species as well as bacteria.

Table 2.2. Fungal and bacterial contaminants (external and internal) isolated from *Avicennia marina* seeds during 21 days of hydrated storage.

Seed tissue		Newly-harvested seds	Stored with pericarp		Stored without pericarp	
			6 days	12-21 days	6 days	12-21 days
Pericarp	**External**	Fungi*	*Fusarium* sp. *Penicillium* sp.	*Fusarium* sp.	-	-
		Bacteria	-	-	-	-
	Internal	Fungi	*Fusarium* sp.	*Fusarium* sp.	-	-
		Bacteria	-	-	-	-
Cotyledon	**External**	Fungi*	*Fusarium* sp. *Penicillium* sp.	*Fusarium* sp.	*Fusarium* sp.	*Fusarium* sp.
		Bacteria	-	-	-	-
	Internal	Fungi	*Fusarium* sp.	*Fusarium* sp.	*Fusarium* sp.	*Fusarium* sp.
		Bacteria				
Embryonic axis	**External**	Fungi*	*Fusarium* sp. *Penicillium* sp.	*Fusarium* sp.	*Fusarium* sp.	*Fusarium* sp.
		Bacteria	-	-	Bacteria	-
	Internal	Fungi	*Fusarium* sp.	*Fusarium* sp.	*Fusarium* sp.	*Fusarium* sp.
		Bacteria	-	-	-	-

*More than two fungal species

Following six days of hydrated storage it was evident that *Fusarium* and *Penicillium* spp. were the dominant fungal contaminants of seeds stored with pericarps. While both genera of fungi were isolated from all the surfaces of seed tissues, only *Fusarium* sp. was found as an internal contaminant. At this stage of the wet storage, no bacterial contamination of seeds stored with coats was evident. In contrast, in the case of seeds stored without pericarps bacteria were isolated from the surface of embryonic axes after six days of storage. *Fusarium* sp. was the only fungal pathogen associated with both cotyledons and embryonic axes of *A. marina* seeds stored for six days without coats.

From 12 days of wet storage onwards, whether seeds were stored with or without pericarps, only *Fusarium* sp. persisted on, and within, seed tissues of *A. marina*. No bacterial contaminants were isolated from the seeds that were stored either with or without pericarps for 12-21 days.

2c.1.3 Scanning Electron Microscopical Study (SEM)

Scanning electron microscopy of newly harvested *A. marina* seeds demonstrated the presence of both fungi and bacteria on the pericarp. These contaminants were observed on both the outer surface of the seed coat (Fig. 2.8) and on the inner side (naturally contiguous with the outer cotyledons) (Fig. 2.9). Although fungi and bacteria were isolated from the surfaces of cotyledons of fresh *A. marina* seeds, scanning electron microscopy revealed no contamination on the cotyledonary surfaces (Fig. 2.10). However, internally, the cotyledonary tissue of freshly harvested material showed fungal structures as well as bacteria (Fig. 2.11), suggesting that the fungal association with the surface of cotyledons could have been localised and thus only occasionally apparent. Micrographs of the embryonic axis of fresh *A. marina* seeds confirmed the presence of fungal hyphae and bacteria (Fig. 2.12).

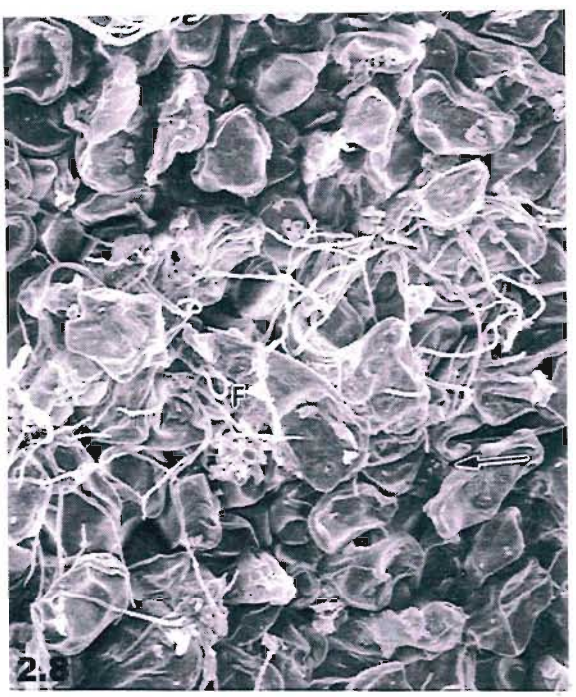
The pericarps of seeds wet stored with coats for six days exhibited considerable fungal proliferation on the external (Fig. 2.13) and inner surfaces (Fig. 2.14). This infection of cotyledon external surfaces often with substantial fungal mats (Fig. 2.15) is presumed to have originated from the heavy infection of the seed coat. Fungal penetration of cotyledonary tissues (Fig. 2.16) appeared to have been achieved via salt glands and/or stomata (Fig. 2.15), and the occasional hyphae within cotyledons seen in fresh material (Fig. 2.11) may also have originated from surface loci of infection. It was not surprising to observe the persistence and proliferation of fungus on the surface of the embryonic axis (Fig. 2.17) of these seeds.

Avicennia marina seeds stored without coats for six days showed abundant fungal proliferation on the surface of the cotyledon (Fig. 2.18) and some degree of what appeared as localised tissue necrosis (Fig 2.19). Figure 2.19 shows fungal hyphae apparently penetrating cotyledonary tissue through salt glands and stomata of the otherwise intact cotyledonary surface. While the adjacent, apparent necrotic area (Fig. 2.19) probably implies severe fungal degradation, possible injury sustained by other means cannot be eliminated. The situation was similar in embryonic axis of these seeds, with mycelial mats (Fig. 2.20) and associated localized necrotic areas evident on the surface as well as internal tissue infection (Fig. 2.21).

Analysis of *A. marina* seeds stored with coats for a minimum period of 12 days revealed considerable fungal contamination of the pericarp, with associated severe tissue necrosis (Fig. 2.22). By day 16 of hydrated storage, the cotyledons of these seeds exhibited fungal proliferation, penetration and some localised necrosis (Fig. 2.23), while advanced necrosis of the cotyledons was evident by 21 days of hydrated storage (Fig. 2.24). This was associated with considerable internal cotyledonary tissue damage (Fig. 2.25). A similar trend was observed in embryonic axes of the seeds stored for more then two weeks,

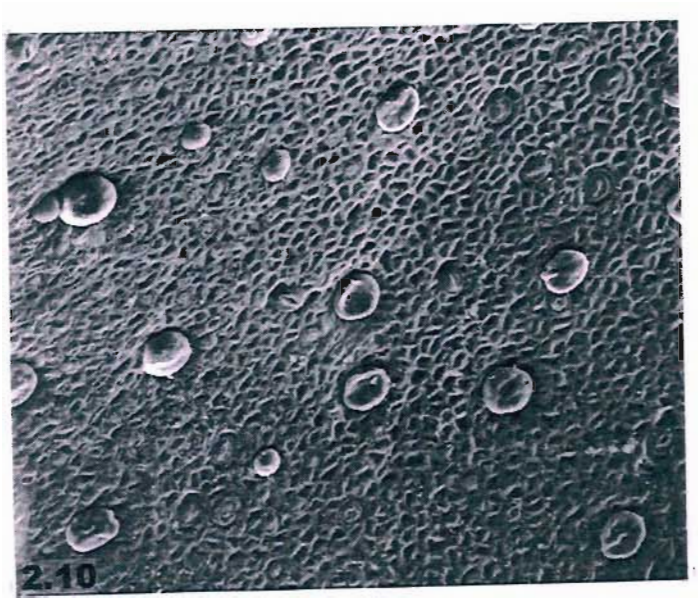
which also exhibited a high degree of surface necrosis (Fig. 2.26) and internal damage (Fig. 2.27).

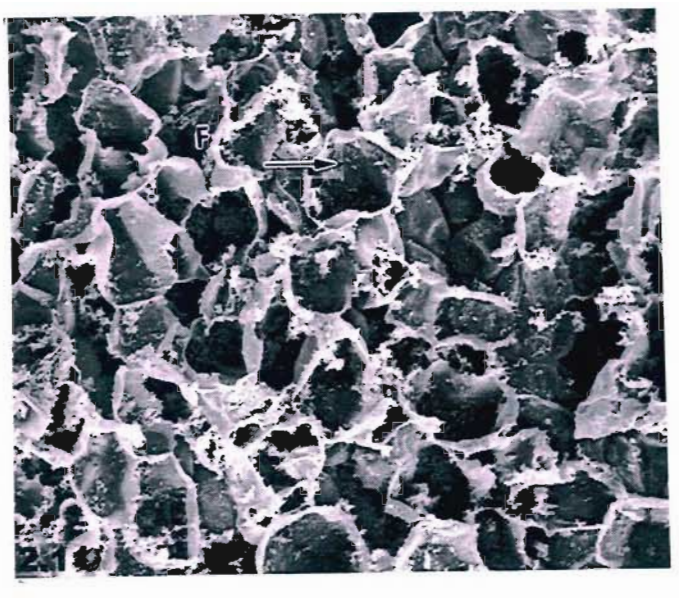
The situation was very similar in *A. marina* seeds stored without coats for a more then 12 days. The cotyledons of these seeds showed a prolific fungal mat on the surface (Fig. 2.28) and advanced internal tissue deterioration (Fig. 2.29). Extensive necrosis and fungal structures were also observed on the surface (Fig. 2.30) and within the tissues (Fig. 2.31) of the embryonic axes of the seeds stored without pericarps for up to 21 days.

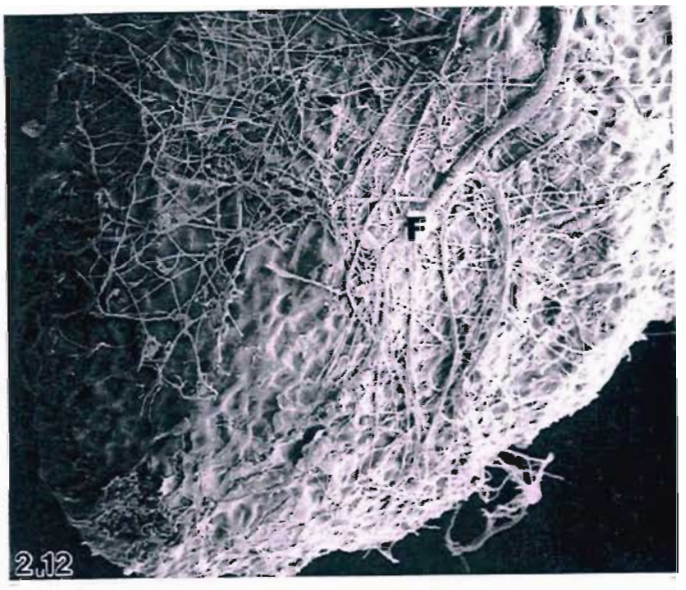


Figs. 2.8 - 2.9. **Scanning electron micrographs of the pericarp of freshly harvested *A. marina* seeds**. The external seed coat surface exhibited fungal and bacterial (arrow) infection (Fig. 2.8 [x 300]). Fungi and bacteria (arrow) were also evident on the inner surface of the pericarp (Fig. 2.9 [x 300]).*

* F, fungal structure

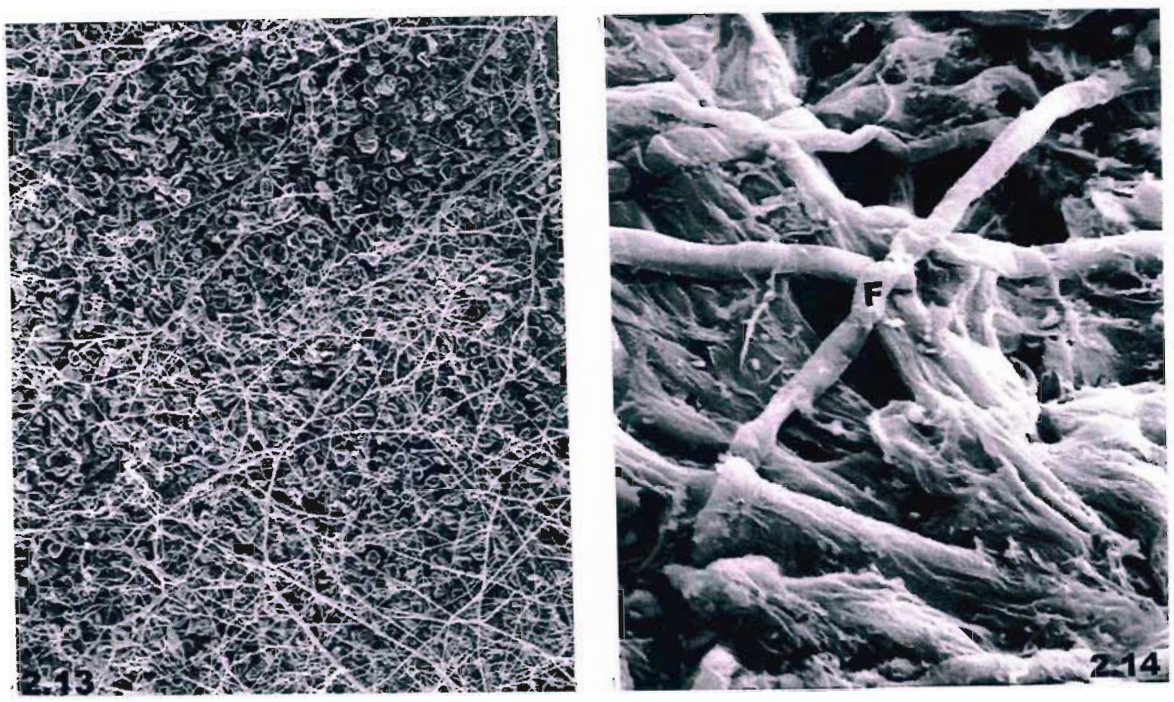






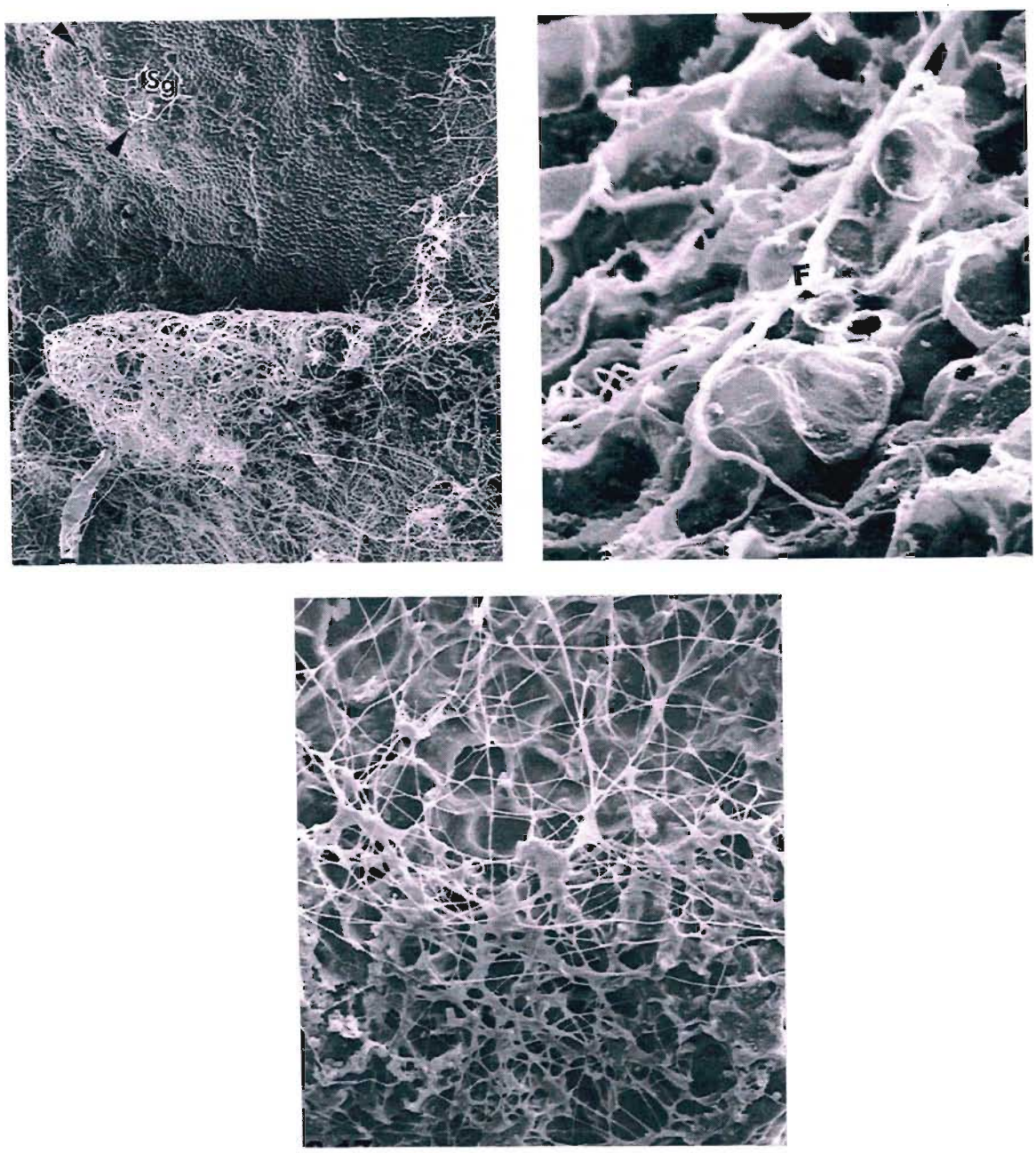
Figs. 2.10 - 2.12. **Scanning electron micrographs of cotyledons and embryonic axis of freshly-harvested *A. marina* seeds**. The external cotyledonary surface showed no apparent microbial presence (Fig. 2.10 [x 200]). However, internally the cotyledonary tissue showed the presence of fungal and bacterial (arrow)) infection (Fig. 2.11 [x 300]). Considerable fungal proliferation and some associated bacteria were also apparent on the embryonic axis surface (Fig. 2.12 [X 100]).*

* F, fungal structure



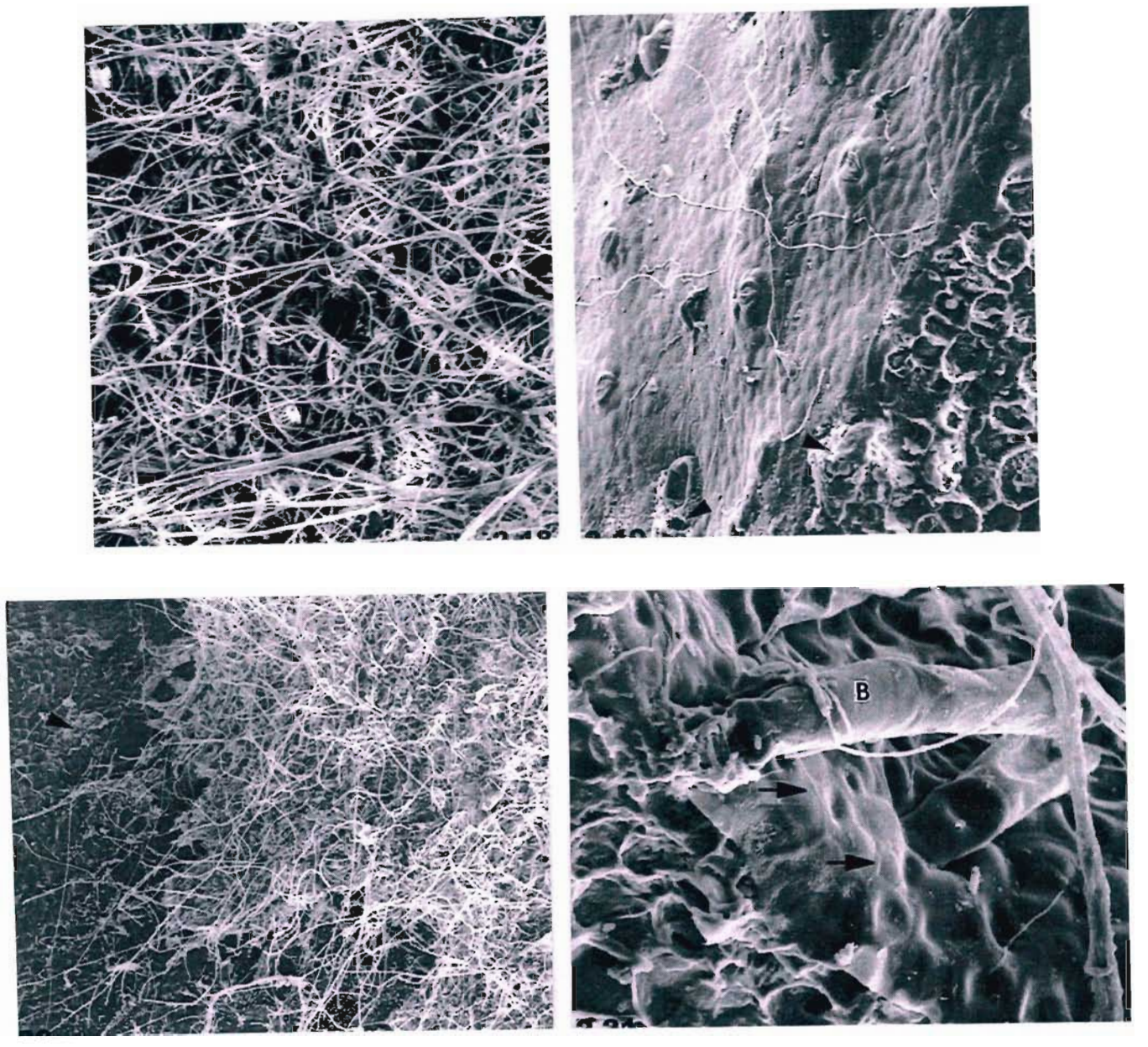
Figs. 2.13 - 2.14. **Scanning electron micrographs of the pericarps of *A. marina* seeds stored with coats for 6 days**. Considerable fungal proliferation had occurred on the external (Fig. 2.13 [x 70]) and the inner seed coat surfaces (Fig. 2.14 [x 1000]).*

* F, fungal structure



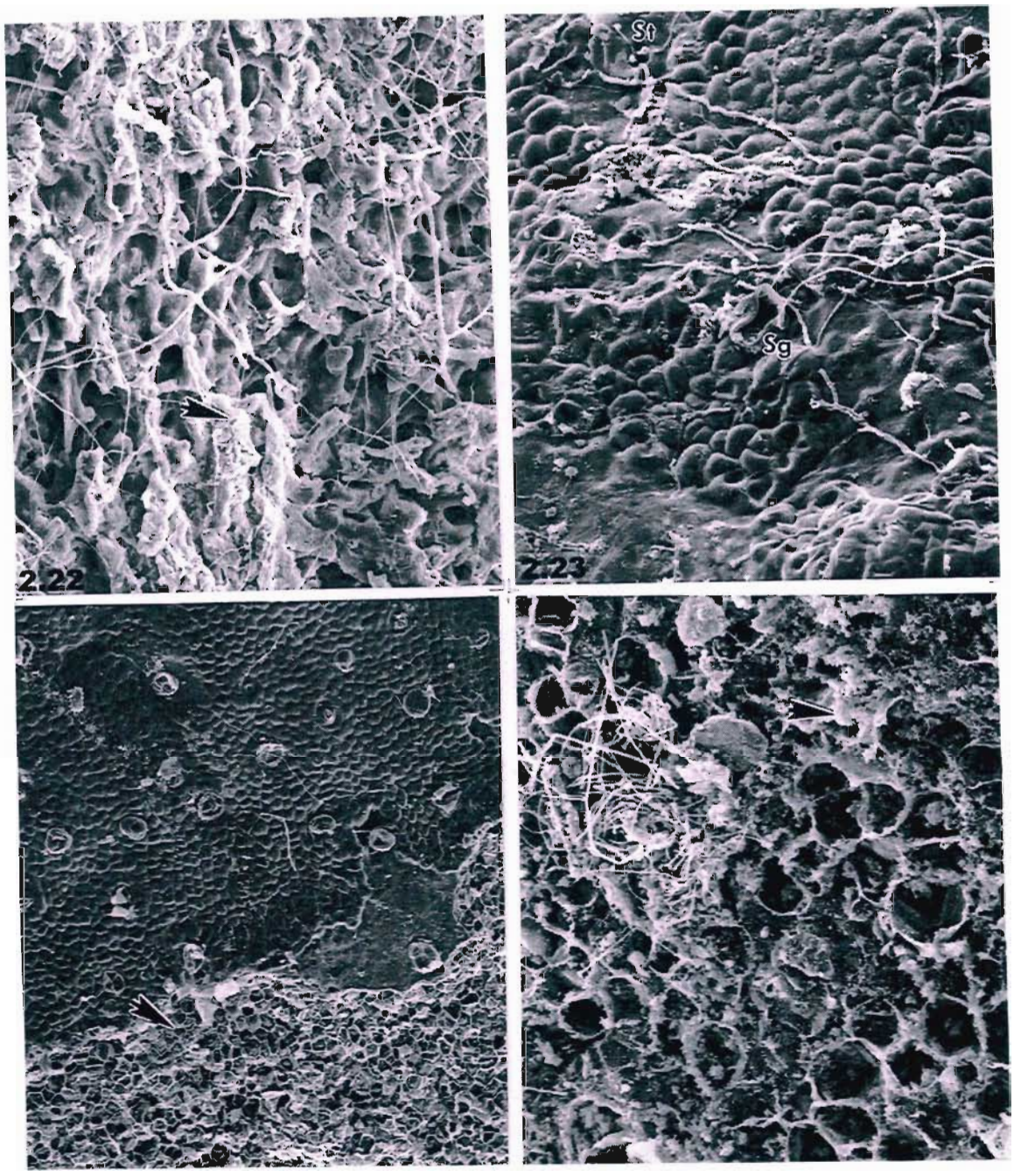
Figs. 2.15 - 2.17. **Scanning electron micrographs of cotyledons and embryonic axes of *A. marina* seeds that were stored with pericarps for 6 days**. A dense fungal mat was observed on the surface of cotyledons. When the fungal mat was lifted, the mycelium was observed penetrating cotyledonary tissues through salt glands (arrowheads, Fig. 2.15 [x 70]). Internal infection was evident in cotyledons (Fig. 2.16 [x 700]) and considerable fungal proliferation infection was also apparent on the surfaces of embryonic axes (Fig. 2.17 [x 500]).*

* F, fungal structure; Sg, salt gland



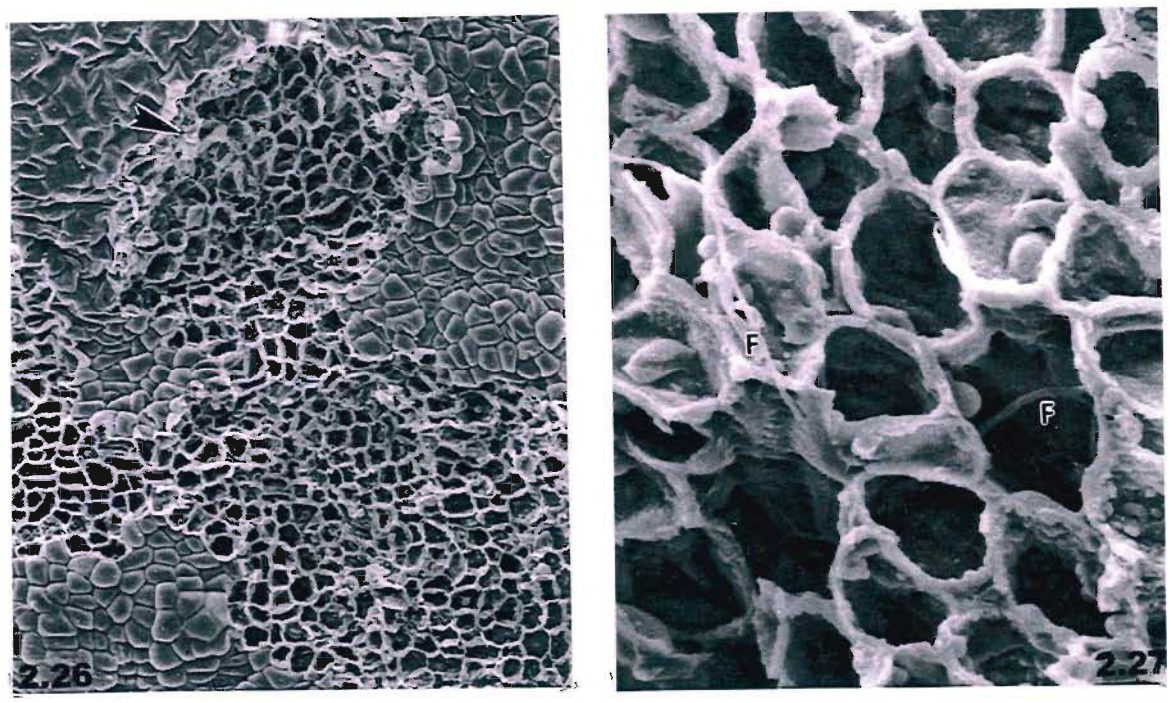
Figs. 2.18 - 2.21. **Scanning electron micrographs of *A. marina* seeds stored without pericarps for 6 days**. The abundant fungal hyphae observed on the surface of the cotyledons (Fig. 2.18 [x 200]) penetrated the internal tissues through salt glands and stomata (Fig. 2.19 [x 300]). Possible localised necrotic areas (arrowhead) were observed on the surface of cotyledons (Fig. 2.19). Some necrosis was also evident on the surface of infected embryonic axes that were infected with microflora (Fig. 2.20 [x 300]). Fungal structures were present inside embryonic axis tissues, growing below the epidermis (arrows). It appeared that penetration of these tissues occurred at the bases of bristles (Fig. 2.21 [x 500]).*

* B, bristle



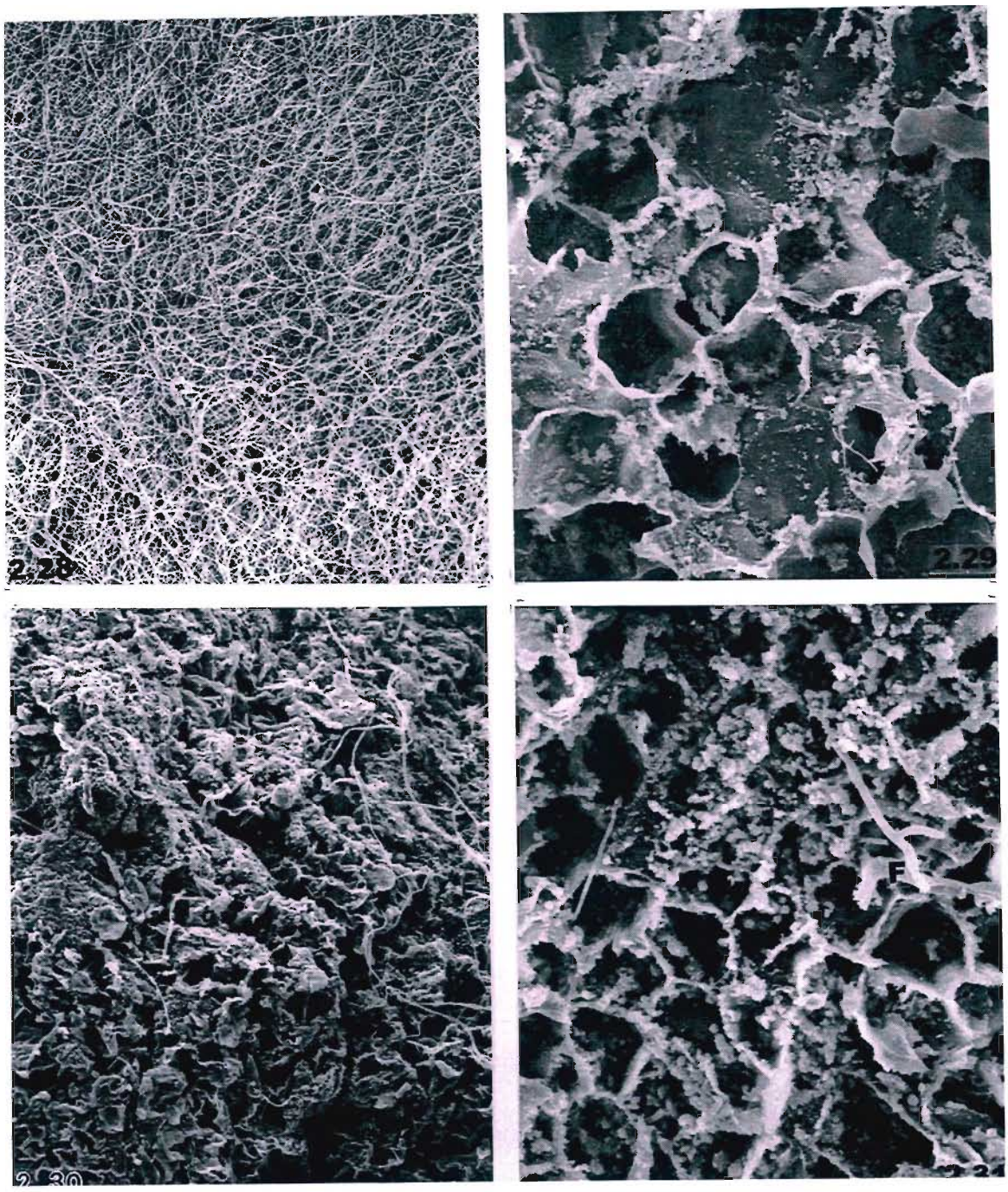
Figs. 2.22 - 2.25. **Scanning electron micrograph of *A. marina* seeds wet-stored with pericarps for more than 2 weeks**. Tissue necrosis (arrowhead) was associated with fungal hyphae on the inner surface of the pericarp of seeds stored for 21 days (Fig. 2.22 [x 200]). In seeds stored for 16 days, a region of localised necrosis is shown on the cotyledonary surface, where hyphae can be seen apparently penetrating salt glands and stomata (Fig. 2.23 [x 300]). Advanced necrosis (arrowhead) was evident on the surface (Fig. 2.24 [x 120]) and within the cotyledons of seeds stored for 21 days (Fig. 2.25 [x 300]).*

* Sg, salt gland; St, stomata



Figs. 2.26 - 2.27. **Scanning electron micrographs of embryonic axes of *A. marina* seeds wet-stored with pericarps for more than 2 weeks.** Advanced necrosis was observed on the surfaces of embryonic axes (Fig. 2.26 [x 200]) and fungal structures were also observed within the tissues (Fig. 2.27 [x 1100]).*

* F, fungal structure



Figs. 2.28 - 2.31. **Scanning electron micrographs of *A. marina* seeds wet-stored without pericarps for more than 2 weeks**. Fungal proliferation (Fig. 2.28 [x 120]) and internal tissue necrosis (Fig. 2.29 [x 600]) were observed on and in cotyledons. The situation was similar for embryonic axis surfaces (Fig. 2.30 [x 250]) and internal tissues (Fig. 2.31 [x 800]).

2c.2 A study of the manipulation of stored seeds in relation to fungal contamination: Experiment 2 – manipulation

Avicennia marina seeds were assessed for germination, fungal contamination and their susceptibility to fungal contamination. In addition, a parallel transmission electron microscopical study was carried out on all seed samples.

2c.2.1 Germination assessment

The germination achieved by newly harvested; clean; infected; and clean stored – infected seeds is shown in Table 2.3. Although detailed data are presented in this table, only the final germination percentage (germination achieved after 12 days) is presently analysed.

All fresh seeds were germinable. Similarly, the seeds stored clean for four (4d clean) and seven days (7d clean) displayed complete germination. In contrast, the seeds that were infected with *F. moniliforme* (infected, Block 1, Table 2.3), showed an abrupt decline in germination, all seeds having zero germinability by the seventh day of hydrated storage. The germination of the seeds that were kept clean in storage decreased to 70% after 21 days of wet storage. These data clearly indicate that fungal contamination was a major contribution to the loss of viability of stored hydrated seeds of *A. marina*. Therefore, it is currently suggested that if the associated mycoflora (external and internal) of stored seeds is maintained at a minimal level, these (and probably other) highly recalcitrant seeds could perhaps be successfully stored for extended, although still not long periods.

To determine whether the seeds become more susceptible to fungal invasion and/or to fungal-induced degradation as they become debilitated in storage, seeds were stored clean for various periods of time, infected with *F. moniliforme* and then re-stored (clean stored – infected seeds). Analysis of the germination

percentages indicates that the seeds stored clean for four days and then infected and re-stored (4d-4 to 4d-10) exhibited a substantially higher viability then those stored for longer periods, e.g. 14d-4 or 16d-4. It was evident that even after eight days from inoculation, seeds that were previously stored clean for four days (4d-8) showed 55% germinability while those inoculated after seven (7d-8) and ten days (10d-8) of clean storage had low germination percentages of 15% and 10%, respectively.

Although 30% of the seeds that were infected after four days of clean storage and stored for a further ten days (4d-10) germinated, none of the equivalent clean stored–infected seeds sampled later during storage (starting from the seventh day) was viable. These results indicate that seeds infected after four days of clean storage were more resistant to fungal degradation than those stored for longer periods. This could be explained by the possibility that the seeds stored clean produced some active defence mechanisms over the four days period that enabled them to minimise the fungal infection. In contrast, it appeared that further into the storage period, seeds lost their ability to counteract mycofloral infection. This could be a consequence of: 1) the postulated production of antifungal compounds (active defence mechanisms) declined during storage; and/or 2) the fungal infection was too aggressive for the already-sensitive stored hydrated recalcitrant seeds (by day 21 of wet storage, 70% of the seeds were viable).

Table 2.3. Germination of the hydrated *Avicennia marina* seeds wet-stored clean and experimentally inoculated with *Fusarium moniliforme*.

Treatment		Codes	Germination (%)			
Storage days prior to inoculation	Storage days following inoculation		Day 3	Day 6	Day 9	Day 12
Fresh	**0**	Fresh	30	55	75	100
	4	4d infected	10	10	40	55
	7	7d infected	0	0	0	0
	10	10d infected	0	0	0	0
	12	12d infected	0	0	0	0
4 days	**0**	4d clean	30	65	80	100
	4	4d-4	25	30	60	70
	6	4d-6	25	30	60	60
	8	4d-8	10	25	55	55
	10	4d-10	10	20	30	30
	12	4d-12	-	-	-	-
7days	**0**	7d clean	35	60	85	100
	4	7d-4	25	35	55	55
	6	7d-6	20	30	45	45
	8	7d-8	0	15	15	15
	10	7d-10	0	0	0	0
	12	7d-12	0	0	0	0
10 days	**0**	10d clean	30	55	85	95
	4	10d-4	20	45	60	65
	6	10d-6	0	30	30	30
	8	10d-8	0	10	10	10
	10	10d-10	0	0	0	0
	12	10d-12	0	0	0	0
12 days	**0**	12d clean	35	60	70	95
	4	12d-4	25	45	50	50
	6	12d-6	0	10	10	10
	8	12d-8	0	0	0	0
	10	12d-10	0	0	0	0
	12	12d-12	0	0	0	0
14 days	**0**	14d clean	30	55	70	95
	4	14d-4	0	0	0	30
	6	14d-6	0	0	0	0
	8	14d-8	0	0	0	0
	10	14d-10	0	0	0	0
	12	14d-12	0	0	0	0
16days	**0**	16d clean	25	55	60	85
	4	16d-4	0	0	0	0
	6	16d-6	0	0	0	0
	8	16d-8	0	0	0	0
	10	16d-10	0	0	0	0
	12	16d-12	0	0	0	0
21 days	**0**	21d clean	35	50	65	70
	4	21d-4	0	0	0	0
	6	21d-6	0	0	0	0
	8	21d-8	0	0	0	0
	10	21d-10	0	0	0	0
	12	21d-12	0	0	0	0

2c.2.2 Fungal contamination

The results of this study are depicted in Table 2.4. Newly harvested seeds harboured a variety of microorganisms on the surface of the embryonic axis and of cotyledons. No contaminants were isolated from internal cotyledonary tissues. Of the genera isolated, *Fusarium* spp. were singled out as representing field fungi. In total, four fungal species were isolated from the surface of embryonic axes and six from cotyledons. Bacterial contamination was also evident, with *Pseudomonas* sp. being isolated from surfaces of fresh cotyledons.

After four days of clean hydrated storage (4d clean), no fungal or bacterial species were evident on/in seed tissues. Further into storage, embryonic axes of clean seeds remained fungal-free throughout the 21-day storage period. While *F. moniliforme* was the predominant fungus on the surface of the cotyledons, no fungal contaminants were found internally, with the exception of cotyledons of seeds stored clean for 21 days (21d clean).

Although *A. marina* seeds were experimentally infected with *F. moniliforme*, it appeared that *A. niger* was also present on embryonic axes of infected seeds stored for four days (4d infected). Thereafter, however (to 12 days of hydrated storage), all samples of the infected seeds exhibited *F. moniliforme* as the only persisting fungal species.

The clean stored – *F. moniliforme*-infected seeds (seeds that were maintained clean for various periods of storage, inoculated and re-stored) harboured this fungus as the dominant and, in most instances, the only external and internal contaminant. There were some exceptions, however: *Trichoderma harzianum* Rifai was isolated from the surface of the cotyledons, six days following the inoculation of the seeds stored clean for four days (4d-6).

Table 2.4. Microbial contamination of hydrated stored seeds of *A. marina*.

Treatment		Codes	Seed tissues		
Storage days prior to inoculation	Storage days following inoculation		Embryonic axis	Cotyledons (* = % of seeds infected) Surface	Internal
Fresh	**0**	Fresh	*Aspergillus niger* *Penicillium glandicola* *Penicillium crustosum* *Fusarium moniliforme*	*Fusarium semitectum (65)** *Curvularia brachyspora* *Aspergillus niger* *Penicillium glandicola* *Penicillium crustosum* *Fusarium moniliforme* *Pseudomonas* sp.	-
	4	4d infected	*A. niger* *F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	7	7d infected	*F. moniliforme*		*F. moniliforme*
	10	10d infected	*F. moniliforme*		*F. moniliforme*
	12	12d infected	*F. moniliforme*		*F. moniliforme*
4 days	**0**	4d clean	-	-	-
	4	4d-4	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	6	4d-6	*F. moniliforme*	*F. moniliforme* *Trichoderma harzianum*	*F. moniliforme*
	8	4d-8		*F. moniliforme*	*F. moniliforme*
	10	4d-10		*F. moniliforme*	*F. moniliforme*
7 days	**0**	7d clean	-	*F. moniliforme (10%)*	-
	4	7d-4	*P. crustosum* *F. moniliforme*	*P. crustosum* *F. moniliforme* *P. glandicola* *F. semitectum*	*F. moniliforme*
	6	7d-6	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	8	7d-8	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	10	7d-10	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	12	7d-12	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
10 days	**0**	10d clean	-	*F. moniliforme (10%)*	-
	4	10d-4	*F. semitectum* *F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	6	10d-6	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	8	10d-8	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	10	10d-10	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	12	10d-12	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
12 days	**0**	12d clean	-	*F. moniliforme (15%)* *P. glandicola*	-
	4	12d-4	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	6	12d-6	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	8	12d-8	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	10	12d-10	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	12	12d-12	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
14 days	**0**	14d clean	-	*F. semitecum (15%)*	-
	4	14d-4	*F. moniliforme* *F. semitecum*	*F. moniliforme* *F. semitectum*	*F. moniliforme*
	6	14d-6	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	8	14d-8	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	10	14d-10	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	12	14d-12	*F. moniliforme*	*F. moniliforme* *P. glandicola*	*F. moniliforme*
16 days	**0**	16d clean	-	*F. moniliforme (20%)*	-
	4	16d-4	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	6	16d-6	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	8	16d-8	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	10	16d-10	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	12	16d-12	*F. moniliforme*	*F. moniliforme* *F. semitectum*	*F. moniliforme*
21 days	**0**	21d clean	-	*F. moniliforme (30%)*	*F. moniliforme*
	4	21d-4	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	6	21d-6	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	8	21d-8	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	10	21d-10	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*
	12	21d-12	*F. moniliforme*	*F. moniliforme*	*F. moniliforme*

Additionally, *Penicillium crustosum* Thom was identified from the surface of the embryonic axes and cotyledons four days after inoculation of the seven-day clean seeds (7d-4). *Fusarium semitectum* Berk. & Rav. was also isolated throughout storage, notably, on the fourth day after inoculation, on the surface of cotyledons of both 12 (12d-4) and 14-days clean stored seeds (14d-4). This fungus was also present on the axis of the 14-days clean stored seeds, sampled after four days from the experimental infection (14d-4).

2c.2.3 Transmission electron microscopy (TEM)

The present study was undertaken on fresh and clean hydrated-stored and clean stored–infected seeds of *A. marina* with microscopical examination being focused on the root primodia of the embryonic axis and on the surface of the cotyledonary tissue. These were areas that generally showed fungal proliferation first during storage of *A. marina* seeds.

The meristematic root primordium cells of freshly harvested *A. marina* seeds were characterised by an appearance of relatively low intracellular activity as suggested by sparse endoplasmic reticulum and the absence of Golgi bodies (100% germination) (Fig. 2.32). The compact cells contained large nuclei with prominent nucleoli. Mitochondria had limited cristal development and a matrix characterised by patchy electron transparent areas (Fig. 2.33). The plastids present in the cytomatrix had no accumulated starch but contained densely staining granular material (Fig. 2.33).

The cells of cotyledons exhibited a cytomatrix that was restricted to a narrow peripheral band by the large central vacuole (Fig. 2.34). The plastids contained large reserves of starch (Fig. 2.35) and nuclei and relatively well-developed mitochondria were also evident (Fig. 2.36).

After four days of clean hydrated storage (4d clean) (100% germination), the meristematic cells of the root primodia showed an ultrastructural condition indicative of some enhancement of intracellular activity accompanied by a very small degree of vacuolation (Fig. 2.37). While the nuclei maintained their shape, an increase in the development of the organelles, particularly mitochondria, that contained well-defined cristae and in the occurrence of Golgi bodies (Figs. 2.38 and 2.39). In addition, plastids maintained the same appearance as the fresh material (Fig. 2.38) and short strands of endoplasmic reticulum were present (Fig. 2.39).

No fungal structures were observed on the surface of the cotyledons of the seeds stored clean for four days (4d clean) (Fig. 2.40), the ultrastructure of which was essentially similar to that of fresh material. Inner membranes of plastids had developed strongly, all showing grana and stroma lamellae typical of functional chloroplasts (Fig. 2.41). There appeared to have been some diminution of starch grain volume compared with the newly-shed state (Fig. 2.35).

In contrast to four days clean seeds (4d clean), the ultrastructural condition of meristematic cells of the root primodia of seeds stored infected for four days (4d infected) (55% germinability) showed some signs of enhanced metabolic activity, but also evidence of incipient deterioration (Figs. 2.42-2.44). There was an abnormal degree of vacuolation (Fig. 2.42), accompanied by development of long ER profiles, indicative of development of the endomembrane system (Fig. 2.44). The mitochondrial cristae were well developed, but had assumed a bulbous appearance compared with those of equivalent 4d clean material, and the matrices of these organelles were generally more evenly dense (Figs. 2.43 and 2.38). Golgi bodies with associated vesicles and polysomes were prominent (Fig. 2.44). There was no visible ultrastructural evidence of nuclear envelope or plasma membrane abnormality (Fig. 2.42). Generally, a high level of protein synthesis was indicated by the abundance of polysomes (Fig. 2.44).

An interesting aspect of the ultrastructure of this tissue was the occasional presence of lipid bodies (Figs. 2.42 and 2.43), which are an unusual feature in root primodial cells of *A. marina*.

Although the agar study demonstrated fungal infection, the surface of the cotyledonary tissue analysed did not present evidence of such structures (Fig. 2.45). However, the cells of the cotyledonary tissue of seeds stored infected for four days (4d infected) revealed ultrastructural changes. Although no obvious membrane damage was evident, it was apparent that the cytoplasm had become more dense and contained darkly-stained, short strands of endoplasmic reticulum; however, the compact mitochondria with well defined cristae appeared unaffected (Fig. 2.45). Vacuoles with occasional membranous inclusions were also observed (Fig. 2.45) in the cotyledonary cells, which had retained well-developed chloroplasts (Fig. 2.46).

When analysing the ultructructural evidence of the root primordium meristematic cells of the seeds stored clean for four days, infected and re-stored for four days (4d-4) (70% germination) (Figs. 2.47-2.49), it was apparent that these tissues were generally comparable with those of seeds stored clean for four days (4d clean) (Figs. 2.37-2.39). A similar degree of vacuolation and of organelle development was observed (Fig. 2.47). In contrast to the cells of four-day stored clean seeds (4d clean), however, the nuclei of some cells of the 4d-4 tissue showed considerable nuclear lobing (Fig. 2.48), indicating possible nucleo-skeleton and/or microfilament derangement; occasional condensed intra-nuclear material, possibly chromatin was seen (Fig. 2.47). Some vacuolar inclusions were observed, as illustrated in Figures 2.48 and 2.49. Similar to the four-day clean treatment (4d clean), the endoplasmic reticulum was present in relatively short strands and vesicles were associated with Golgi bodies (Fig. 2.49). Some mitochondria were characterised by well-defined cristae while others had electron transparent regions and abnormal inner membrane formations (Figs. 2.48 and 2.49) and plastids were observed to contain darkly-

stained material (Fig. 2.48). In contrast to seeds clean stored for four days (4d clean), but similar to those stored infected for the same period (4d infected), occasional lipid bodies were evident (Fig. 2.49).

Although at day four after infection of seeds that had previously been clean-stored for four days (4d-4) fungal structures were present on the surface of the cotyledons (Fig. 2.50), the ultrastructure of the cotyledonary tissue remained similar to that of seeds stored clean for four days (4d clean) (Figs. 2.40 and 2.41). However, there was some depletion of starch reserves in the chloroplasts (Fig. 2.51).

At eight days following the infection of clean seeds that were stored for four days (4d-8) (55% germination), the subcellular organisation of the root primordium meristematic cells provided evidence of ongoing intracellular activity (Fig. 2.52), although some small degree of abnormality persisted, such as irregularity of the nuclear profile (Fig. 2.52). While some mitochondria showed a dense matrix (Fig. 2.52), others had prominent electron transparent matrical regions (Fig. 2.53). The degree of vacuolation was not very different from root primidium cells of seeds stored clean for four days (4d clean) (Fig. 2.52). The plastids had maintained a dense appearance (Fig. 2.52), but in some cases contained electron transparent regions, indicating a degree of dispersal of this (unidentified) material, which normally occurs as a post-harvest event (Figs. 2.53 and 2.58).

Some areas of the surface of the cotyledons of seeds sampled eight days following infection after four days clean storage (4d-8) harboured fungal structures (Fig. 2.54), and degradation was observed in the tissues underlying these areas (Fig. 2.55). The cotyledon tissues with no evident fungal infection showed ultrustructure indicative of ongoing intracellular metabolic activity (Fig. 2.56), although one notable abnormality was that of chloroplasts that developed compressed, darkly-stained stroma regions (Fig. 2.56).

After seven days of clean, hydrated storage (7d clean) (100% germination), the meristematic root primordium cells contained dense mitochondria that showed well-characterised crista development (Figs. 2.57 and 2.58). The endoplasmic reticulum appeared as short profiles and the Golgi bodies exhibited well-stacked cisternae and associated vesicles (Figs. 2.57 and 2.58). It appeared that although many plastids retained their dense content, some developed starch grains (Fig. 2.57). In other cases, the plastidial dense material had largely disappeared, facilitating visualisation of internal features, including a degree of inner membrane formation (Fig. 2.58).

No fungal presence could be found on the surface of the cotyledons of seeds stored clean for seven days (7d clean) (Fig. 2.59). The ultrastructural analysis of these tissues revealed an increased intracellular activity with strong development of organelles, exemplified by mitochondria with particularly well-defined cristae (Fig. 2.60), the occurrence of polysomes and the presence of closely-associated profiles of endoplasmic reticulum (Fig. 2.61).

In contrast to seeds stored clean for seven days (7d clean), the ultrustructure of the meristematic primordium of embryonic axes of the seeds stored infected for seven days (7d infected) showed an advanced degree of degradation (0% germination) (Figs. 2.62-2.65). It was apparent that while some areas of the embryonic axis had groups of extensively damaged cells, indicating tissue necrosis (Fig. 2.62), other cells had apparently maintained overall integrity, but showed several signs of aberration (Fig. 2.63). These cells had developed an extensive degree of vacuolation, with many of the vacuoles becoming confluent and thus forming larger compartments. In addition, nuclear lobing was also evident (indicating perhaps some abnormality in the nuclear skeleton and/or at microfilament level) and mitochondria had lost the internal organisation, electron transparent characterising the matrices of these organelles (Fig. 2.63). Long profiles of endoplasmic reticulum were present peripherically in the cytoplasm (Fig. 2.64) and many Golgi bodies were present, having numerous associated

vesicles (Fig. 2.65). As for the tissues of the four day infected seeds (4d infected; Figs. 2.42 and 2.43) and those of seeds infected at four days of clean storage and sampled four days following infection (4d-4) (Fig. 2.49) lipid bodies were also noticed (Fig. 2.63).

The cotyledonary tissues of *A. marina* seeds stored infected for seven days (7d infected) exhibited an abundance of fungal structures (Figs. 2.66 and 2.67). Deterioration of cell walls, with cytoplasmic derangement and dark staining of the tissues was observed and the cells underlying the fungal structures showed complete breakdown (Figs. 2.66 and 2.67).

Meristematic cells of embryonic axes of *A. marina* seeds stored clean for ten days (10d clean) (95% germination) showed little sign of abnormality (Figs. 2.68-2.70). With some exceptions, the cells of this tissue remained essentially similar to those of the seeds clean-stored for seven days (7d clean) (see Figs. 2.57 and 2.58). A small degree of vacuolation and some intra-nuclear material (similar to that seen in some cells of 4d-4 material [Fig. 2.47]) were observed (Fig. 2.68). Signs of intracellular activity were demonstrated by the presence of Golgi bodies (Fig. 2.69) and longer profiles of endoplasmic reticulum (Fig. 2.68). While mitochondria with well-defined cristae had developed matrical electron-transparent areas (Figs. 2.68 and 2.69), the plastids (some still retaining dense material, and some not) exhibited sizeable starch grains (Figs. 2.69 and 2.70). In addition, the presence of lipid droplets was noted (Figs. 2.68 and 2.69).

The ultrastructure of the cotyledonary tissue of the seeds stored clean for ten days (10d clean) revealed highly differentiated and metabolically active cells comprising the mesophyll (Figs. 2.71 and 2.72). Both chloroplasts and mitochondria were exceptionally well-developed, with the latter showing many cristae and a dense matrix, typical of a high respiratory rate.

Micrographs of the axis tissues of *Avicennia* stored infected for ten days (10d infected) (0% germination), showed that a marked general deterioration had occurred. In addition to large confluency of vacuoles, an almost amorphous cytoplasm was observed (Fig.2.73). While almost complete loss of mitochondrial organisation and intra-nuclear definition had occurred, there was also degeneration of the plastids and the endomembrane system, the long endoplasmic reticulum profiles and the nuclear envelope appearing dilated (Fig. 2.74). Also, Golgi bodies were not in evidence.

The fungal infection of the cotyledons of the seeds stored infected for ten days (10d infected) demonstrated by the agar study was confirmed by the ultrastructural analysis. Fungal structures were associated externally with the cotyledonary tissues (Fig. 2.75) and internally. Intracellular cell degradation of the seed tissue was advanced (Fig. 2.76).

In contrast to the seeds stored clean for ten days (10d clean), seeds that were sampled four days following inoculation after ten days clean storage (10d-4) (65% germination) showed some ultrastructural changes. Meristematic cells of embryonic axes developed larger vacuoles, presumably by confluence of smaller compartments (Fig. 2.77). Although the nucleus remained apparently normal, some lobing had occurred and a general loss of the organisation of organelles was apparent (Figs. 2.77 and 2.78). Golgi bodies had dilated cisternae, and the short profiles of endoplasmic reticulum also appeared abnormal, sometimes exhibiting significant dilation (Fig. 2.78). Mitochondria with electron-transparent matrical regions and occasional abnormal inner membrane formations and plasmalemma withdrawal from the cell wall (Fig. 2.78) accentuated the signs of increased cell deterioration.

The above-mentioned symptoms of abnormality were more dramatic at the cotyledonary level, where advanced deterioration was observed (Figs. 2.79). Relatively normal chloroplasts were present, but the cytoplasm was abnormally

dense and contained organelles that had lost their internal organisation (Fig. 2.79).

In contrast to seeds that were stored clean for 10 days (10d clean), seeds of 10d-8 samples (10% germination) demonstrated marked degree of ultrastructural deterioration that included large vacuoles formed by fusion of individual vacuoles (Fig. 2.80), abnormal endoplasmic reticulum with markedly dilated cisternae (Figs. 2.80 and 2.81) and highly irregular nuclear profiles (Fig. 2.81). At the cotyledonary level, extensive cell deterioration was evident (Fig. 2.82).

After 12 days of clean storage (12d clean) (95% germination), an enhancement of the intracellular activity of meristematic cells of the embryonic axis was observed (Fig. 2.83). Cells that contained only small vacuoles, presented a metabolically active appearance, typified by a well-defined endomembrane system (Golgi bodies and short ER profiles) and elongated mitochondria. Some mitochondria, however, did show an occasional abnormal inner membrane formation (Fig. 2.83). Inner membranes were clearly visible within plastids; however, some nuclear profiles had become slightly irregular (Fig. 2.83).

The surface of the cotyledons of seeds stored clean for 12 days (12d clean) revealed no seed-associated mycoflora (Fig. 2.84). The highly metabolically active cells of this tissue contained extremely well-developed chloroplasts with highly organised internal membranes and starch grains, as well as mitochondria characterised by a dense matrix and prominent cristae (Fig. 2.84).

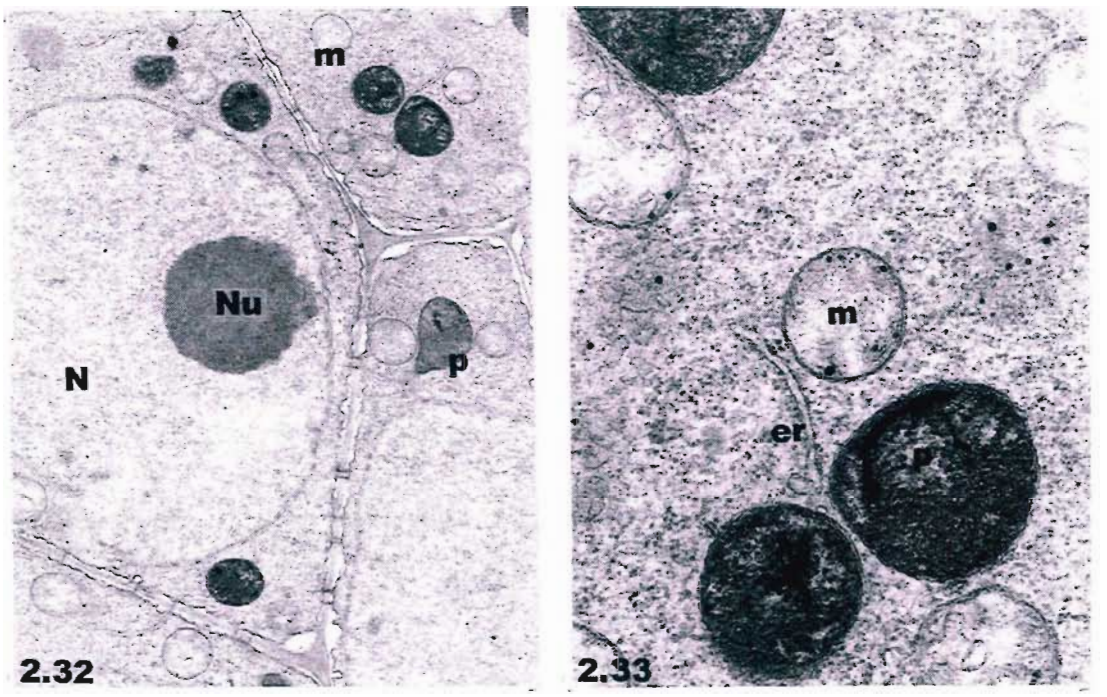
As expected, after 12 days of infected storage (12d infected) (0% germination), both the embryonic axes and the cotyledons of *A. marina* seeds were critically degraded, complete loss of internal organisation being observed in both tissue types (Figs. 2.85 and 2.86).

After 21 days of clean storage (21d clean) (70% germination), the general condition of the meristematic cells of the embryonic axes showed signs of incipient deterioration. The seeds where fungal infection could not be discerned had retained ultrastructural integrity but with some signs of abnormality (Fig. 2.87). The cells were more vacuolated than was the situation earlier during storage (for example in 10d clean seeds [Fig. 2.68]) and had mitochondria, some of which were markedly elongated (Fig. 2.88); however, other mitochondrial cross-sections revealed occasional abnormal membrane formations (Figs. 2.87). Long endoplasmic reticulum profiles characterised root primordium cells, but possible derangement of the nucleo-skeleton was indicated by nuclear lobing (Fig. 2.87). In contrast to axis cells of the apparently uninfected tissues, the equivalent cells of axes where fungi could be observed showed marked deterioration, with many membranous vacuolar inclusions (suggested to indicate autophagy of damaged intracellular components), vacuolar confluency and marked electron-transparency of mitochondrial matrices (Fig. 2.89).

Cells of cotyledons of seeds with no apparent associated mycoflora (Fig. 2.90) had maintained their integrity, with well-developed mitochondria although some showed occasional abnormal inner membrane formations (Fig. 2.91). The prominent starch grains that typified cotyledonary chloroplasts earlier during storage of clean seeds had been depleted (Fig. 2.91), indicating utilisation or loss of this reserve during the 21-day hydrated storage period. There were some signs of stress, however: the endoplasmic reticulum profiles appeared somewhat dilated and the cytoplasm was less compact than observed earlier during storage of clean seeds. In other seeds, however, fungal infection of cotyledons had occurred (Fig. 2.92) and advanced necrosis was noticed in some cells of the cotyledonary tissues of these seeds (Fig. 2.93). Densely-staining, small globules were contained within the vacuoles and it appeared that an amorphous denser material surrounded some intravacuolar fungal structures

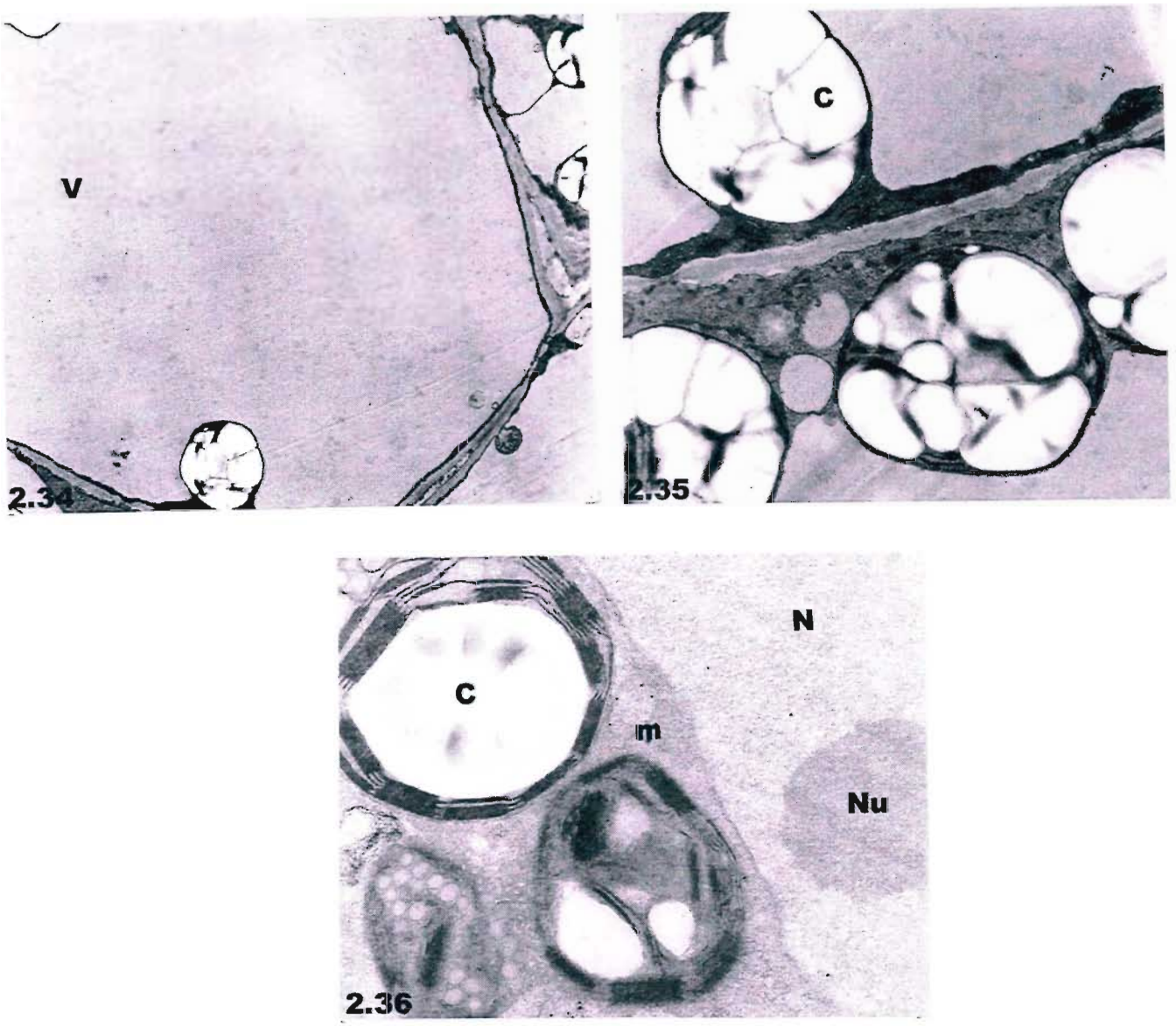
(Fig. 2.92) which could have been produced either by the fungus, as a protection mechanism or by the plant cell as a reaction to fungal presence.

When considering the infection process, it was apparent that *F. moniliforme* produced a fungal mat on the surface of the seed tissues from which penetration hyphae developed (Fig. 2.15). These hyphae penetrated the plant cell walls directly though the natural openings offered by stomata and salt glands (Figs. 2.15, 2.19 and 2.94). At the site of infection, some residue of some sort was observed (Figs. 2.23, 2.75, 2.94 and 2.95). This material could have been produced by the invading fungus, perhaps a manifestation of enzymes (i.e. the fungus produced enzymes or toxins facilitating the infection process) or by the seed in response to fungal infection. Subsequent to penetration, the fungal structures continued to colonise the seed tissues by intra- and intercellular growth (Fig. 2.96 and 2.97). Aggressive fungal growth was accompanied by cell and tissue necrosis (Fig. 2.98).



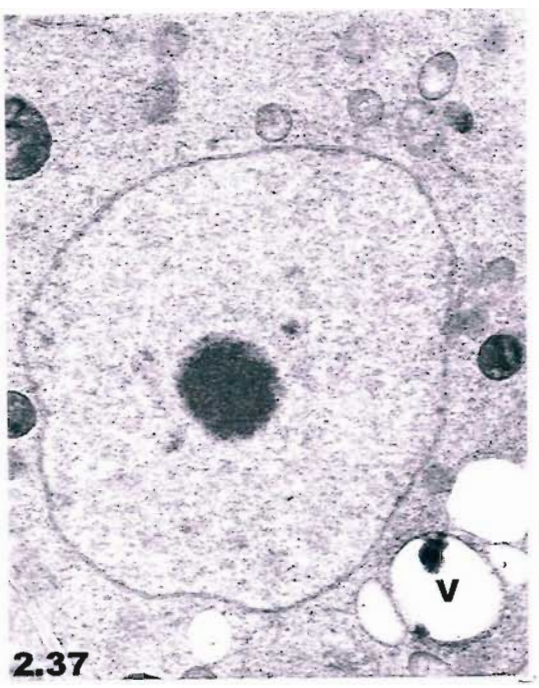
Figs. 2.32 - 2.33. **The ultrastructure of tissues of newly harvested seeds of *A. marina*.** Meristematic root primordium cells showed large nuclei with prominent nucleoli (Fig. 2.32 [x 8000]) and spherical mitochondria with limited crista development and electron-transparent regions (Fig 2.32 and 2.33 [x 25 000]). Plastids were typically electron-dense and short scattered profiles of endoplasmic reticulum occurred (Fig. 2.33).*

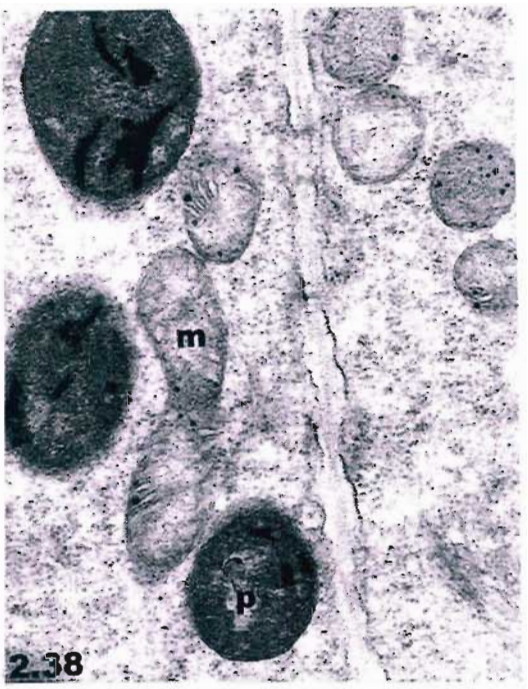
* er, endoplasmic reticulum; N, nucleus; Nu, nucleolus; m, mitochondrion; p, plastid

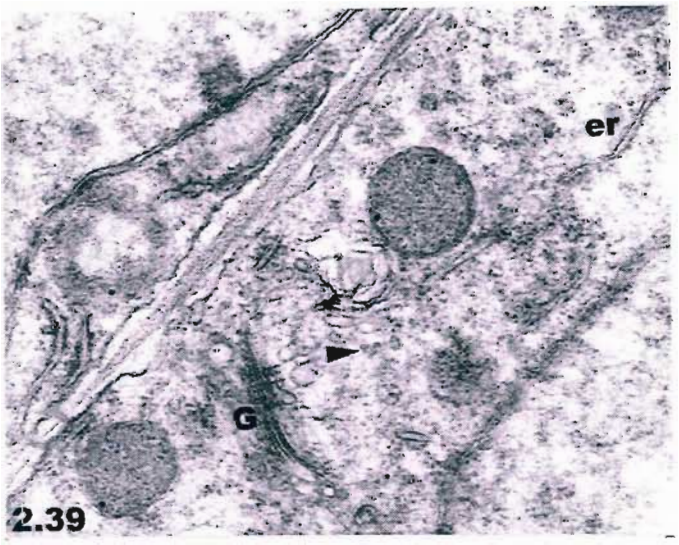


Figs. 2.34 - 2.36. **The ultrastructure of cotyledon tissues of newly harvested seeds of *A. marina*.** Cells were highly vacuolated (Fig. 2.34 [x 2000]) with starch-containing chloroplasts showing highly organised internal membranes (Figs 2.35 [x 10 000] and 2.36 [x 12 000]). These cells also contained prominent nuclei and well developed mitochondria (Fig. 2.36).*

* C, chloroplast; m, mitochondrion; N, nucleus; Nu, nucleolus; V, vacuole

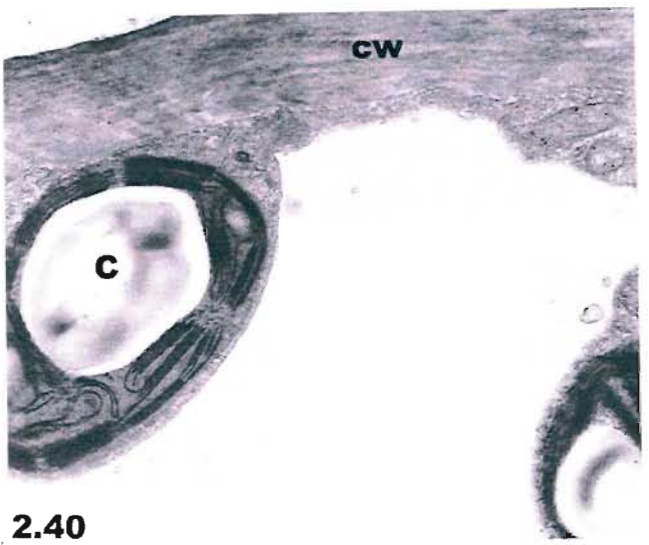






Figs. 2.37 - 2.39. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 4 days (4d clean)**. Small vacuoles had formed in the root primordium meristematic cells (Fig. 2.37 [x 8000]). Cells were characterised by well-developed mitochondria and dense plastids that were similar to those of fresh material (Fig. 2.38 [x 20 000]). Relatively short profiles of endoplasmic reticulum, polysomes (arrowhead) as well as Golgi bodies were prominent (Fig. 2.39 [x 25 000]).*

* er, endoplasmic reticulum; G, Golgi body; m, mitochondrion; p, plastid; V, vacuole



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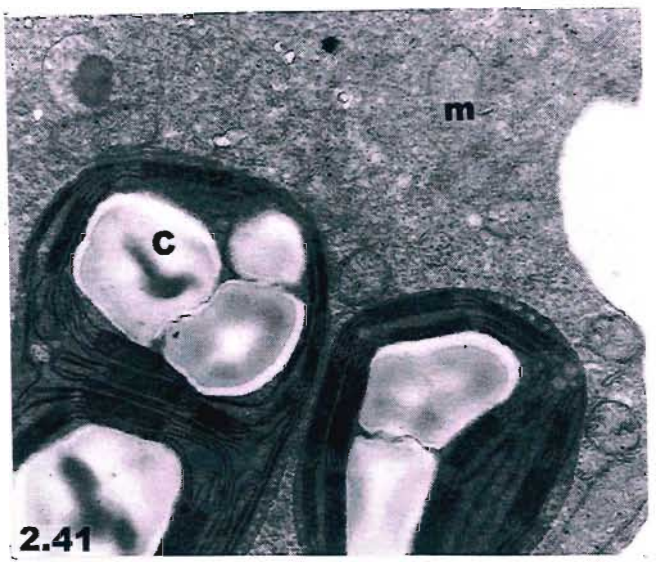

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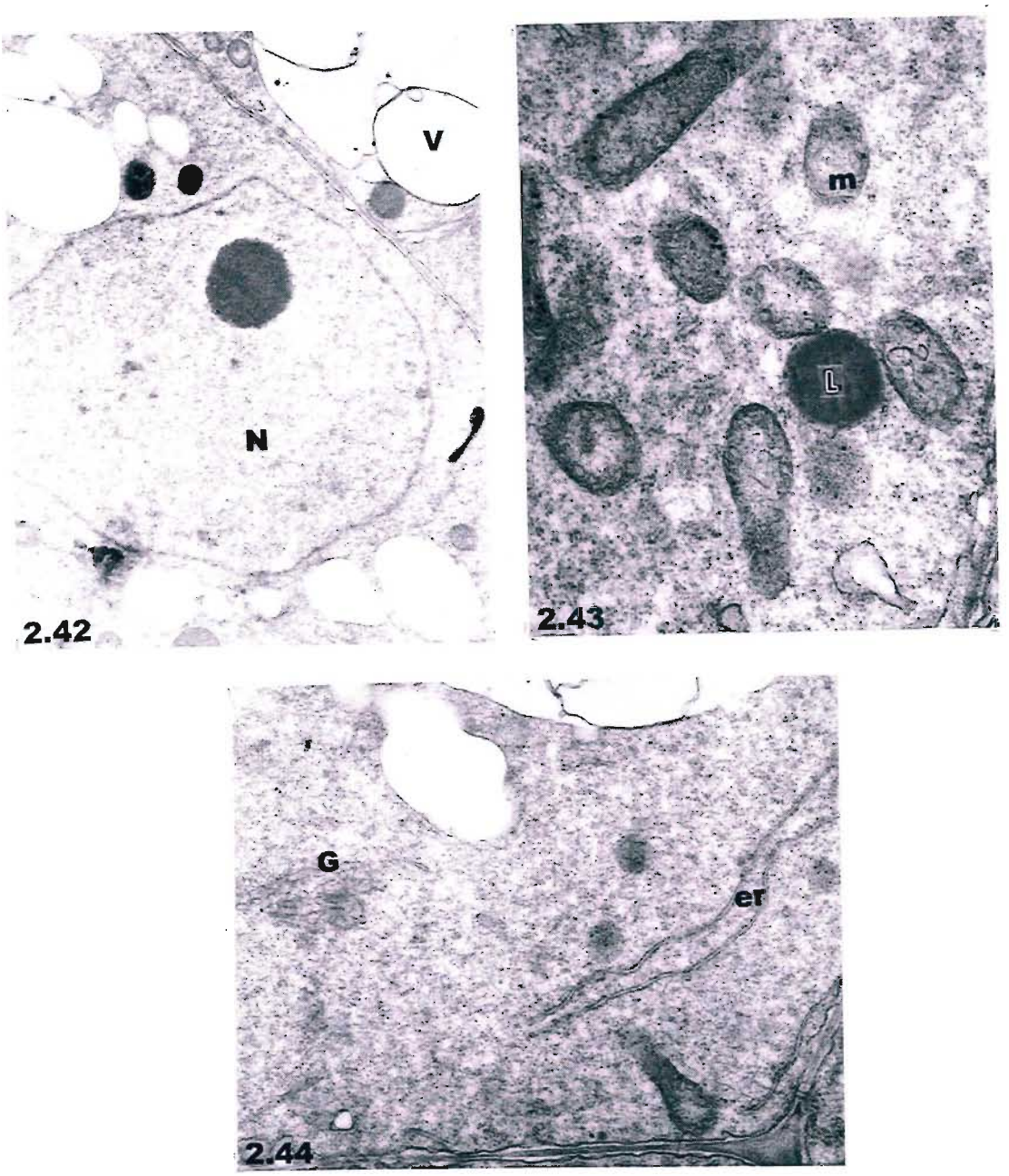
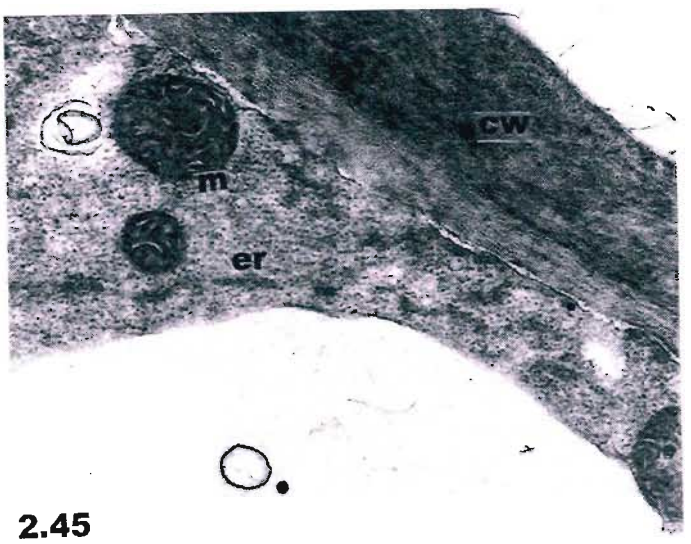
Figs. 2.40 - 2.41. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 4 days (4d clean)**. Cotyledon cells maintained a high level of organisation not visibly different from that characterising the newly shed condition, with mitochondria and chloroplasts being well developed (Fig. 2.41 [x 15 000]). No fungal structures were observed on the cotyledonary surface (Fig. 2.40 [x 10 000]).*

* C, chloroplast; cw, cell wall; m, mitochondrion



Figs. 2.42 - 2.44. **The ultrastructure of tissues of seeds of *A. marina* stored infected for 4 days (4d infected)**. Meristematic cells of root primordia showed a high degree of vacuolation and contained nuclei with apparently intact nuclear envelopes (Fig. 2.42 [x 6000]). Mitochondria had well-defined, but somewhat bulbous cristae and occasional contiguous lipid bodies were observed (Fig. 2.43 [x 2000]). Long profiles of endoplasmic reticulum were evident and frequent Golgi bodies and polysomes occurred (Fig. 2.44 [x 12 000]).*

* er, endoplasmic reticulum; G, Golgi body; L, lipid body; m, mitochondria; N, nucleus; V, vacuole



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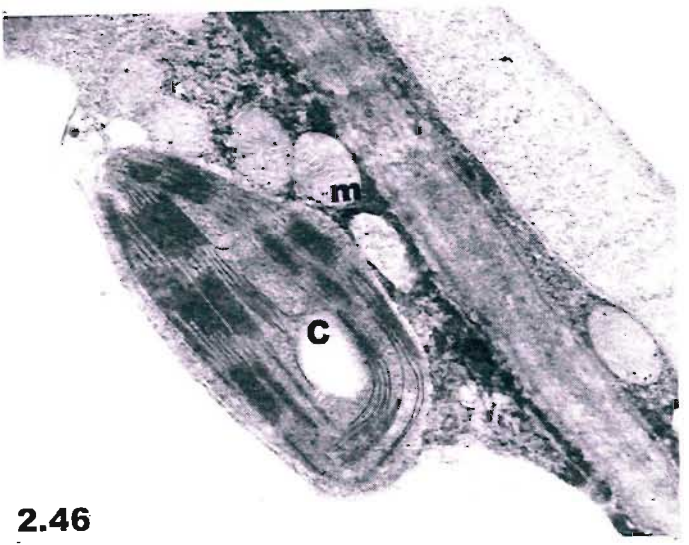
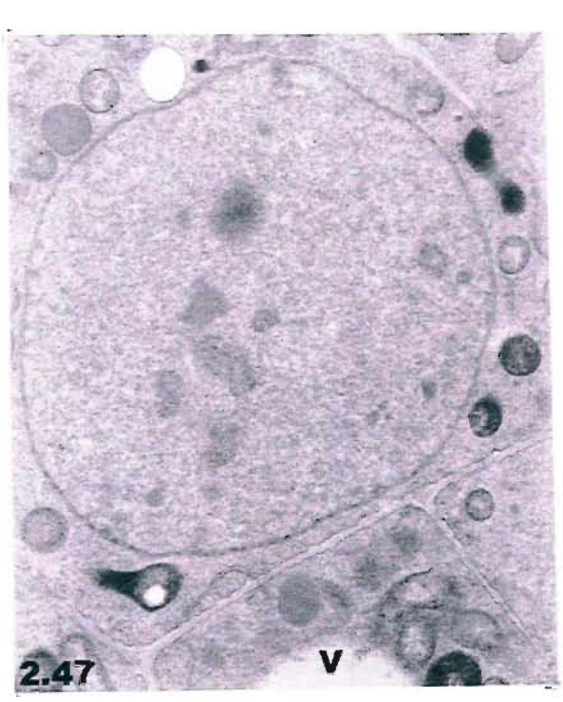
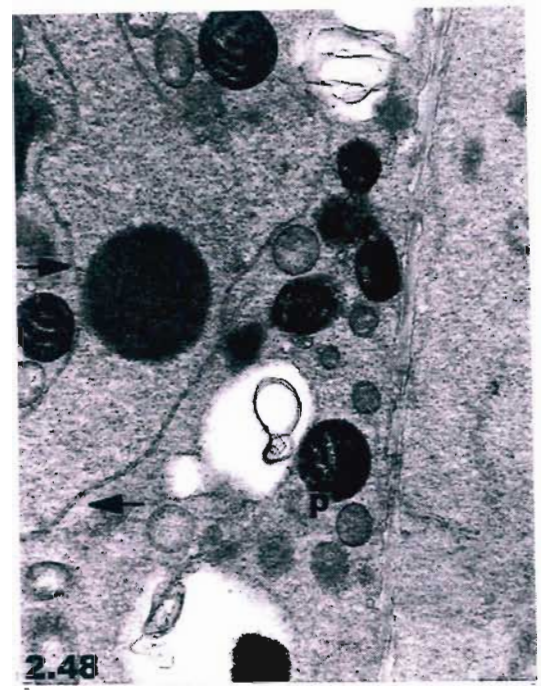


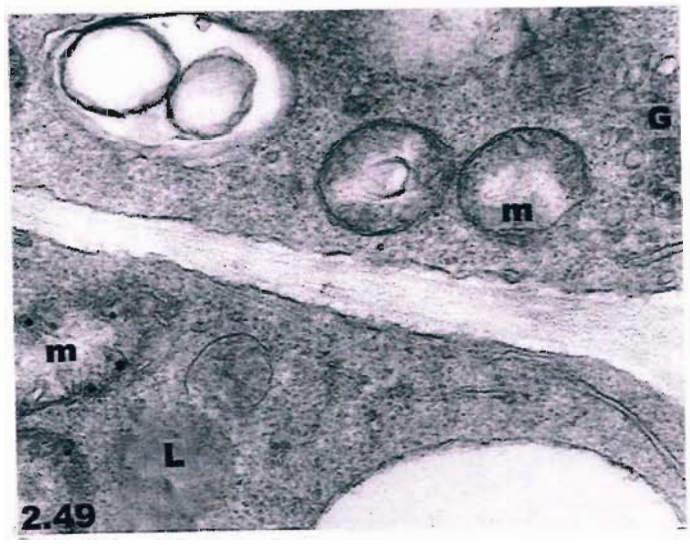
2.46

Figs. 2.45 - 2.46. **The ultrastructure of tissues of seeds of *A. marina* stored infected for 4 days (4d infected).** No fungal structures were evident at cotyledonary level. Cotyledonary cells contained dense mitochondria with well-defined cristae, darkly-staining short strands of endoplasmic reticulum (Fig. 2.45 [x 14 000]) and well-developed chloroplasts (Fig. 2.46 [x 6000]). A few membrane inclusions were evident within vacuoles (Fig. 2.45).*

* C, chloroplast; cw, cell wall; er, endoplasmic reticulum; m, mitochondrion

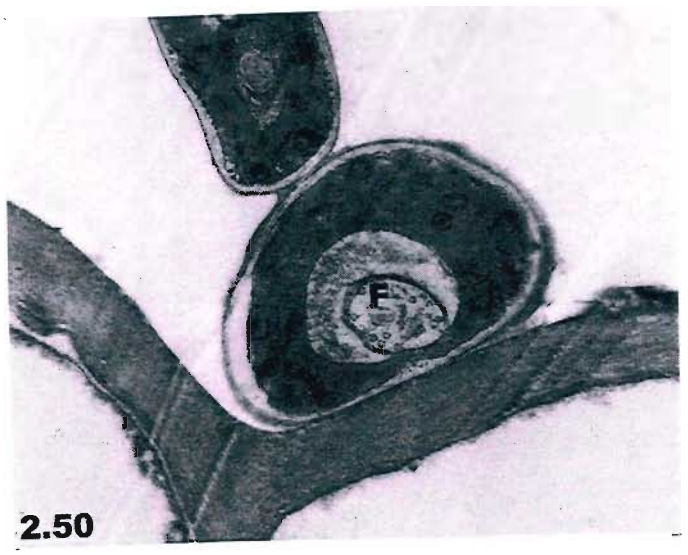


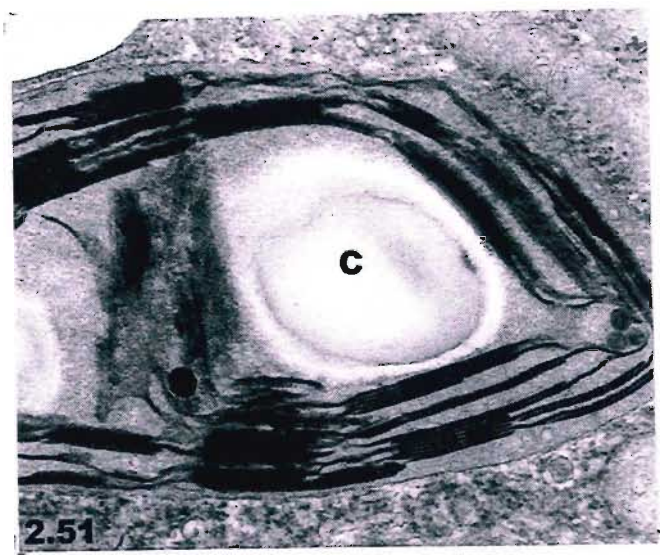




Figs. 2.47 - 2.49. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 4 days, inoculated and re-stored for a further 4 days (4d-4)**. Meristematic cells of the root primordium were not visibly much different from the 4d clean seeds (Fig. 2.47 [x 8000]). They were characterised by a small degree of vacuolation and nuclei that displayed some intra-nuclear material, possibly chromatin (Fig. 2.47). Some degree of nuclear lobing and vacuolar inclusions were evident in some cells (arrows) (Fig. 2.48 [x 8000]). Endoplasmic reticulum and Golgi bodies were evident and while some mitochondria showed cristae, others exhibited abnormal internal membrane formations and electron transparent regions (Fig. 2.49 [x 30 000]). Plastids contained darkely-stained material (Fig. 2.48) and the occasional presence of lipid bodies was observed (Fig. 2.49]).*

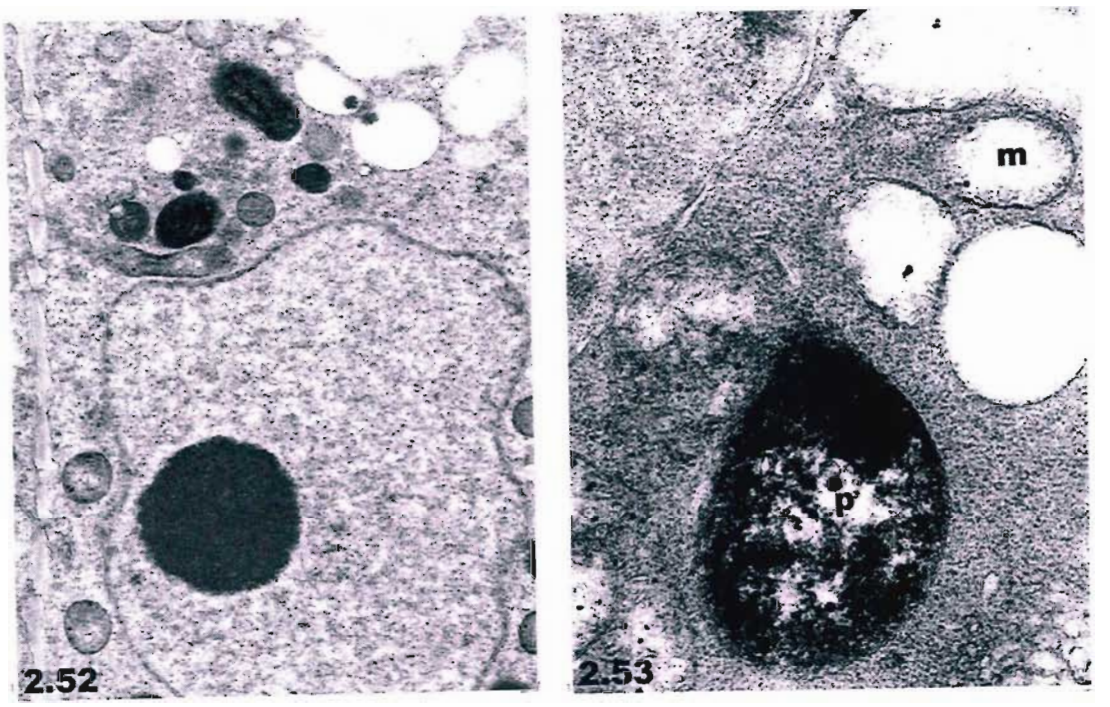
* G, Golgi body; L, lipid body; m, mitochondrion; p, plastid; V, vacuole





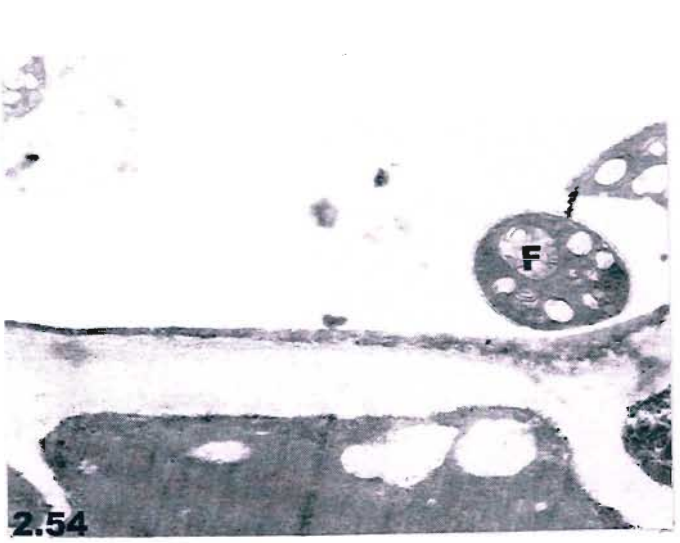
Figs. 2.50 - 2.51. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 4 days, inoculated and re-stored for a further 4 days (4d-4).** Fungal structures were present on the surface of cotyledons (Fig. 2.50 [x 8000]) that had nevertheless retained apparently normal ultrastructure. Some starch depletion was observed in chloroplasts (Fig. 2.51 [x 2000]).*

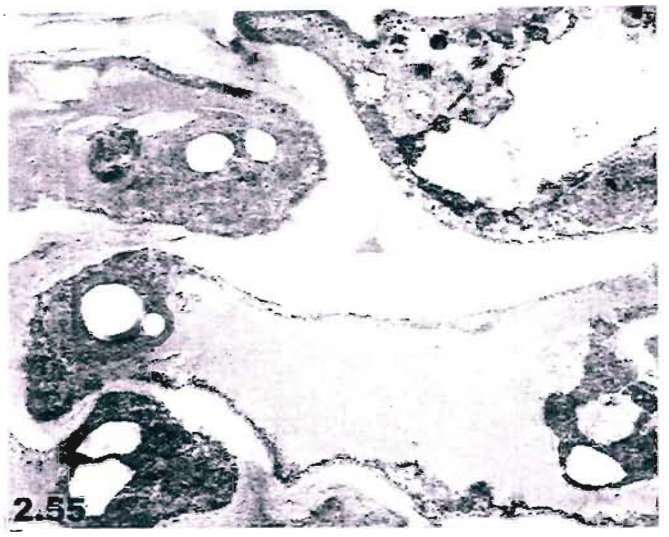
* C, chloroplast; F, fungal structure

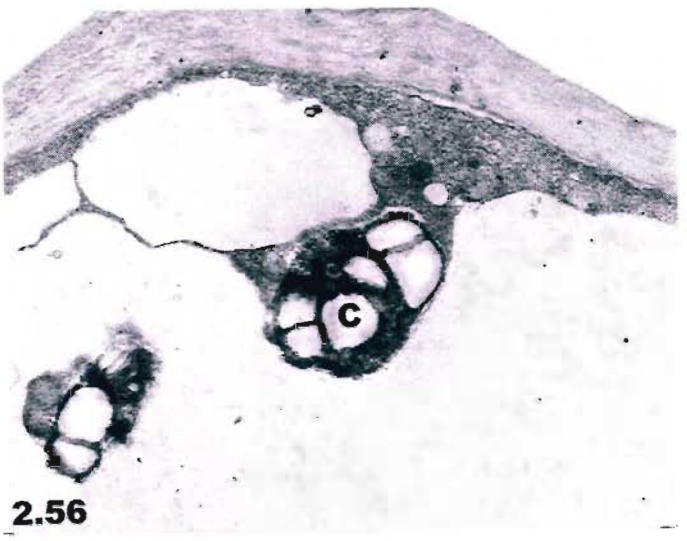


Figs. 2.52 - 2.53. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 4 days, inoculated and re-stored for a further 8 days (4d-8).** The organisation of the root primordium meristematic cells suggested ongoing intracellular activity (Fig. 2.52 [x 8000]), with some indication of abnormality such as irregularity of the nuclear profile, the occasional mitochondria showing prominent electron-transparency and degradation of some plastid content (Fig. 2.53 [x 25 000]).*

* m, mitochondrion; p, plastid

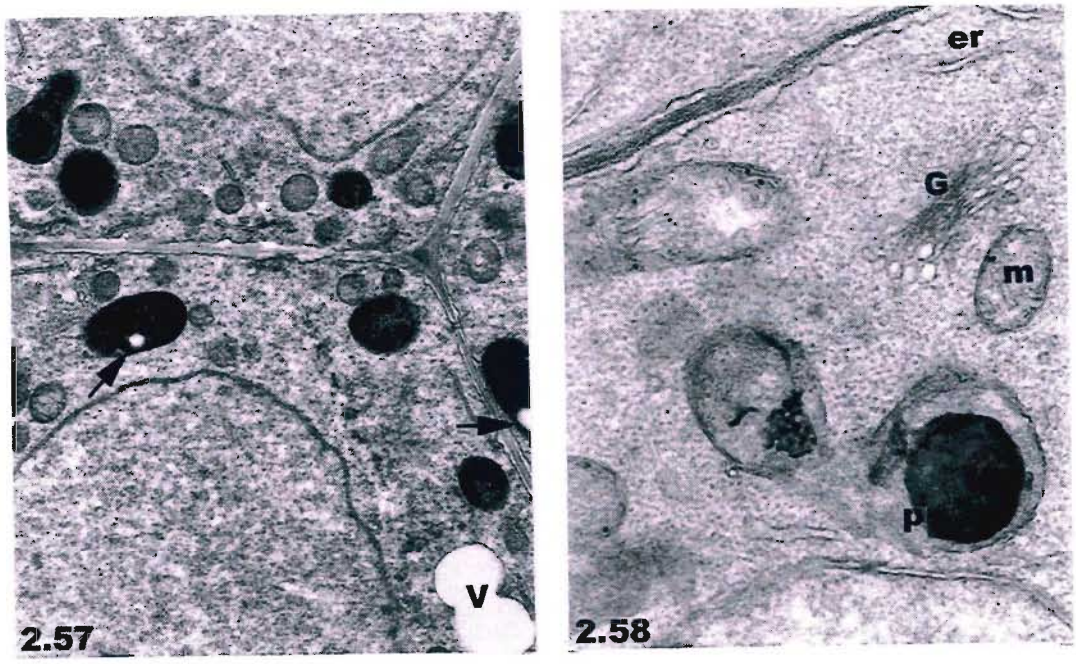






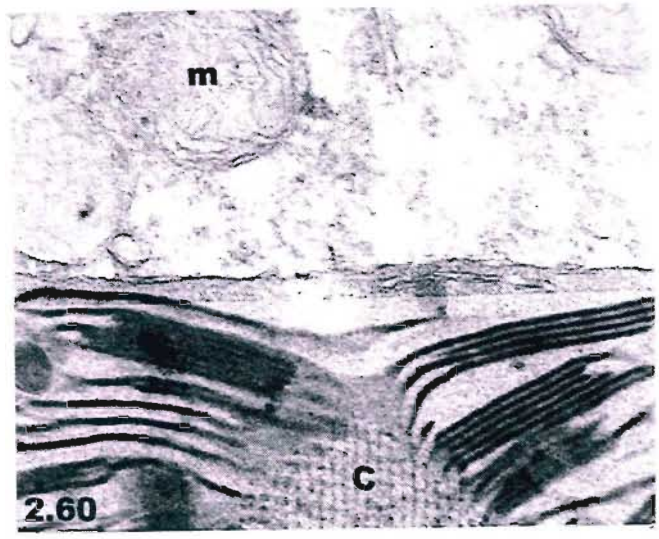
Figs. 2.54 - 2.56. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 4 days, inoculated and re-stored for a further 8 days (4d-8).** Degradation of cotyledon epidermal cells could be observed where fungal structures were juxtaposed externally (Fig. 2.54 [x 5000]). While the tissues underlying the fungal hyphae showed damage (Fig. 2.55 [x 4000]), other cells demonstrated ultrastructure suggestive of ongoing metabolic activity. However, there were signs of disturbance in these cells, e.g. to the chloroplast structure (Fig. 2.56 [x 6000]).*

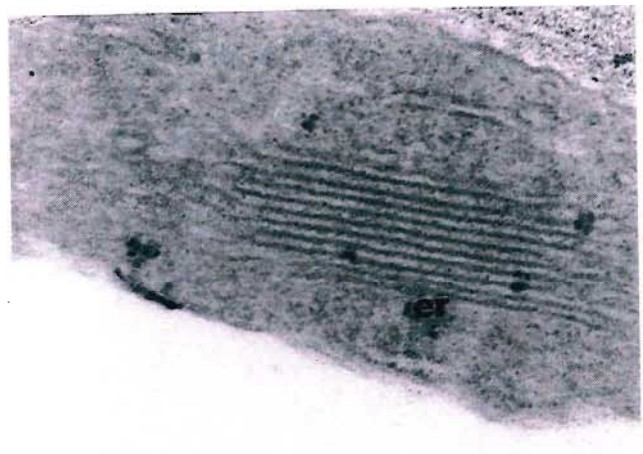
* C, chloroplast; F, fungal structure



Figs. 2.57 - 2.58. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 7 days (7d clean).** The meristematic root primordium cells showed some formation of small vacuoles (Fig. 2.57 [x 8000]). The compact mitochondria had evident cristae and Golgi bodies showed well-defined cristernae and associated vesicles and some plastids from which most of the (unidentified) dense material had disappeared (Fig. 2.58 [x 20 000]). Endoplasmic reticulum was observed to occur as relatively short profiles, and some starch accumulation (arrows) was evident within the dark plastid stroma (Fig. 2.57).*

* er, endoplasmic reticulum; G, Golgi body; m, mitochondrion; p, plastid; V, vacuole

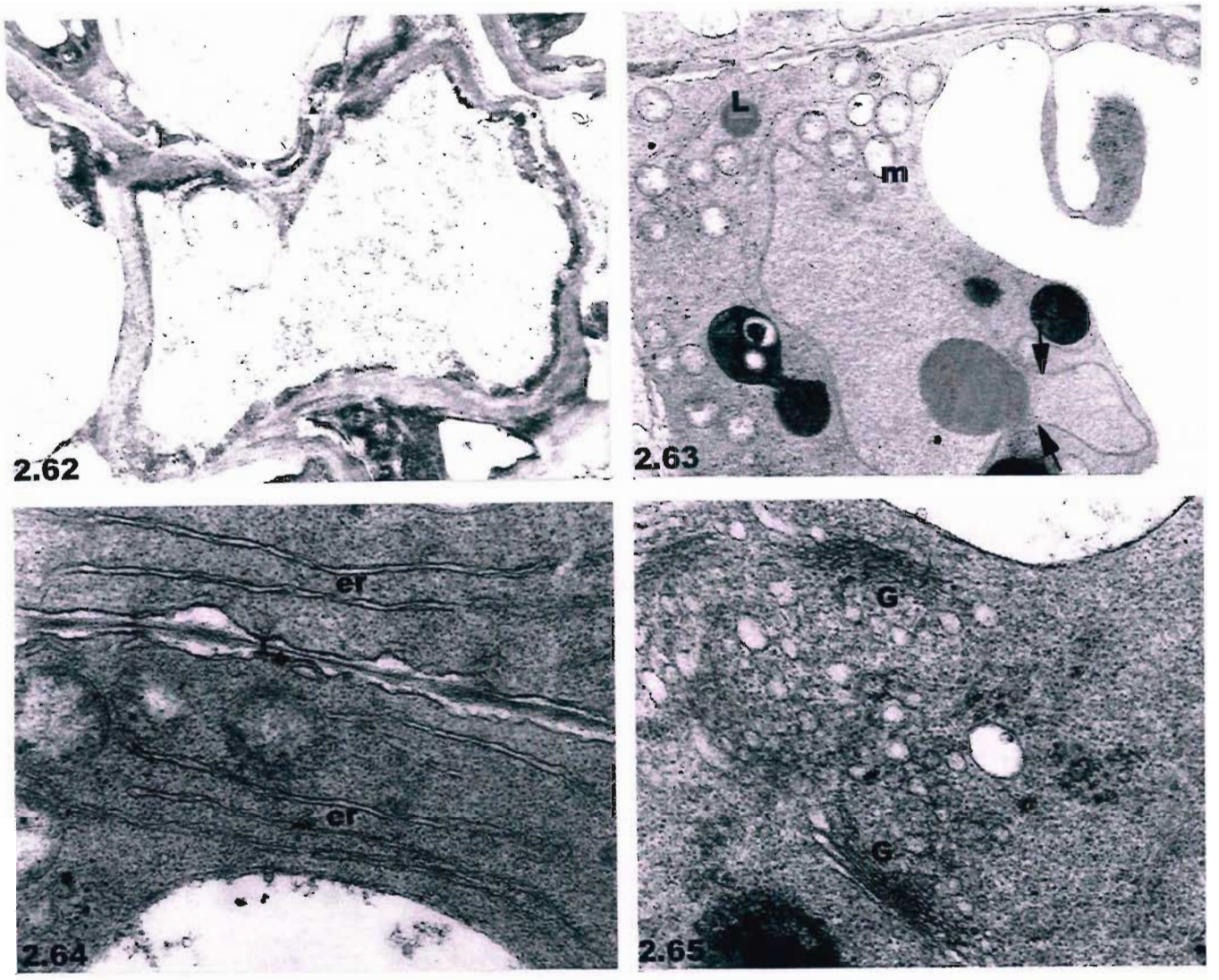




2.61

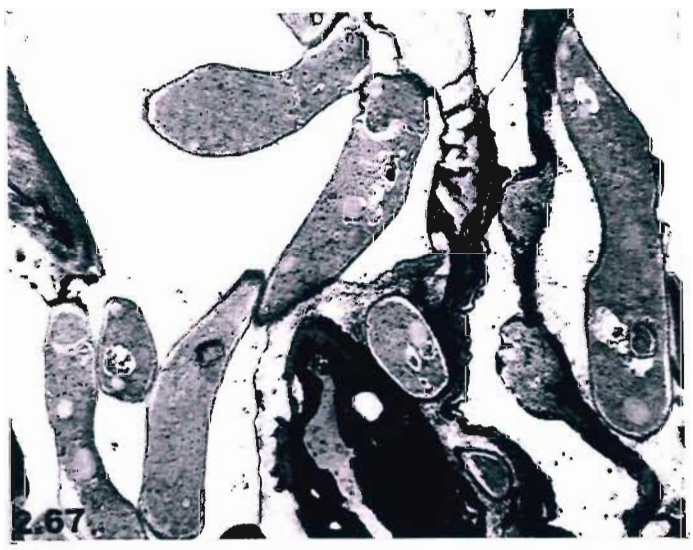
Figs. 2.59 - 2.61. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 7 days (7d clean).** No fungal structures were observed on the cotyledonary surface (Fig. 2.59 [x 5000]). The cotyledonary cells appeared normal but showed signs of increased metabolism such mitochondria with well-developed cristae, highly organised thylakoid structure and the occurrence of polysomes (arrowhead) (Fig. 2.60 [x 40 000]) and closely associated profiles of endoplasmic reticulum (Fig. 2.61 [x 80 000]).*

* C, choloroplast; cw, cell wall; er, endoplasmic reticulum; m, mitochondrion



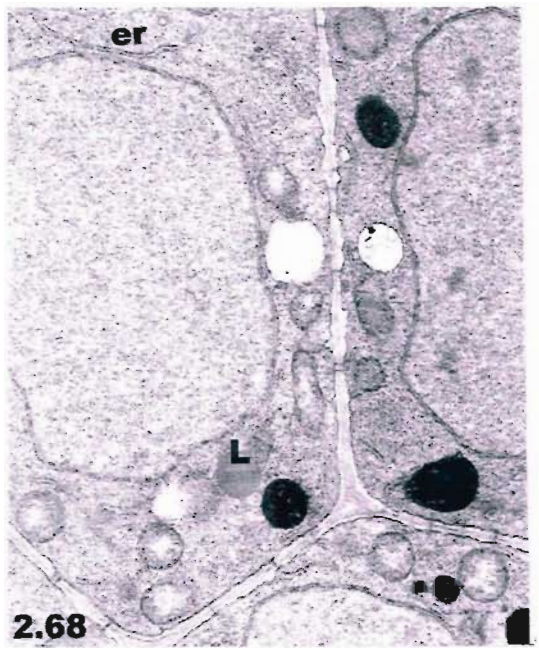
Figs. 2.62 - 2.65. **The ultrastructure of tissues of seeds of *A. marina* stored infected for 7 days (7d infected)**. Some root primordium cells of the embryonic axis showed advanced damage (Fig. 2.62 [x 6000]) while others had maintained integrity but showed marked abnormalities (Fig. 2.63 [x 6000]). Most of these cells had developed confluent, large vacuoles, nuclear lobing (arrows) and mitochondria that had lost internal organisation (Fig. 2.63). Long strands of peripherically-situated endoplasmic reticulum were also observed (Fig. 2.64 [x 25 000]). A few lipid bodies (Fig. 2.63) and numerous well-developed Golgi bodies were also present in these cells (Fig. 2.65 [x 35 000]).*

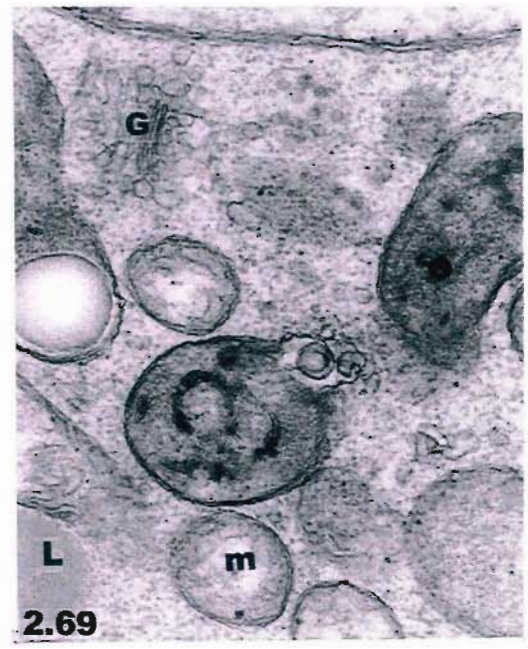
* er, endoplasmic reticulum; G, Golgi body; L, lipid body; m, mitochondrion

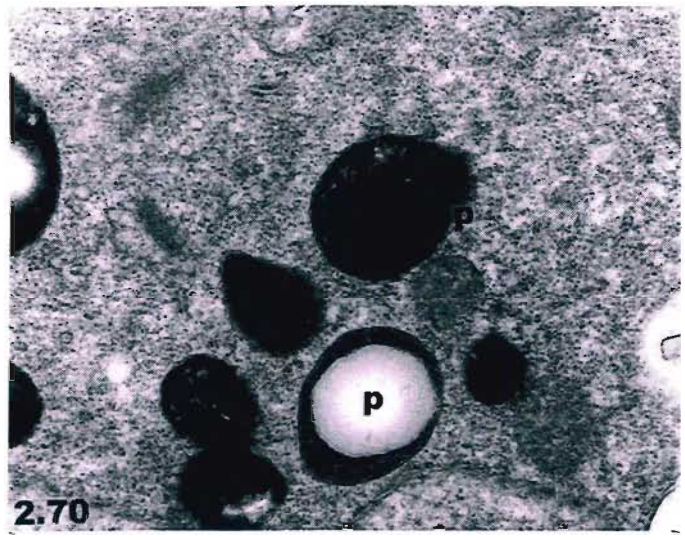


Figs. 2.66 - 2.67. **The ultrastructure of tissues of seeds of *A. marina* stored infected for 7 days (7d infected).** Abundant fungal proliferation was evident (Fig. 2.66 [x 5000]). The fungus had penetrated the internal cotyledonary tissues that were extensively damaged (Fig. 2.67 [x 2500]).*

* F, fungal structure

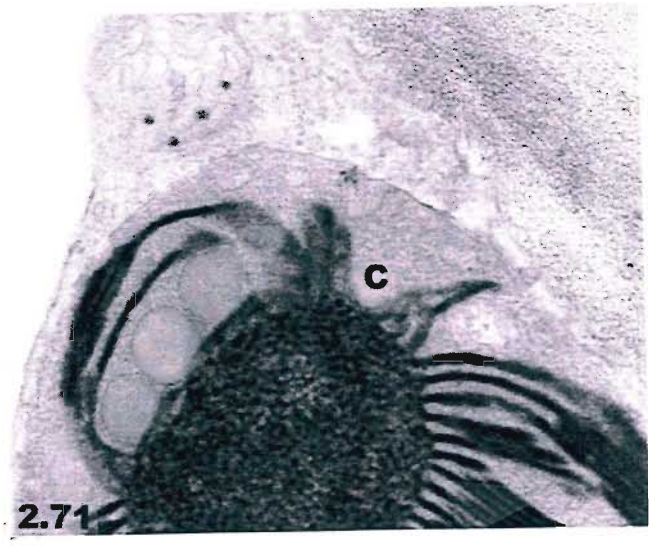






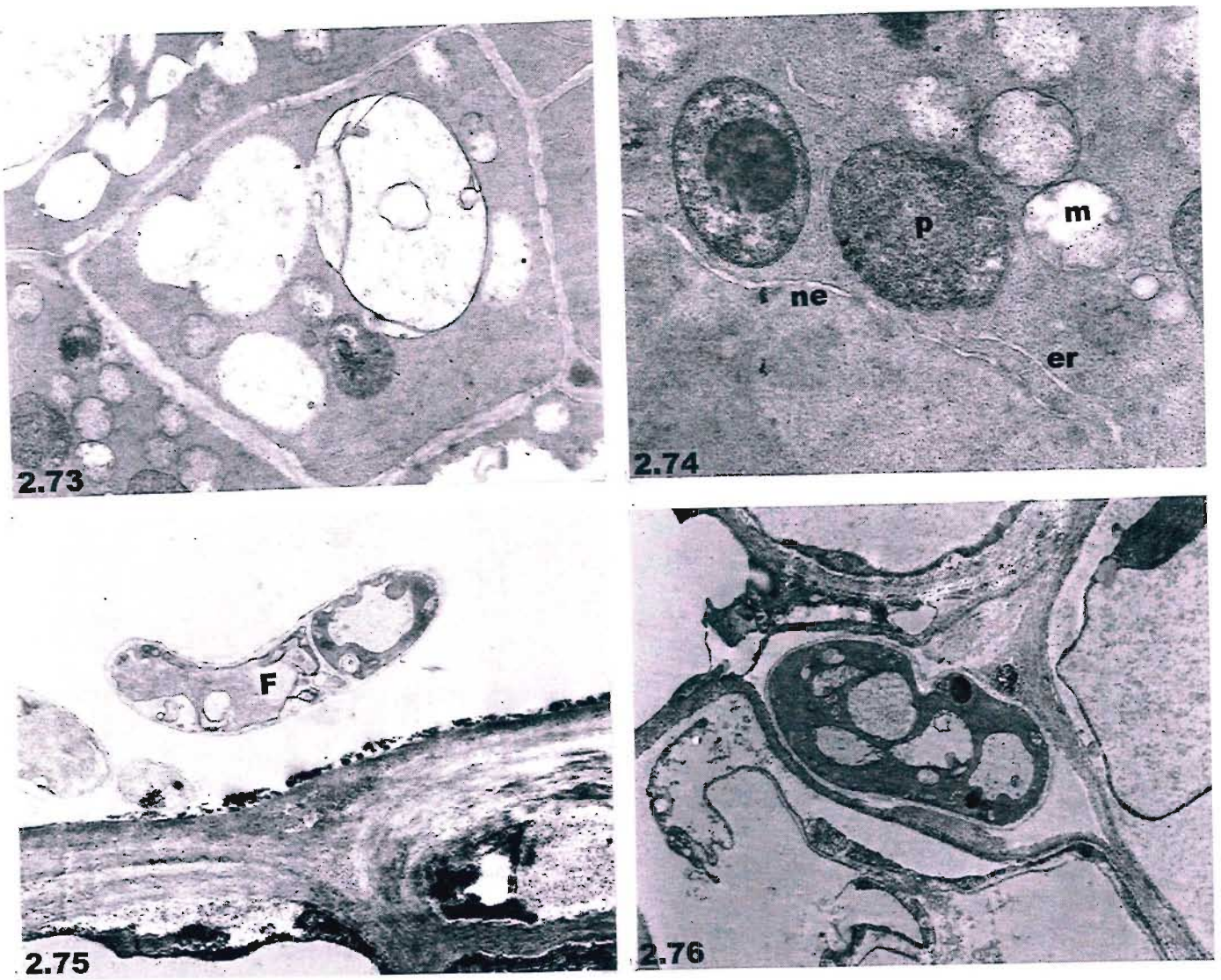
Figs. 2.68 - 2.70. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 10 days (10d clean).** Meristematic root primordium cells of the embryonic axes in which vacuolation had remained minimal, showed little sign of abnormality (Fig. 2.68 [x 8000]). Most mitochondria had dense matrices although some showed electron-transparent matrical regions (Fig. 2.69 [x 25 000]). Prominent starch deposits were visible in some plastids (Figs. 2.69 and 2.70 [x 15 000]). Longer endoplasmic reticulum (Fig. 2.68), Golgi bodies and some lipid bodies could be seen (Fig. 2.69).*

* er, endoplasmic reticulum; G, Golgi body; L, lipid body; m, mitochondrion; p, plastid



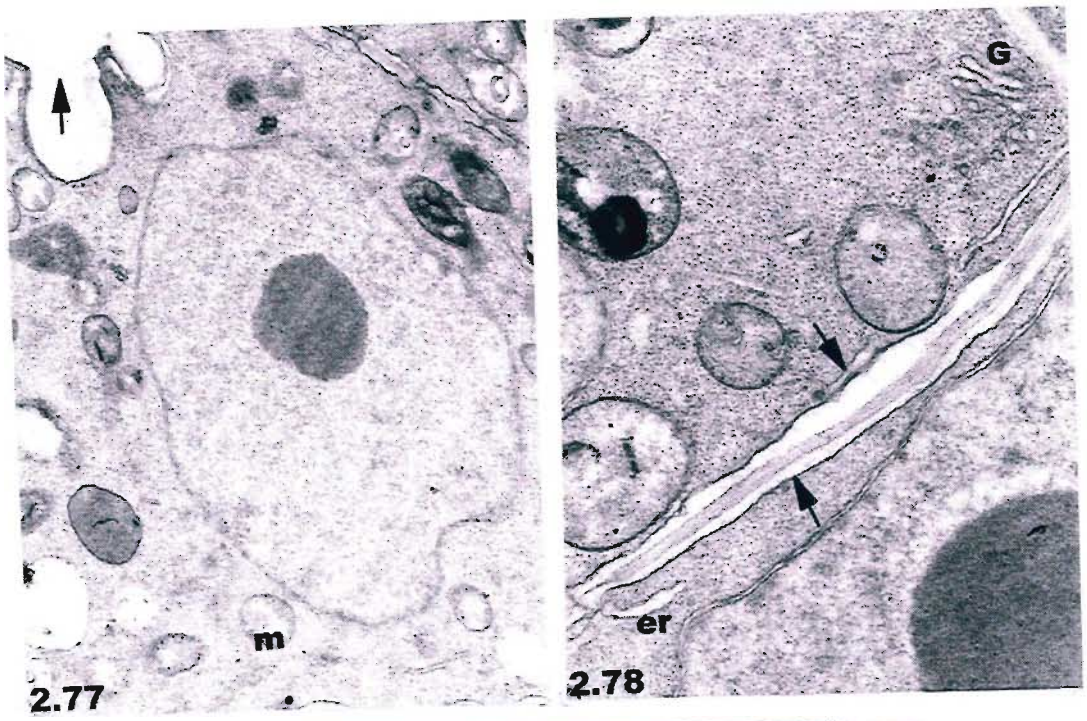
Figs. 2.71 - 2.72. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 10 days (10d clean).** The cotyledonary cells showed signs of active metabolism, having retained well-developed chloroplast (Fig. 2.71 [x 40 000]) and dense mitochondria with prominent cristae (Fig. 2.72 [x 30 000]).*

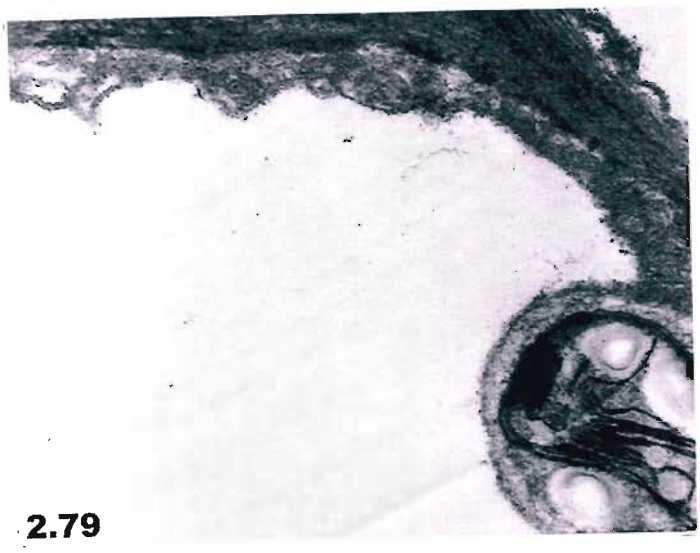
* C, chloroplast; m, mitochondrion



Figs. 2.73 - 2.76. **The ultrastructure of axis tissues of seeds of *A. marina* stored infected for 10 days (10d infected).** Signs of marked deterioration, including the degree of vacuolation and fusion of individual vacuoles, and an almost amorphous cytoplasm were observed (Fig. 2.73 [x 8000]). Mitochondria and plastids appeared internally degraded and the nuclear envelope and long endoplasmic reticulum profiles appeared dilated (Fig. 2.74 [x 20 000]). Figs 2.75 and 2.76 show aspects of the cotyledon cells. Fungal proliferation had occurred resulting in many elements of the mycelium being visibly associated with the cotyledonary tissues (Fig. 2.75 [x 5000]). Intracellular cotyledonary cell degradation was advanced (Fig. 2. 76 [x 4000]).*

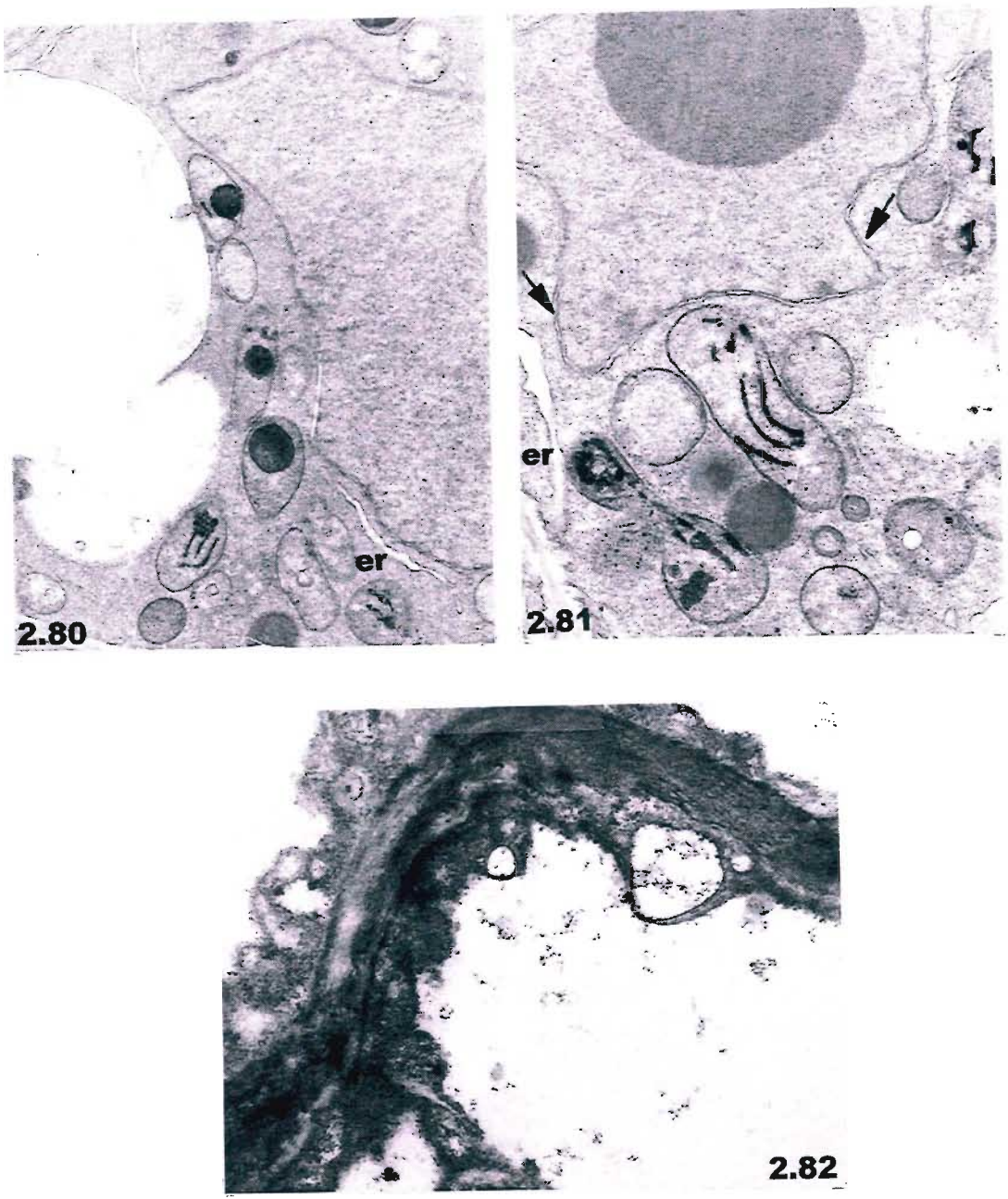
* er, endoplasmic reticulum; F, fungal structure; m, mitochondrion; ne, nuclear envelope; p, plastid





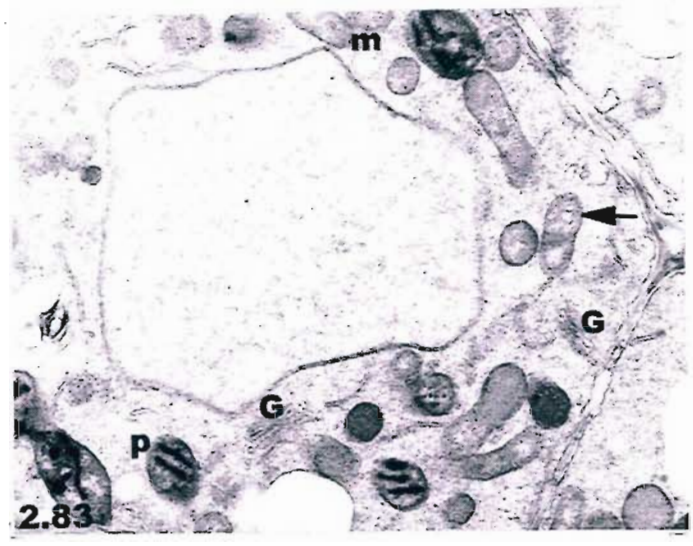
Figs. 2.77 - 2.79. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 10 days, inoculated and re-stored for a further 4 days (10d-4).** Meristematic root primordium cells showed ultrastructural change with some degree of vacuolation and some confluency (abnormal fusion) of these compartments (arrow) (Fig. 2.77 [x 8000]). Mitochondria with electron-transparent patches and occasional abnormal inner membrane formations (Fig. 2.77) and Golgi bodies with unusually loosely–associated cisternae, were observed. Plasmalemma withdrawal (arrows) and short, sometimes dilated, profiles of endoplasmic reticulum occurred (Fig. 2.78 [x 15 000]). At cotyledonary level, advanced degradation was observed (Fig. 2.78 [x 8000]).*

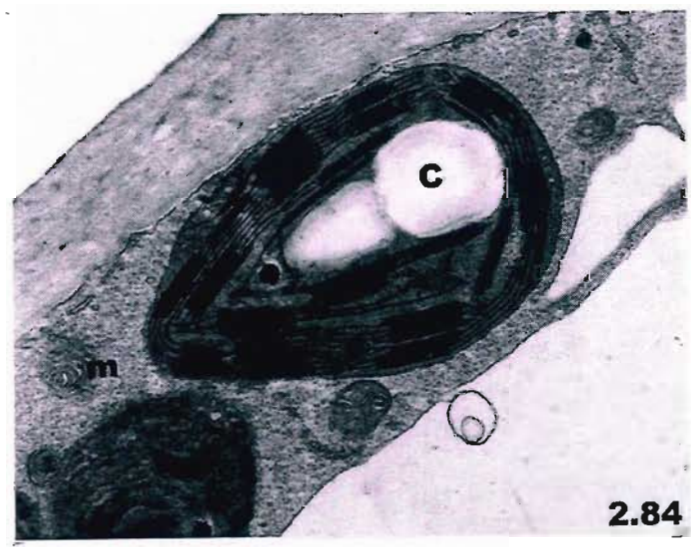
* er, endoplasmic reticulum; G, Golgi body; m, mitochondrion



Figs. 2.80 - 2.82. **The ultrastructure of tissues of seeds of *A. marina* stored cleaned for 10 days, inoculated and re-stored for a further 8 days (10d-8).** Signs of deterioration in the root primordium cells, including the degree of vacuolation and fusion of individual vacuoles, as well as dilation of endoplasmic reticulum profiles (Fig. 2.80 [x 8000]). Markedly abnormal nuclear profiles (arrows) were also observed (Fig. 2.81 [x 15 000]). At cotyledonary level, extensive ultrastructural necrosis was evident (Fig. 2.82 [x 8000]).*

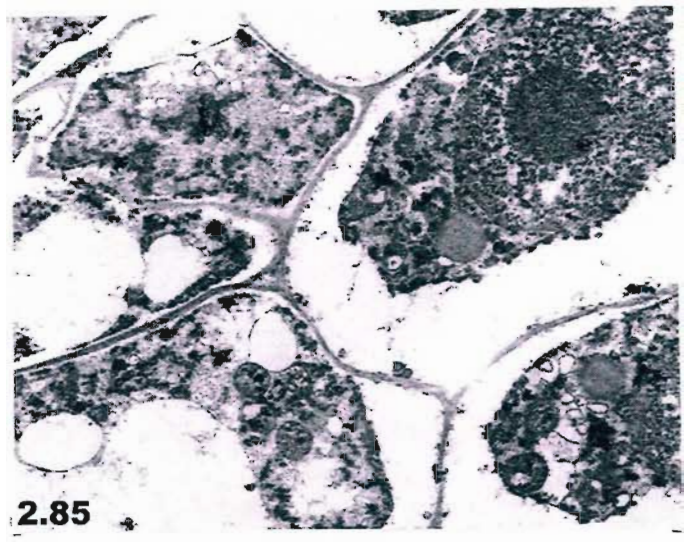
* er, endoplasmic reticulum

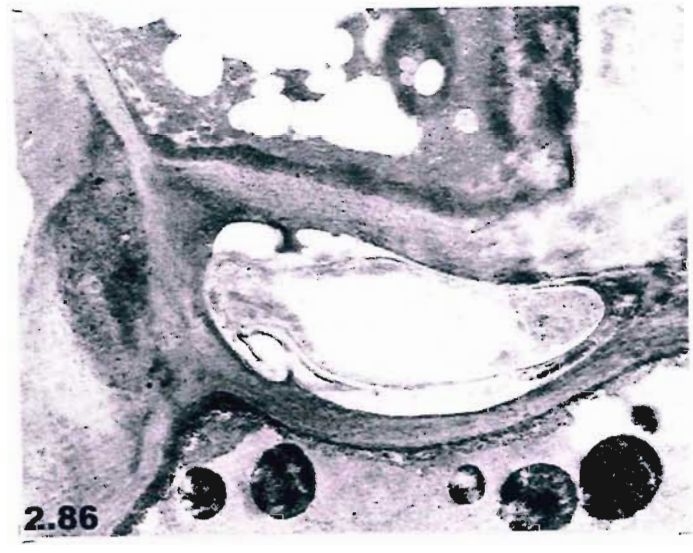




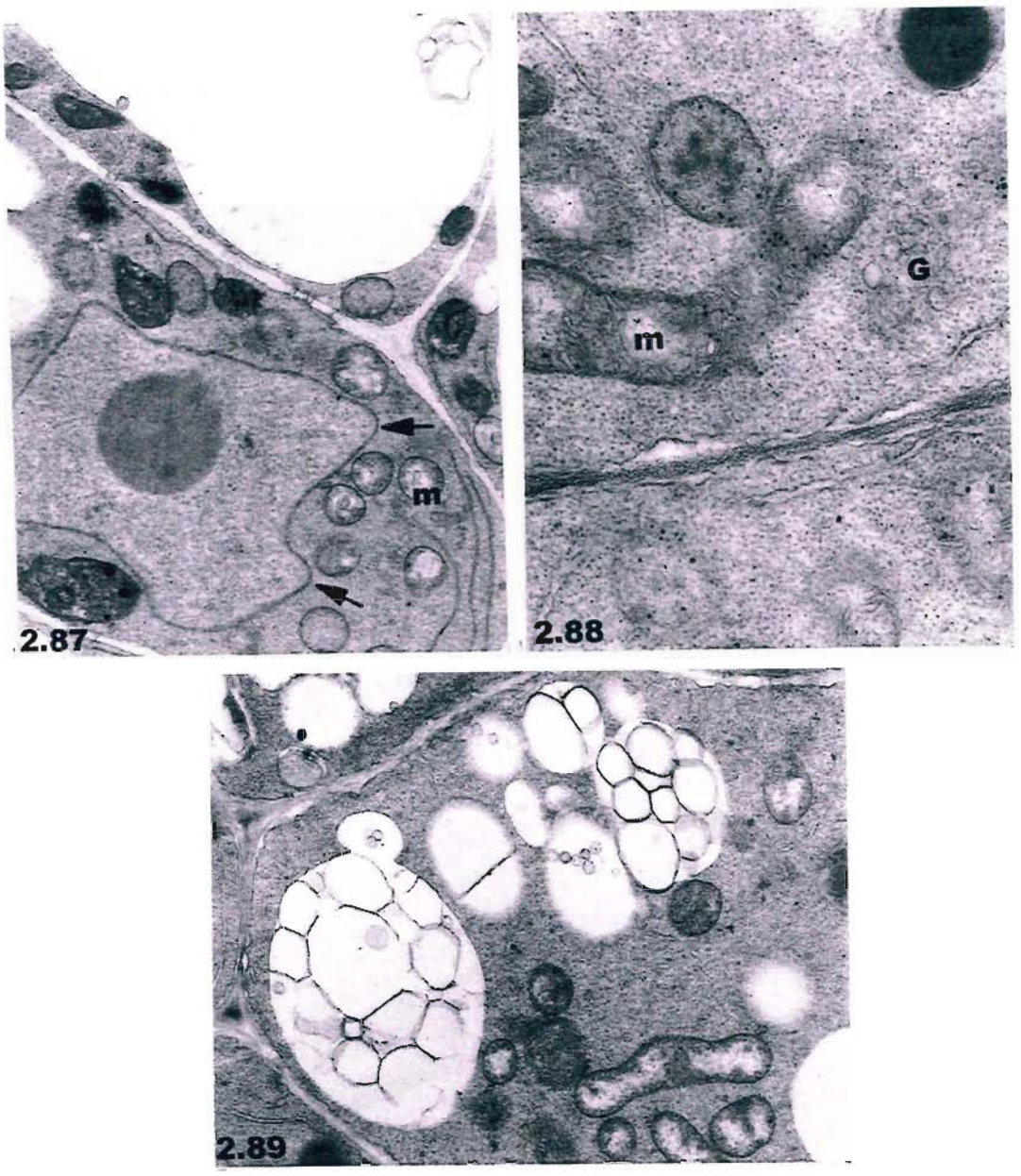
Figs.2.83 - 2.84. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 12 days (12d clean).** Meristematic root primordium cells of the embryonic axes showed a slight degree of vacuolation and well-defined elements of the endomembrane system. Inner membranes had become clearly-defined within plastids (arrowhead), and generally well–developed mitochondria were seen. Some mitochondria showed abnormal inner membrane formation (arrow). As illustrated, however, some nuclei had become slightly irregular in profiles (Fig. 2.83 [x 8000]). No associated fungal structures were evident on cotyledonary epidermal cells that appeared ultrastructurally normal and highly metabolically active Fig. 2.84 [x 15 000]).*

* C, chloroplast; G, Golgi body; m, mitochondrion; p, plastid



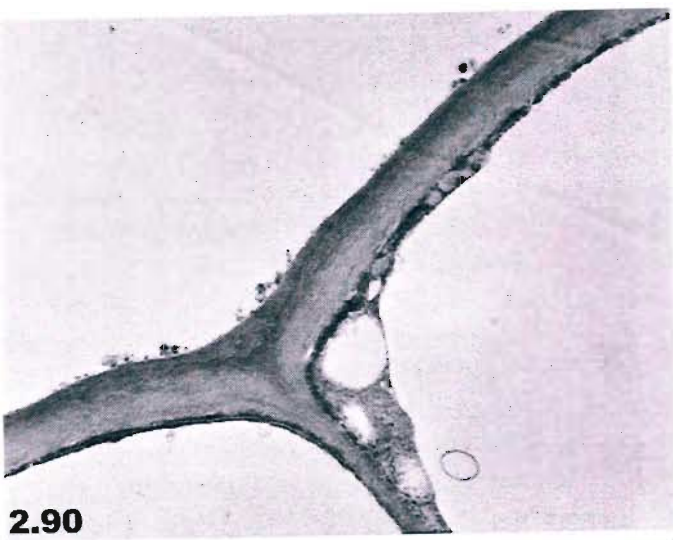


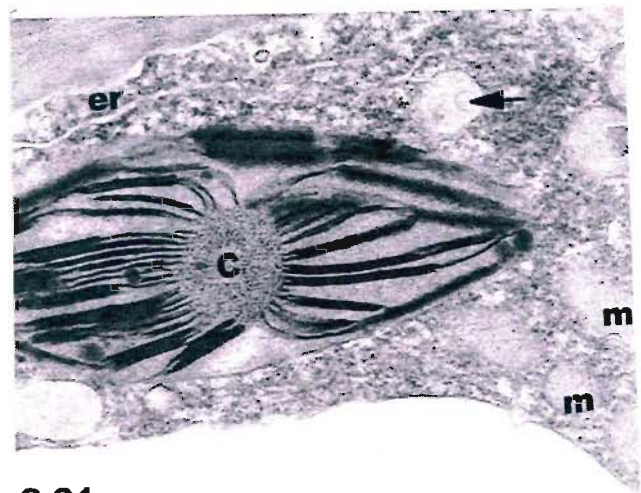
Figs. 2.85 - 2.86. **The ultrastructure of tissues of seeds of *A. marina* stored infected for 12 days (12d infected).** Extensive cell deterioration was observed in both meristematic root primordium cells (Fig. 2.85 [x 6 000]) and cotyledonary cells (Fig. 2.86 [x 10000]), both tissues types showing complete loss of internal organisation (Fig. 2.86).

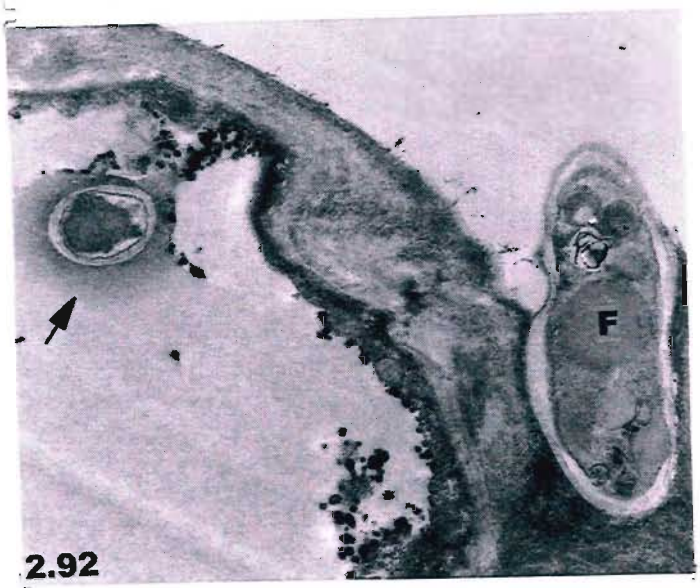


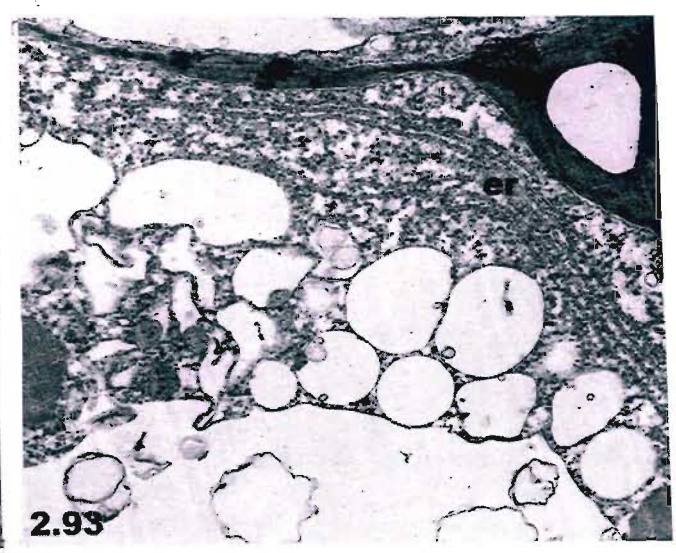
Figs. 2.87 - 2.89. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 21 days (21d clean).** Root primordium meristem cells of embryonic axes of seeds where fungal contamination could not be discerned had become more vacuolated during hydrated storage and showed evidence of ongoing metabolism; however, there were signs of abnormality, e.g. onset of nuclear lobing (arrows) and abnormal membrane formations in some mitochondria (Fig. 2.87 [x 8000]). Vesicles indicating the presence of Golgi bodies (out of the plane of the section) and an elongated, well-developed mitochondrion are illustrated in Figure 2.88 (x 20 000). Deterioration was observed in the seeds where fungal infection could be observed and a marked electron-transparency of mitochondrial matrices occurred (Fig. 2.89 [x 8000]). As in the case illustrated, often, confluent vacuoles containing a considerable amount of membraneous material were observed (Fig. 2.89).*

* G, Golgi body; m, mitochondrion



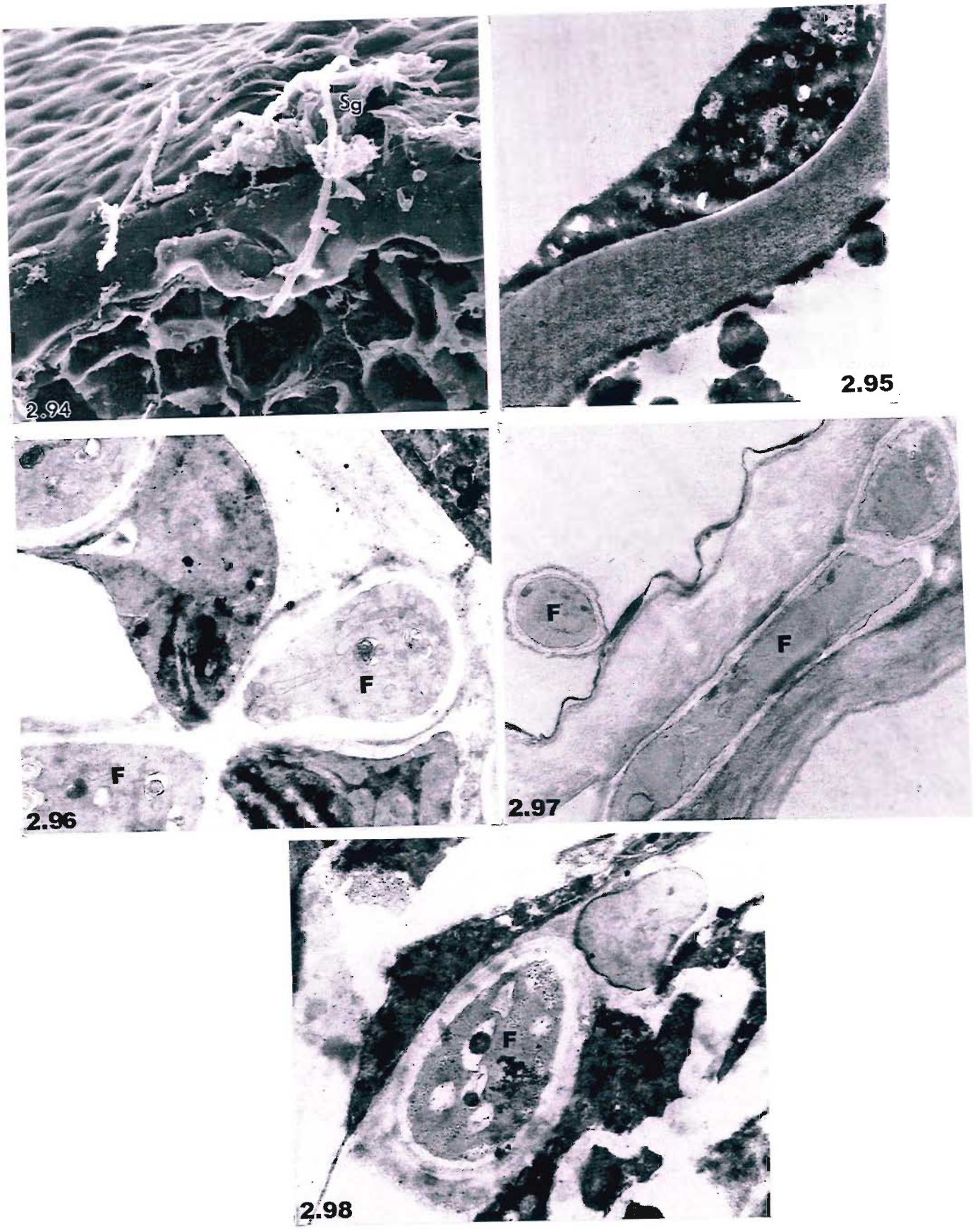






Figs. 2.90 - 2.93. **The ultrastructure of tissues of seeds of *A. marina* stored clean for 21 days (21d clean).** The cotyledons without associated visible fungal structures (Fig. 2.90 [x 8000]) showed intact cells having chloroplasts with depleted starch reserves and well-developed mitochondria that did, however, show the occasional abnormal inner membrane formation (arrow). Profiles of endoplasmic reticulum appeared slightly dilated and the ground cytoplasm was less compact than observed earlier during storage of clean seeds (Fig. 2.91 [x 15 000]). The cotyledonary cells that were penetrated by fungal structures (Fig. 2.92 [x 10 000]) showed densely-staining, small globules largely contained within the vacuoles. Considerable vesiculation and an advanced degree of deterioration of the cytoplasm were observed (Fig. 2.93 [x 2500]). The endoplasmic reticulum in the cell illustrated is surprisingly well-organised (Fig. 2.93). Some fungal structures, apparently within vacuoles, were surrounded by amorphous, slightly denser regions (arrow) (Fig. 2.92).*

* C, chloroplast; er, endoplasmic reticulum; F, fungal structure



Figs. 2.94 – 2.98. **The fungal infection process**. Fungal hyphae penetrated plant tissue through salt glands as illustrated by SEM (Fig. 2.94 [x 500]). Some residue was observed on the surfaces of the penetrated epidermal cells (Figs. 2.94 and 2.95 [x 10 000]). The fungal hyphae continued to invade the internal tissue (Fig. 2.96 [x 15 000]) by intra- and intercellular growth 2.97 [x 10 000]). Advanced cell necrosis was observed in cotyledonary tissues with high degree of infection (Fig. 2.98 [x 15 000]).*

* F, fungal structure; Sg, salt gland

2d. DISCUSSION

The only conservation strategy for intact recalcitrant seeds is the strictly short-term option of wet-storing them at water contents essentially unchanged from those of newly-harvested seeds, and at the minimum temperature tolerated (Chin, 1980; King and Roberts, 1980; Berjak *et al.*, 1989; Berjak and Pammenter, 2001). These minima however, must be relatively high in the case of tropical recalcitrant seeds (which are chilling sensitive), such as *Avicennia marina* (Lewis, 2003). Even in the case of temperate recalcitrant seeds that are highly hydrated, storage at below 0°C temperatures is excluded.

The storage conditions necessary for retention of viability of recalcitrant seeds and other non-orthodox seeds are also those that favour fungal proliferation (Berjak, 1996). In addition, any attempts to manipulate these seeds, for example, to lower their water content to prevent ongoing germinative metabolism, could be stressful enough to stimulate the deleterious effects of seed-associated mycoflora (Drew *et al.,* 2000).

While there is ample circumstantial evidence that fungal proliferation is almost invariably associated with recalcitrant seeds during storage under high relative humidity conditions, this has been systematically recorded in only a few cases. Those studies focus on temperate acorns (Murray, 1974; Delatour, 1978), seeds of commercial rubber, the tropical species *Hevea brasiliensis* (Dalbir-Singh and Singh, 1990), and a variety of tropical, subtropical and temperate species (Mycock and Berjak, 1990; Sutherland *et al.*, 2003). However, intrinsic questions about the effects of seed-associated fungi and their possible role in the curtailed wet-storage lifespan of recalcitrant seeds, and the responses of the seeds themselves have not been investigated.

The present study has shown that if the proliferation of the associated mycoflora can be curtailed, then the hydrated storage lifespan of *A. marina* seeds is

extended. Untreated seeds of *A. marina* have previously been reported to be infected with *Aspergillus*, *Penicilium* and *Fusarium* species as well as by bacteria (Mycock and Berjak, 1990, Goddard, 1994), the bacteria persisting during storage. In the present investigation, *Fusarium* spp. predominated in, and on, seed tissues during hydrated storage, confirming previous observations. The absence of storage fungi is not surprising. This group of fungi that mainly comprises xerotolerant species of *Aspergillus* and *Penicilium*, are generally active in the tissues of orthodox seeds in air-dry storage (Christensen and Kaufmann, 1974; McLean and Berjak, 1987), when the competition imposed by the field fungi is curtailed by the low water activity of seeds stored at low RH. In contrast however, field fungi such as species of *Fusarium, Cladosporium* and *Alternaria* require relatively high seed water contents in order to proliferate, which are conditions that are also necessary to maintain viability of recalcitrant seeds in hydrated storage (Berjak, 1996).

In the study of the inherent fungal infection, scanning electron microscopy of tissues of freshly harvested, revealed fungal hyphae and bacteria to the external surface of the seed coat of untreated *A. marina* seeds. It had been proposed previously that removal of the pericarp might extend seeds storage lifespan by eliminating the source of fungal inoculum (Goddard, 1994). The results of current investigation, failed to support this hypothesis, since survival, assessed as germinability of seeds stored with and without pericarp was consistently similar. While scanning electron microscopy demonstrated an absence of contamination of the surface of axis and cotyledonary tissues, agar isolation studies suggested that a fungal inoculum might be present within the tissues of newly harvested seeds.

Fungi can gain access to the seed tissues at any time from flowering to the post-shedding stage. As is the case for orthodox seeds, recalcitrant seeds might be internally infected by fungi by systemic transmission via the parent plant, as has been described for developing maize (Mycock and Berjak, 1992;

Kabeere *et al.*, 1997), or through the stigma-style continuum during flowering (Marsh and Payne, 1984). If fungal infection does occur during seed development, then the mycelium has the opportunity to become established deep within seed tissues, making eradication difficult (Sutherland *et al.*, 2003).

In contrast to the situation of untreated seeds, periodic treatment with the fungicide Previcur (active ingredient propamocarb-HCl) immediately following surface sterilisation with NaOCl reduced the proportion of seeds from which fungi could be isolated from 65% to zero, an effect that persisted for at least 4 days (Table 2.4). This particular treatment, however, was fungistatic rather than fungicidal, as the presence of *F. moniliforme* became apparent on the cotyledonary surfaces after 7 days of storage despite periodic reapplication of Previcur. *Fusarium moniliforme* has previously been shown to be the dominant fungal species during storage of a variety of recalcitrant seeds of southern African origin and is considered to be particularly aggressive (Mycock and Berjak, 1990).

Nevertheless, despite the persistence of *F. moniliforme* on cotyledonary surfaces, it was only after 21 days of hydrated storage that actual invasion of the cotyledonary tissues was manifested, which was accompanied by a decline in seed viability to 70%. Interestingly, and perhaps correlatively, *F. moniliforme* was isolated from 30% of the seeds after 21 days of storage. In contrast to the survival data of the clean material, when initially surface-sterilised treated seeds were experimentally infected with *F. moniliforme*, none survived in wet-storage for 7 days, and 45% had already lost viability after 4 days.

The hydrated storage lifespan of Previcur-treated seeds (clean seeds) was extended at least three-fold compared with the experimentally infected seeds in the present study. The experimentally infected seeds were, however, drastically affected by inoculation with *F. moniliforme* even when compared with untreated seeds: in the latter case after pericarp removal and without surface sterilisation

or treatment with any fungicidal or fungistatic compounds, the seeds survived better during hydrated storage, but none remained viable for as long as 16 days (Fig. 2.7). It is probable that it is only when the spectrum of fungal species originally associated with these seeds (Table 2.3) becomes dominated by *F. moniliforme* (Mycock and Berjak, 1990) that significant deleterious effects on viability are manifested.

However, hydrated storage lifespan of *A. marina* seeds can be prolonged considerably beyond 21 days. Motete *et al.* (1997) and Pammenter *et al.* (1997) have shown that encapsulation of surface-sterilised seeds in a custom-prepared alginate gel maintained seed viability at a high level for 70-80 days. While ultrastructural studies suggested that metabolism in the gel-encapsulated seeds had slowed, Motete *et al.* (1997) could not identify suppression of any specific metabolic process, nor were there indications that water stress was involved in the rapid viability loss of unencapsulated seeds, or alleviated in those that had been gel-encapsulated. Those authors did, however, report a notable reduction in fungal proliferation on the encapsulated seeds and in the number of seeds that became visibly infected. Since the 1997 studies of Motete *et al.* and Pammenter *et al.,* other research has established the alginate gel to be completely inhibitory of *in vitro* growth of fungal species associated with tropical recalcitrant seeds (Myeza[1], unpublished data).

Thus, seed-associated mycoflora have a significant effect in curtailing the hydrated lifespan of recalcitrant seeds. If appropriate and effective fungicidal treatments can be implemented, and provided there is no inaccessible inoculum deep within these tissues, the lifespan of recalcitrant seeds in hydrated storage may be significantly extended.

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The second question is whether hydrated recalcitrant seeds become more susceptible to fungal attack with increased storage time. The present data show that the viability of clean seeds remained unchanged (100-95%) during 14 days of hydrated storage, after which there was a decline to 70% by 21 days. At the other extreme, when seeds were experimentally infected immediately after they had been treated to remove the original associated mycoflora, germinability declined to 55% within 4 days, and by 7 days of hydrated storage, all had lost viability. However, if the seeds were stored clean for 4-10 days prior to being experimentally infected with *F. moniliforme*, the adverse effects of the fungus were reduced, although it should be noted that the improved resilience of the seeds was greatest after 4 days storage.

For example, viability of the 4d-4 seeds (storage for 4d clean followed by 4 d after inoculation) was 70%, compared with 55% germinability of those not stored prior to infection and sampled after 4 days (4 day infected). Seeds stored for 4 days prior to infection and subsequently maintained for 8 and 10 days (4d-8 and 4d-10) retained 55% and 30% viability, respectively, whereas those inoculated immediately after harvest had totally lost viability when they were sampled after 7 days of hydrated storage. The beneficial effects of hydrated storage prior to experimental infection continued to be manifested in seeds that had been maintained in the clean state for 7 and 10 days prior to experimental infection. The enhanced resilience of the seeds stored clean prior to experimental infection was, however, effectively lost after 12 days, when post-inoculation viability retention was essentially similar to the seeds that were infected immediately (4d infected and 12d-4 with 55% and 50% viability, respectively). Seeds stored clean for 14 days were more susceptible to the effects of the fungus after 4 day (14d-4) than were the 4d infected seeds, and those stored for 16 days prior to inoculation did not survive a subsequent 4 days of storage (16d-4).

It is implicit from the present results that after the relatively short period of 4 days of storage, during which the clean seeds were effectively free from extraneous stress imposed by an associated mycoflora, they become more resilient to such effects when inoculated with *F. moniliforme*. Farrant *et al.*, (1986) showed that *A. marina* seeds initiate germinative metabolism and become more metabolically active during 4 days of hydrated storage, an observation verified by the current study. It might thus be argued that concomitantly the seeds, which are essentially seedlings after 4 days, acquire an enhanced ability to counteract the effects of associated mycoflora. This resilience that did, however, decline somewhat, was maintained for up to 10d of clean storage, during which the ultrastructure indicated maintenance of activity and intracellular integrity in both axis root primordia and cotyledonary tissues.

While the resilience of seeds stored clean (particularly for 4 days, but seemingly for as long as 10 days) might simply be the outcome of their enhanced metabolic status compared with the newly harvested state, the possibility of the concomitant development of inherent defence mechanisms, and/or a greater ability for inducible mechanisms to be entrained must be considered. Healthy vegetative plant tissues have an arsenal of weapons from physical barriers and inherent mechanisms and processes, to inducible compounds such as phytoalexins, which counteract the deleterious effects of fungi (Hammerschmidt, 1999). From the present study, the ability of *A. marina* seeds to resist a virulent fungal pathogen, while minimal at harvest, seems to be rapidly acquired. This would account for the resilience of the majority of the clean seeds in surviving the 21-day storage period, despite the persistence of some associated mycoflora. In the context of the sampling intervals presently used, this ability was most effective after 4 days of clean storage but gradually declined under the stress of prolonged maintenance in storage. Consequently, seeds clean-stored for longer periods gradually became more vulnerable to *F. moniliforme*, and, after 14 days, were more susceptible than in the newly harvested condition.

Therefore, the answer to the second question posed – whether hydrated recalcitrant seeds become more susceptible to fungal attack with increasing storage time – is more complex than simply a matter of yes or no. In long-term storage, seeds exhibit heightened vulnerability to fungal attack. In short-term storage, at least for *A. marina*, seeds show increased resilience as a transient feature.

The final question to be asked is whether it is possible to differentiate fungal-induced deteriorative changes from those inherent degenerative events that may be the inevitable outcome of prolonged wet-storage of recalcitrant seeds (Pammenter *et al.*, 1994, Smith and Berjak, 1995). There is no doubt that all recalcitrant seeds stored hydrated ultimately die (Pammenter *et al.*, 1994). However, in the light of the present results, and those reported for gel-encapsulated *A. marina* seeds (Motete *et al.*, 1997; Pammenter *et al.*, 1997), it is necessary to re-examine the ultrastructural features previously accepted as signs of inherent deterioration.

Studies on several different species have shown that intracellular and metabolic enhancement occur in the early stages of hydrated storage, culminating in events such as cell division and the onset of substantial vacuolation, which are indicative of ongoing germination (Berjak *et al.*, 1984; 1989; Farrant *et al.*, 1985, 1989; Pammenter *et al.*, 1994). Intracellular degeneration occurs thereafter (Farrant *et al.*, 1989) and ultimately viability is lost, despite there having been no significant dehydration (King and Roberts, 1980; Suszka and Tylkowski 1980; Tompsett 1984, 1992; Farrant *et al.*, 1985, Fu *et al.*, 1990; Pritchard *et al.*, 1995). However, the timing of the onset of deterioration is variable, as shown for *A. marina* in the present study and in the investigations of Motete *et al.* (1997) and Pammenter *et al.* (1997), compared with other reports for this same species which indicate that declining vigour culminating in viability loss may be initiated in as little as 6 days (Farrant *et al.*, 1986).

Previous studies on hydrated *A. marina* seeds showed that vacuoles that formed in the course of germination became markedly confluent after 12 days, and internal disorganisation of the mitochondria had occurred by that stage (Farrant *et al.*, 1986). In contrast, in the present study, vacuolar confluency and mitochondrial degeneration were seen by 10 days in hydrated storage – but only in material that had been experimentally infected prior to storage. In contrast, clean-stored seeds sampled after 10 days exhibited neither a marked degree of vacuolation – and there were no indications of abnormal confluency of these organelles – nor of mitochondrial deterioration (Figs. 2.68-2.70). Even after 21 days, some clean-stored seeds showing no signs of fungal proliferation had well-organised root primordium (meristem) cells and no indication of vacuolar confluency (Fig. 2.84).

It is noteworthy that in these seeds on which fungal proliferation was observed in the 21 day clean-stored material, the mycelium was not associated with the axis, although root primordium cells were adversely affected (Fig. 2.89). Considering that uninfected seeds did not show symptoms of damage, the observation of such damage in visibly infected seeds suggests that axis deterioration mediated by *F. moniliforme* was effected at a distance. Also, in the studies where the cotyledons were examined by TEM, no assertion can be made that the material was free of associated fungus as only very limited areas are visualised in any one section. The suggestion that the fungus can induce reactions from a distance is supported by the scanning electron microscopical study that showed localised necrotic cells in areas adjacent to the infection site (Figs. 2.19 and 2.24) and by the ultrastructural evidence of deterioration of cotyledonary cells that did not demonstrate associated fungal structures (Figs. 2.79 and 2.82).

However, root primordium meristem cells of seeds that were infected after germinative metabolism had proceeded during clean storage, showed very similar symptoms to those described by Farrant *et al.* (1986). For example, in

the 10d-8 material, marked vacuolation and confluency of these organelles occurred (Figs. 2.80 and 2.81) and both mitochondria and plastids showed degenerative changes. Even more advanced deterioration was exhibited by the root primordium cells after 7 days if seeds had been stored in the infected state from the outset (Figs. 2.62, 2.63 and 2.73, 2.74). Several of the observed ultrastructural changes presently described as deteriorative for tissues of *A. marina* have also been reported by other workers (Berjak *et al.,* 1989; Motete *et al.*, 1997). Besides an increase in vacuolation, including fusion of these organelles, and mitochondrial and plastid aberrations, abnormalities also include plasmalemma withdrawal, swelling of endomembrane cisternae generally, and, 'unstacking' of the Golgi cisternae. Pammenter *et al.* (1994) expressed the opinion that extensive vacuolation of cells of the embryonic axis implies a requirement for additional water during storage of recalcitrant seeds. In the short-term, water could be withdrawn from the cotyledons to support this requirement in the axis cells. However, Pammenter *et al.* (1994) postulated that, in the long-term, unless additional water is provided to the seeds, cells may undergo a water stress, as a consequence of which regulatory biochemical reactions may become aberrant and/or arrested. As a consequence, free radicals, which normally would be quenched in fully hydrated tissues, could accumulate, resulting in peroxidation of lipids and proteins with subsequent membrane damage (Hendry *et al.,* 1992; Leprince *et al.,*1990b). Pammenter *et al.* (1994) have proposed that such free radical damage, and the inability to repair the resultant damage, might be a major contributing factor to the loss of viability of recalcitrant seeds in storage.

McLean (1994a) has postulated that the excessive membrane damage observed in the aflatoxin B_1–treated plant cells may have arisen as a result of free radical damage. This proposal is, therefore, not dissimilar from that of Pammenter *et al* (1994) to explain the loss of viability of recalcitrant seeds that are stored hydrated, but with no access to extraneous water. At present, it is not possible to distinguish the basis of such events – i.e. whether if free radicals

arise, they emanate from unbalanced metabolism in the stressed seeds and/or as a result of fungal activity.

A feature that has not been highlighted by other researchers working on recalcitrant seeds is that of apparent heightened endomembrane system activity that was observed throughout the hydrated storage period in meristematic root primordium cells of the embryonic axis: this occurred notably in seeds stored for 4d clean (Fig. 2.39), 7d clean (Fig. 2.58), 7d infected (2.65), 10d clean (Fig. 2.69), 10d-4 (2.78), 12d clean (2.83) and 21 d clean (2.88). The presence of well-defined endoplasmic reticulum and the prevalence of Golgi bodies that had many associated vesicles could be a reflection of intensive protein synthesis by the ER-associated ribosomes, followed by transport of such proteins in ER-derived vesicles to be processed through Golgi bodies. The well-developed Golgi-associated vesicles that were observed in the clean-stored material could transport various proteins elsewhere in the cell, including secretion to the cell exterior by exocystosis (Wolfe, 1995; Becker *et al.,* 2003).

If it is virtually impossible to obtain fungal-free seeds, is it possible to differentiate fungal-induced deteriorative changes from those that are direct consequence of wet storage? Several of the ultrastructural observations of the tissues of seeds of declining viability may (in part) be fungal-induced (by extracellular enzymes and/or toxins). In extensive studies on the effects of several mycotoxins on the ultrastructure of root tips of *Zea mays* embryos, a combination of features was frequently observed: an increase in the degree of vacuolation (size and number), membrane damage, a decrease in mitochondrial cristae and a reduction in the polysome population (McLean *et al.* 1992, 1994a, 1994b).

A feature that has not been reported by other researchers investigating the loss of viability of stored recalcitrant seeds, but which may be useful indicator of fungal presence, is that of abnormal formation and location of lipid bodies. In

the present investigation, lipid deposits apparently not initially present in fresh material, were consistently observed during storage, and may reflect a disruption of normal lipid biochemical pathways. In fungal-infected and aflatoxin-treated plant tissues, abnormally large lipid bodies and an increase in their frequency has been postulated to be a fungal-induced phenomenon (Russell *et al*, 1982; McLean *et al.* 1992, 1994b, 1994c). Nuclear lobing, another abnormality that was demonstrated by some experimentally infected seeds (Figs. 2.48, 2.63 and 2.81), has been linked to programmed cell death during plant-microbe interactions (Kosslak *et al.*, 1997). Similarly, densely-staining small globules contained within the vacuoles of the cotyledonary cells as presently observed, have also been suggested to be part of a plant defence reaction to pathogen invasion (Benhamou, 1996; Hu *et al.*, 2003).

While gel-encapsulated *A. marina* seeds, which appeared to harbour no fungal mycelium at all, ultimately developed similar symptoms accompanying inherent viability decline (Motete *et al.*, 1997; Pammenter *et al.*, 1997), these appeared only after some 50 days or more in hydrated storage. In the study by Farrant *et al.* (1986), similar symptoms were seen after approximately 12 days and, in the present study on clean seeds, were noted in some axis samples after only 21 days (21 days clean) in storage when fungal presence was concomitantly manifested. It is noteworthy that in studies by Farrant *et al.* (1986; 1989), the fungicide Benlate® was applied at the outset as powder to the damp seed surfaces. Such application inevariably results in uneven distribution of the fungicide, which can also have no significant effect upon inoculum borne below the thick pericarp. In the present investigation, seeds were sprayed with an atomised solution of Previcur, which not only gives an even application to the external (cotyledon) surfaces, but it is highly effective in its access to the axis in *A. marina* seeds when the pericarp has been removed.

The major differences shown in the timing of the onset of the subcellular deteriorative symptoms that accompany viability loss may be related to the

absence or presence of seed-associated fungi. If absent, axis (and cotyledon) cells maintain a highly ordered ultrastructure for a much-extended period of storage lifespan, compared with the early intracellular deterioration accompanying viability loss that occurs rapidly in the presence of fungi.

Thus, while it may not be possible to differentiate unequivocally between the symptoms of inherent intracellular deterioration and that induced by seeds-associated fungi in axis meristem cells – at least in the case of *A. marina* seeds in hydrated storage – it seems possible to discriminate between the causes in terms of the timing of their effects.

Chapter 3

Biochemical responses of wet-stored seeds of *Avicennia marina* infected with *Fusarium moniliforme*[#]

1. Assessment of possible antifungal activity of *A. marina* seeds during hydrated storage[*]
2. Detection of ß-1,3-glucanase and chitinase activities of wet-stored *A. marina* seeds[**]

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3a. INTRODUCTION

Plants need to defend themselves against attack from fungi, bacteria, viruses, nematodes and insects. These pathogens can be divided into those that kill the host and feed on the contents (necrotrophs) and those that require a living host to complete their life cycle (biotrophs and hemibiotrophs) (Dangl and Jones, 2001).

There are necrotrophs that attack a broad range of hosts, cell death often being induced by enzymes and/or toxins targeted to specific substrates. *Pythium* sp. and *Botrytis* sp. are examples of such fungal necrotrophs (Duffy *et al.,* 2003). In contrast, other necrotrophs, such as *Cochliobolus carbonum*, produce host-selective toxins that are effective only within a narrow range of plants. Plants attacked by these pathogens become resistant through the loss or alteration of the target of the toxin or through detoxification (Hammond-Kosack and Jones, 1997).

Biotrophic and hemibiotrophic pathogens invade the living host cells and affect host metabolism to help their own growth and reproduction. To establish a compatible interaction, these pathogens have to avoid recognition, suppress activation of plant defence or counter-defend activated defence by detoxification of antimicrobial compounds (Schulze-Lefert and Panstruga, 2003). The biotrophic mode of action is represented by the frequent formation of green islands on senescing leaves surrounding the infection site of fungal rusts and mildew. While biotrophs generally cause disease on only one or few related plant species and do not kill the host, hemibiotrophic fungi such as *Phytophthora* and *Colletrotrichum* kill surrounding plant cells during later stages of infection (Hammond-Kosack and Jones, 1997).

Natural plant protection against such a variety of pathogens is partly based on a number of constitutive barriers already existing in the plant before the actual

attack. The result of the combination of all these barriers is known as constitutive resistance. In addition, when attacked, plants can also activate various protective mechanisms, phenomenon referred to as induced or acquired resistance.

- **Constitutive resistance**

Constitutive resistance factors, also known as preformed factors, are morphological and chemical entities present in plants prior to pathogen attack. The cuticle is considered to be a morphological constitutive first line of defence against pathogens. Constitutive antifungal substances are pre-infection plant metabolites that exist in their biologically active forms or as inactive precursors and are activated only by pathogenic presence (Osbourn, 1996; Walton, 1997). While in some plants, they are normally present in concentrations high enough to inhibit most fungi, in others, the concentration of these antifungal compounds may normally be low but could increase significantly upon infection to inhibit fungal growth (Grayer and Harborne, 1994).

It is believed that some preformed compounds are present in the outer layers of the plant tissues but generally these compounds are compartmentalised predominantly in vacuoles and other organelles of healthy plants (Prusky and Keen, 1993; Osbourn, 1996). Therefore, the concentrations that are encountered by an invading fungus will depend on the extent to which the fungus causes tissue damage (Prusky, 1997). Biotrophs may avoid the release of preformed compounds by minimising the damage caused by infection, whereas nectrotrophs are likely to cause substantial release of these constitutive compounds. In addition, the nature and also the level of preformed antifungal compounds to which a pathogen is exposed could vary, depending on host age and environmental conditions (Osbourn, 1996).

Although citrus plants may be heavily infected with *Penicillium digitatum and P. italicum*, their fruits are resistant to these pathogens during growth by the

release of citral, a preformed compound that, however, decreases in older fruits, allowing decay to occur rapidly (Ben-Yehoshua *et al.*, 1995; 1997; Rodov *et al.*, 1995). Many kinds of preformed antifungal compounds were also found in the peel of pomelo fruits, increasing their resistance to disease after harvest (Kumpoun *et al.*, 1997). Resistance to *Alternaria alternata* and *Colletotrichum gloeosporioides* in unnripe mango fruits and avocado was facilitated by preformed compounds (Droby *et al.*, 1987; Prusky *et al.*, 1990, 1997; Kobiler *et al.*, 1993). Gramineceous plants also produce constitutive antifungal compounds. These include saponins in oats (Walton, 1997), an alkaloid in barley and fatty acids in rice (Grayer and Harborne, 1994).

Other studies have demonstrated the possible involvement of preformed proteins in plant resistance. Cell walls of unrelated plants such as apples and tomato (Stotz *et al.*, 1994) have proteins that could inhibit polygalacturonases (PG) of fungal origin (Yao *et al.*, 1995). Although the presence of pre-formed antifungal compounds has been demonstrated in diverse plants, it is very seldom that the pathogen attack is actually stopped through using only constitutive resistance mechanisms (Osbourn, 1996). Generally, the constitutive systems are followed by initiation of induced resistance but it is also likely that both preformed and induced antifungal compounds occur simultaneously, as is the case in certain fruits such as lemon where four preformed antifungal compounds act as the first line of defence against *Penicillium digitatum* while the phytoalexin, scoparone, is considered another line of defence (Adikaram *et al.*, 1995).

- **Induced resistance**

Induced cellular defence prevents further microbial colonisation after structural and/or constitutive barriers have been breached. Programmed cell death is the first response to pathogen invasion and occurs in cells that are in direct contact with the pathogen (Jones, 2001). Secondary responses are induced in adjacent cells surrounding the infection site and are an immediate response to diffusible

signal molecules called elicitors. A macroscopic manifestation of pathogen-induced programmed cell death is the hypersensitive reaction. If pathogenic infection is still present, systemic acquired resistance (SAR) is then hormonally induced throughout the entire plant (Hammerschmidt, 2003a).

Pathogen recognition occurs rapidly and results in several early responses including membrane aberrations (lipid peroxidation, K^+ and Cl^- efflux, Ca^{2+} influx and depolarisation of membranes), activation of kinase cascades and the generation of reactive oxygen species (also called an oxygen burst) (Jabs *et al.*, 1997; Montesano *et al.*, 2003). In later stages, changes in plant cell ultrastructure such as condensation and vacuolation of the cytoplasm while retaining functional mitochondria have been observed. While endonucleases degrading DNA and RNA could also be induced, cleavage of chromosomal DNA into large fragments is also common (Greenberg, 1997).

Through these events, the pathogen could become trapped in dead cells, thereby preventing it from spreading from the site of infection. In addition to these local responses, to inhibit further pathogen invasion, the surrounding cells change their cell wall composition (which inhibits possible penetration) and will also synthesise *de novo* antimicrobial compounds such as phytoalexins (Hammerschmidt, 1999) and pathogenesis-related (PR) proteins (Greenberg *et al.*, 1994; van Loon and van Strien, 1999).

If infection still occurs, then signal molecules (elicitors) elicit secondary responses in nearby cells that lead to the establishment of systemically acquired resistance.

The origin of elicitors can be of pathogen source (exogenous) and compounds released from plants by the direct action of the pathogen (endogenous) (Nishi, 1994). Exogenous elicitors are of a variety of compound classes such as oligosaccharides, peptides, proteins and lipids (West, 1981; Nishi, 1994; Mikes

et al, 1998; Nurenberger and Nennstiel, 1998; Montesano *et al.*, 2003). Endogenous signal molecules include salicylic acid (SA), jasmonic acid (JA) and ethylene (Druner *et al.*, 1997; Murphy *et al.*, 1999). Salicylic acid has been shown to play a central role as a signalling molecule involved in both local defence reactions and in the induction of systemic resistance. It regulates many pathogenesis-related genes, including those encoding for PR1 and most acidic PR proteins, many of which are antifungal hydrolases targeted to the plant cell wall (Pieterse and van Loon, 1999). Jasmonic acid has been found to be essential in a number of defence mechanisms against tobacco hornworm larvae, the fly *Bradysia*, and the fungus *Phythium mastophorum* (Reymond and Farmer, 1998). Low concentrations of JA induces proteinase inhibitors, thionin, osmotin, proline-rich cell wall protein and also different enzymes involved in plant defence reactions such as phenylalanine ammonia-lyase (PAL). The induction of such proteins in plants suggests a possible role of JA in halting or minimising pathogenic growth (Sticher *et al.*, 1997). In addition to SA and JA, ethylene could be involved in plant resistance by inducing some PR proteins such as β-1,3-glucanase and chitinase (Sticher *et al.*, 1997). Other studies have demonstrate the ethylene regulation of many different processes in plants and its implication in defence responses, by controlling the amplitude and development of disease symptoms after inoculation with virulent bacteria or fungal pathogens (Mur *et al.*, 1997; Moore *et al.*, 1999).

In addition to SA, JA and ethylene, other molecules such as reactive oxygen species (ROS) are believed to be involved in plant defence signalling (Montesano *et al.*, 2003). The oxidative burst, a rapid production of large amounts of ROS, is one of the earliest observable aspects of the plant defence strategy (Goodmann, 1994). Most cells have the ability to produce and detoxify ROS. In normal conditions, ROS are evident in cells as inevitable by-products formed as a consequence of successive one-electron reduction of molecular O_2. Generally, cells maintain low levels of ROS through protective mechanisms (Wojtaszek, 1997a), but when under microbial attack, these protective

mechanisms are overridden by a rapid oxidative burst that produces ROS. These ROS function as protectants against invading pathogen and also signal the activation of further plant defence reactions such as systemic acquired resistance (SAR) (Bolwell and Wojtaszek, 1997; Wojtaszek, 1997b).

The time needed for the establishment of SAR depends on both the plant and the type of pathogen. The induction of SAR could vary from seven hours after microbe attack (Smith and Metraux, 1991) to two-three weeks (Cohen and Kuc, 1981). Once established, SAR can last over weeks and consists of an array of mechanisms including lignification and structural barriers, production of antifungal compounds/phytoalexins and pathogenesis-related (PR) proteins (Sticher *et al.*, 1997).

There is evidence that lignification plays an important role in plant disease resistance in many different ways but little is known about its role in induced resistance (Morschbacher *et al.*, 1990; Carver *et al.*, 1994). Lignin is normally an important constituent of woody plant cell walls. This biological polymer could possibly make the plant cell wall more resistant to degradation by enzymes produced by an invading pathogen. In addition, a lignified wall could also act as a barrier that prevents free nutrient flow to the pathogen. Also, lignin precursors have a toxic effect directly on the pathogen or they can bind to fungal cell walls, making them more rigid and impermeable, hence minimising pathogen invasion and/or uptake of nutrients (Sticher *et al.*, 1997). Through stopping the direct pathogen advance inside plant tissues or by slowing down penetration, the lignification process allows the plant to activate further defence mechanisms such as production of antifungal compounds/phytoalexins and PR-proteins.

Phytoalexins are low molecular weight antimicrobial secondary metabolites that are synthesized in a highly localised area around the site of infection and are either absent from, or present only in negligible amounts, in healthy plants

(Whitehead and Threlfall, 1992). Phytoalexin accumulation is classically associated with the hypersensitive response (Hammerschmidt, 2003b).

The chemical structures of many phytoalexins that have been isolated so far from a wide range of plants (but not from plants with recalcitrant seeds) appears be diverse (see details in Table 2 in Introduction) (Hammerschmidt, 2003c). While some plants synthesize more than one chemical class of phytoalexin, other plants belonging to a particular family produce phytoalexins via a common pathway and thus the chemical structure of these compounds, whilst diverse within the plant kingdom, tends to be uniform within a plant family (Hammerschmidt, 1999). For example, all phytoalexins from cruciferous plants such as *Brassica rapa, Rahanus sativus* and *Camelina sativa* have been reported to posses a similar chemical structure, an indol or oxindole nucleus with an appendage containing one or two sulphur atoms (Rouxel *et al.*, 1995). Phytoalexins from Vitaceae seem to also constitute a rather restricted group of molecules (viniferins and resveratrol) belonging to stilbene family (Jeandet *et al.*, 2002).

Although there are few studies where the phytoalexin concentration of the site of infection has been quantified, the role of phytoalexins in inhibiting pathogen development in some host tissue-pathogen interactions is still unclear (Hammerschmidt, 2003c). However, the extensive number of papers published on the elicitation and accumulation of phytoalexins in diverse plants suggest that phytoalexins and PR-proteins are the most common plant defence mechanisms.

Whereas induction of phytoalexins and cell wall rigidification are local defence reactions, accumulation of pathogenesis-related (PR) proteins extends into non-infected plant tissues that, upon challenge, exhibit acquired resistance (van Loon, 1997). Pathogenesis-related proteins have been classically divided into five groups, PR-1, -2, -3, -4 and –5, based on serological and amino acid

sequence analysis. In addition, a further nine groups of proteins have been suggested for inclusion as PR-proteins, bringing the total to fourteen groups (van Loon and van Strien, 1999). Each of the classical five groups has one basic subclass that is present in the plant cell vacuole and one acidic subclass that is generally found in the extracellular space (Selitrennikoff, 2001). Although each group has members with antifungal activity, the mechanisms of antifungal action of only the PR-2 and PR-3 groups of proteins have been clearly identified.

PR-2 proteins (β-glucanases) have β-1,3-endoglucanase activity *in vitro*. Plant β-glucanases have been classified into three structural classes that differ in sequence from each other by ≈ 40-50% (Ward *et al.*, 1991). The class I are basic proteins that have been localised in the vacuole of mesophyll and epidermal cells (Keefe *et al.*, 1990). Class II and III β-glucanases are acidic, extracellular isoforms (Payne *et al.*,1990). The antifungal activity of plant β-1,3-endoglucanases occurs by PR-2 proteins hydrolysing the structural β-1,3-glucan that is present in the fungal cell wall, especially at the hyphal apex of filamentous fungi where the glucan is most exposed. This mechanism results in weakening of pathogen cell walls, eventually leading to cell lysis and death (Selitrennikoff, 2001).

PR-3 proteins (chitinases) have shown *in vitro* chitinase activity and have been divided into five groups: class I contain a cysteine-rich domain of 40 amino acids; class II have similar amino acid sequence to class I proteins; class III do not have any similarity to any other classes; class IV proteins resemble class I but are smaller and class V resemble the amino acid sequence of bacterial exochitinases (Selitrennikoff, 2001). PR-3 proteins that are endochitinases weaken pathogen cell wall through cleavage of cell wall chitin polymers. Not surprisingly, PR-2 (β-1,3-endoglucanases) and PR-3 (chitinases) proteins act synergetically in inhibiting fungal growth (Selitrennikoff, 2001).

Both β-1,3-endoglucanases and chitinases have been reported from a variety of monocotyledonous and dicotyledonous plants, in different tissues and life-stages, including seeds of tomato (Wu *et al.*, 2001), tobacco (Leubner-Metzger *et al.*, 1996), barley (Jacobsen *et al.*, 1990), maize (Neucere *et al.*, 1991; Huyuh *et al.*, 1992) and cucumber (Majeau *et al.,* 1990). However, their occurrence in recalcitrant seeds has not been investigated.

The current study was undertaken to investigate possible defence mechanisms of the highly recalcitrant seeds of *Avicennia marina* when infected with *Fusarium moniliforme* during hydrated storage. The presence of constitutive and induced compounds as well as of β-1,3-endoglucanases and chitinases was assessed, evaluated and related to the short-term storage responses of these seeds.

3b. MATERIALS AND METHODS

3b.1 Seed sampling

The present study was undertaken on fresh and clean hydrated-stored and clean stored–infected seeds of *A. marina*. Embryonic axis and cotyledonary tissue were obtained from seeds sampled as described in section 2b.3.2 (fresh, four day stored clean [4d clean], ten days stored clean [10d clean], ten days stored infected [10d infected], eight days after infection of clean seeds stored for four days [4d-8] and eight days after infection of clean seeds stored for ten days [10d-8].

3b.2 Biochemical assessment of possible antifungal activity

3b.2.1 Extractives from axes and cotyledonary material

The axes and cotyledons were first separated from the seed. They were then extracted with methanol on a Labcon shaker for four days yielding on average, after evaporation of solvent, 33 g of crude axis extracts and 323 g of crude cotyledon extracts.

3b.2.2 Column and thin layer chromatography

Column chromatography is commonly used for separation of compounds from crude extracts (Ingham, 1980). Thin layer chromatography (T.L.C.) and anisaldehyde spray reagent are used for detection of compounds in fractions obtained during column chromatography. Column chromatographic separation of the crude extracts was achieved using hexane:ethyl acetate followed by dichloromethane:methanol step gradients (on the same column and extract) as the mobile phase during the separation (Table 3.1). Twenty fractions of 40 ml each were collected for each solvent ratio (Table 3.1).

Silica gel (0.2 mm) containing fluorescent indicator (F_{254}) on aluminium-backed plates (Merck: Art 5554) was used for T.L.C. analysis and silica gel (Merck: Art 9385) was used for column chromatography.

The T.L.C. plates were developed using anisaldehyde:conc. H_2SO_4:methanol [1:2:97] as spray reagent, followed by heating.

Table 3.1. Solvent systems used for the separation of compounds from crude extracts via column chromatography

Solvents	Ratio	Fraction number
hexane:ethyl acetate	19 : 1	1 - 20
	18 : 2	21 - 40
	16 : 4	41 - 60
	12 : 8	61 - 80
100% ethyl acetate		81 - 100
dichloromethane:methanol	19 : 1	101 - 120
	18 : 2	121 – 140
	16 : 4	141 – 160
	12 : 8	161 – 180
	8 : 12	181 – 200
	2 : 18	201 – 220
100% methanol		221 – 240

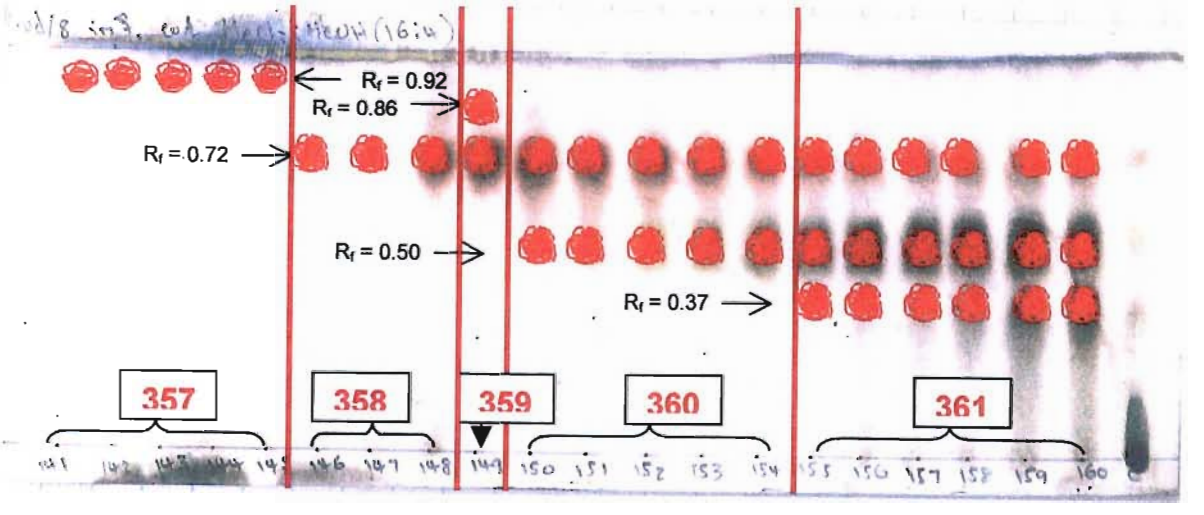
A total of 1440 fractions (240 fractions x 6 treatments) was collected.

During T.L.C. separations, R_f values were used to detect the presence of possible compounds. An R_f value is the ratio of the distance travelled by the compound to that of the solvent front (Stock and Rice, 1974) (for example, in Fig, 3.1, compound 3 had the R_f value of 0.72 = 4.7cm/6.5cm). R_f values are dependent upon the stationary phase, the mobile phase (the eluant), the

polarity of the compounds and also the conditions under which the T.L.C. was carried out (saturation inside vessel, angle of the T.L.C. plate and amount of solvent used). It is therefore difficult to reproduce R_f values. An R_f value from a repeated T.L.C. chromatogram may be as different as ± 0.10 of the initial R_f value.

Subsequent to determination of R_f values, the 1440 fractions were combined into 368 samples based on their R_f values in a particular solvent system (Tables 3.2-3.25). For example, in the cotyledon extract of 10d-8 seeds that was eluted with dichloromethane and methanol (16:4), five compounds were evident from their R_f values (Fig. 3.1). Fractions 141 to 145 contained the compound with R_f value of 0.92 and were combined as sample 357. Fractions 146 to 148 were combined to form sample 358 as they contained one compound with R_f value of 0.72. While sample 359 was represented only by fraction 149 with two compounds with R_f values of 0.86 and 0.72, sample 360 was a result of combining fractions 150-154 with two compounds, 0.72 and 0.52. Fractions 155 to 160 contained three compounds with R_f values of 0.72, 0.50 and 0.37 and were combined as sample 361.

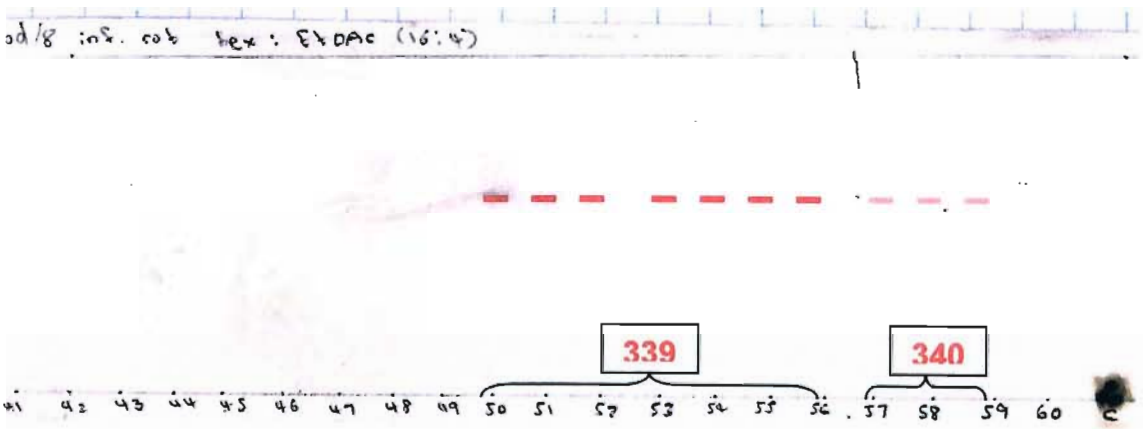
Fig. 3.1. Chromatogram of 10d-8 cotyledon extract eluted with dichloromethane and methanol in a 16:4 ratio (for easier interpretation, each R_f value was highlighted).



Different compounds may have similar R_f values. In these cases, the colour of the compound as indicated by the spray reagent may be used as a tool to differentiate between different compounds. Thus, in Fig. 3.2, sample 339 and 340, both contained a compound with the same R_f, but in each sample, the compound developed a different colour on the T.L.C.

The number of compounds detected for any treatment is the total number of all the samples. When the number of compounds is less than the number of samples (e.g. Table 3.2: 10d-8 had 3 samples with 2 compounds), this is a result of over-interpretation of the T.L.C. data (based on colour and R_f values). It is likely that some of the separate samples are in reality not different.

Fig. 3.2. Chromatogram of 10d-8 cotyledon extract eluted with hexane and ethyl acetate in a 16:4 ratio (for easier interpretation, each R_f value was highlighted).



3b.2.3 Detection of antifungal activity on thin layer chromatograms

Bio-assay methods using *Cladosporium* for visualisation of growth inhibitory substances on thin layer chromatograms have become standard procedures for the detection of antifungal compounds in plant extracts. This fungus is widely used as its dark mycelium renders obvious zones of inhibition (Homans and Fuchs, 1970).

the case of the current study, however, *Fusarium moniliforme* was used to ect possible antifungal activity of the 368 combined fractions. This fungus ; a white, light-coloured mycelium, similar to the T.L.C. plate background, king it difficult to identify inhibition zones or even to visualise the mycelium wth. To overcome this, an adapted method was used (Woodward and arce, 1985):

3b.2.3.1 Culture of *Fusarium moniliforme*

Cultures of *F. moniliforme* isolated from *A. marina* seeds were sub-cultured in a mineral and 30% aqueous glucose solution (10:1). After incubation for 14 days at 22 °C, mycelial mats were removed and fragmented in 500 ml fresh mineral/glucose solution, using an MSE top-drive homogeniser (full speed for 30 seconds).

Each of the 368 combined fractions (details in section 3b.3) was spotted (three times) on T.L.C. plates. The plates were then sprayed with the resultant suspension of spores and mycelium and incubated in moist, sterile chambers at 25 °C for five days.

Mineral solution:

- KH_2PO_4 7 g
- Na2HPO4.2H_2O 3 g
- KNO_3 4 g
- MgSO4.7H_2O 1 g
- NaCl 1 g
- Tap water 1000 ml

3b.2.3.2 Detection of fungal mycelium and growth inhibition zones

The mycelium growth and/or growth inhibition was visualised in ultraviolet (UV) light, using a fluorescence brightener Calcoflour White M2R (Cyanamid, Sigma, St. Louis, MO, USA), that has high affinity for β-linked carbohydrate polymers including those in fungal cell walls (Peterson *et al.*, 1981).

The incubated T.L.C. plates were sprayed with a 0.15 g/300 ml solution of Calcoflour in 0.1M phosphate buffer, pH 8.0. When illuminated with UV light (254 and/or 366 nm), the fungal mycelium had a light-coloured fluorescence while the zones of fungal inhibition appeared as darker areas.

3b.2.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H NMR and ^{13}C NMR spectra were recorded with a 400 MHz Varian Unity Inova spectrometer using either $CDCl_3$ or CD_3OD as solvent. The ^{1}H NMR spectra were recorded at 400 MHz and ^{13}C spectra at 100 MHz. The spectra were referenced against the central line of the deuteriochloroform signal at δ_c 77.0, the deuteriochloroform singlet at δ_H 7.24, the deuteriomethanol signal at δ_c 49.0 or the deuteriomethanol signal at δ_H 3.34, using TMS (tetramethylsilane) as an internal standard.

3b.3 Detection of β-1,3-glucanase and chitinase activity

3b.3.1 Protein extraction and determination

Protein extraction was carried out using embryonic axes of *A. marina* seeds that have been treated as described previously in Material and Methods, Chapter 2. The only exception was that enzyme activities were tested in seeds stored infected for eight days (8d infected) instead of 10 days (10d infected).

A modified method Farrant *et al.* (1992a) was used to alleviate adverse effects of polyphenolics:

- Frozen axes of *A. marina* (0.5 g) were ground in liquid nitrogen to a fine powder
- One gram of insoluble polyvinylpyrrolidone (Sigma, St. Louis, MO, USA) was mixed with the sample
- This was then transferred into a centrifuge tube and suspended in 7 ml of 50 mM Tris-HCl buffer (pH 7.3) containing 10 mM β-mercaptoethanol and 2 mM phenylmethylsulfonyl fluoride.
- The suspension was mixed thoroughly for one minute and incubated on ice for 10 minutes to extract the soluble proteins.
- After centrifugation for 30 minutes at 15 000 g, the supernatant that contained the total soluble protein was collected.

A. marina seeds contain low molecular mass sugars that are a major form of carbohydrate reserve (Farrant *et al.*, 1992a). Thus, to remove the interference caused by these low molecular mass sugars, the supernatant was purified by gel filtration on a PD-10 column packed with Sephadex G-25 (Pharmacia, Germany) and used for enzyme determination.

The protein concentration was determined in triplicate using the Bradford (1976) method, using Bradford reagent (Bio-Rad, Philadelphia, PA, USA) and bovine gamma globulin (Bio-Rad) as the standard.

3b.3.2 β-1,3-Glucanase assay

This assay was carried out according to Fink *et al.* (1988) by measuring the rate of reducing sugar production with laminarin (Sigma) as the substrate.

A standard curve relating absorbance at 540 nm to glucose concentration was used to calculate specific β-1,3-glucanase activity, which was expressed as mg glucose mg^{-1} protein min^{-1}.

3b.3.3 Chitinase assay

Chitinase activity was determined using the Wirth and Wolf (1990) method and was determined spectrophotometrically with dye-labelled CM-Chitin RBV (Loewe Biochemica GmbH, Sauerbach, Germany) as substrate.

The absorbance (A_{550nm}) was taken as a measure of enzyme activity and was expressed as A_{550nm} mg^{-1} protein min^{-1}.

All enzyme assays were performed in triplicate. The statistical programme Sigma Plot 4.0 (Statistical Solutions, Saugus, MA, USA) was used for calculating standard deviations of treatment means.

3c. RESULTS

3c.1 Fraction combination and assessment of possible compounds present during different extract elutions

When axes extracts were eluted with hexane and ethyl acetate (19:1) (Table 3.2), their fractions combined into two samples per treatment, except the 4d clean (only one sample) and three samples of 10d-8. While the 4d-8 sample demonstrated only one possible compound and 10d-8, two, all other samples appeared to produce three compounds, each.

Table 3. 2. Elution of axis extracts with hexane and ethyl acetate in a 19:1 ratio

TREATMENT	AXES			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	11-13, 14-20	1,2	3	0.72 0.52 0.43
4d clean	15-20	47	3	0.68 0.54 0.43
10d clean	10-14, 15-20	95, 96	3	0.58 0.43 0.12
10d infected	12-15, 17-20	160, 161	3	0.92 0.75 0.62
4d-8	14-16, 17-20	236, 237	1	0.73
10d-8	10, 11-13, 14-20	300, 301, 302	2	0.68 0.54

With hexane:ethyl acetate (18:2) as the mobile phase, the fractions of axis extracts of 10d clean, 10d infected and 4d-8 were each combined to form three samples, in contrast to the two samples each from fresh, 4d clean and 10d-8 extracts (Table 3.3). Four (fresh, 4d clean, 10d infected and 4d-8) out of six treatments demonstrated the presence of three possible compounds/treatment.

Table 3. 3. Elution of axis extracts with hexane and ethyl acetate in a 18:2 ratio

TREATMENT	AXES			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	21-30, 32-40	3, 4	3	0.90 0.55 0.41
4d clean	21-34, 35-40	48a, 48b	3	0.46 0.30 0.14
10d clean	21-23, 24-28, 29-40	97, 98, 99	2	0.55 0.30
10d infected	21-26, 27-34, 35-40	162, 163, 164	3	0.65 0.45 0.19
4d-8	23-25, 31-34, 37-40	238, 239, 240	3	0.59 0.48 0.38
10d-8	21-31, 32-40	303, 304	1	0.47

When hexane and ethyl acetate in 16:4 ratio was used as the solvent system (Table 3.4), with exception of 4d clean and 10d infected treatments that were combined into one and four samples, respectively, three samples were formed by the fractions of the rest of the treatments. Four and five compounds were observed in 4d clean and 10d infected treatments. In contrast, each of the 10d clean, 4d-8 and 10d-8 samples produced only two compounds.

During elution with hexane and ethyl acetate (12:8), only one sample was combined from fractions for fresh and 4d clean material, in contrast to 10d-8 fractions where fractions were combined to form six samples. While 4d clean, 10d infected and 4d-8 samples produced four compounds each, 10d clean and 10d-8 demonstrated the presence of only two compounds (Table 3.5).

Table 3. 4. Elution of axis extracts with hexane and ethyl acetate in a 16:4 ratio

TREATMENT	AXES			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	44-49, 50-56, 57-60	5,6,7	3	0.75 0.46 0.38
4d clean	41-60	49	4	0.97 0.87 0.73 0.45
10d clean	41-48, 49-54, 55-60	100, 101, 102	2	0.48 0.38
10d infected	41-50, 51-53, 54-55, 56-60	165, 166, 167, 168	5	0.97 0.78 0.63 0.52 0.22
4d-8	43-50, 51-53, 54-60	241, 242, 243	2	0.56 0.40
10d-8	41-53, 54-55, 56-60	305, 306, 307	2	0.65 0.54

Table 3. 5. Elution of axis extracts with hexane and ethyl acetate in a 12:8 ratio

TREATMENT	AXES			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	61-72	8	3	0.75 0.63 0.48
4d clean	61-68	50	4	0.77 0.67 0.52 0.21
10d clean	61-65, 66-73, 74-80	103, 104, 105	2	0.65 0.56
10d infected	61-65, 66-68, 69-71, 72-80	169, 170, 171, 172	4	0.77 0.62 0.57 0.19
4d-8	61-71, 72-80	244, 245	4	0.99 0.83 0.74 0.54
10d-8	61-67, 71-72, 73-74, 75, 76, 77-80	308, 309, 310, 311, 312, 313	2	0.87 0.66

In 100% ethyl acetate (Table 3.6), the fractions of 4d-8 and 10d-8 treatments were each combined into a relatively high number of samples (five). The lowest number of samples was observed in fresh and 4d clean material. The 4d clean material demonstrated two compounds while the rest of the treatments showed the presence of three or four compounds.

All axis extracts eluted similarly in dichloromethane and methanol in a 19:1 ratio (Table 3.7). However, fraction combination appeared to be different from formation of one sample in 4d-8 treatment to four samples in 10d infected. While fresh and 4d-8 samples produced only two and one compound, respectively, three to four compounds were evident for the rest of the treatments.

Table 3. 6. Elution of axis extracts with 100% ethyl acetate

TREATMENT	AXES			
	Fraction combination	**Sample no.**	**Number of compounds**	**R_f value**
Fresh	91-92, 93-100	9, 10	4	0.90 0.79 0.66 0.56
4d clean	88-100	51	2	0.92 0.56
10d clean	81-88, 89-91, 92-94, 95-100	A, B, C, D	4	0.94 0.77 0.56 0.46
10d infected	88-91, 92-94, 95-97, 98-100	173, 174,175, 176	4	0.90 0.78 0.66 0.47
4d-8	81-88, 89-91, 92-94, 95-97	246, 247, 248, 249, 250	4	0.92 0.81 0.70 0.52
10d-8	90, 91, 92, 93, 94-96	314, 315, 316 317, 318	3	0.94 0.85 0.70

Table 3. 7. Elution of axis extracts with dichloromethane and methanol in a 19:1 ratio

TREATMENT	AXES			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	101-109, 110-120	11, 12	2	0.48 0.37
4d clean	101-105, 106-118, 119-120	52, 53, 54	4	0.63 0.52 0.44 0.27
10d clean	101-110, 118-120	106, 107	4	0.48 0.36 0.26 0.13
10d infected	101-106, 107-110, 117-118, 119-120	177, 178, 179, 180	3	0.41 0.26 0.15
4d-8	101-120	251	1	0.16
10d-8	101-108, 109-112 113-120	319, 320, 321	3	0.62 0.46 0.35

When eluted with dichloromethane and methanol in a 18:2 ratio (Table 3.8), the fresh material yielded only one sample. In addition, the fractions of 4d clean combined to produce only two samples and the fractions of the rest of the treatments formed at least three to four samples. It was observed that 4d clean and 4d-8 each produced only two compounds while the other treatments showed evidence of three-four compounds.

Fraction combination varied in dichloromethane and methanol in a 16:4 ratio: from formation of one sample in the fresh material to four in 10d clean, 4d-8 and 10d-8 treatments. A similar situation was observed in the number of possible compounds, the sample of fresh material producing only one compound while 4d-8 showed evidence of five compounds (Table 3.9).

Table 3. 8. Elution of axis extracts with dichloromethane and methanol in a 18:2 ratio

TREATMENT	AXES			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	127-140	13	3	0.34 0.23 0.14
4d clean	121-130, 131-140	55, 56	2	0.25 0.14
10d clean	121-126, 127-133, 134-136, 137-140	108, 109, 110, 111	4	0.95 0.72 0.51 0.42
10d infected	121-129, 130-131, 132-135, 136-140	181, 182, 183, 184	4	0.72 0.47 0.33 0.18
4d-8	125-131, 132-135, 136-140	252, 253, 254	2	0.51 0.19
10d-8	125-129, 130-131, 132-140	322, 323, 324	4	0.88 0.74 0.45 0.28

Table 3. 9. Elution of axis extracts with dichloromethane and methanol in a 16:4 ratio

TREATMENT	AXES			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	142-160	14	1	0.28
4d clean	141-145, 146-153, 154-160	57, 58, 59	3	0.96 0.27 0.12
10d clean	141-143, 144-145, 146-152, 153-160	112, 113, 114, 115	2	0.79 0.58
10d infected	141-149, 150-160	185, 186	3	0.60 0.32 0.22
4d-8	141-148, 149-150, 151-153, 154-160	255, 256, 257, 258	5	0.87 0.73 0.49 0.33 0.19
10d-8	141-150, 151-153, 154-157, 158-160	325, 326 327, 328	3	0.79 0.60 0.42

Two to four samples were formed by the combined fractions in all samples that eluted in dichloromethane and methanol (12:8) (Table 3.10). The highest number of compounds was evident in the 10d clean axes extract. In contrast, fresh, 4d clean and 10d-8 produced a maximum of two possible compounds.

Table 3.10. Elution of axis extracts with dichloromethane and methanol in a 12:8 ratio

TREATMENT	AXES			
	Fractions combination	Sample no.	Number of compounds	R_f value
Fresh	161-168, 169-180	15, 16	2	0.53 0.41
4d clean	161-167, 168-174, 175-180	60, 61, 62	2	0.73 0.64
10d clean	162-167, 168-180	E, F	4	0.89 0.71 0.39 0.15
10d infected	161-168, 169-170, 171-174&176, 177-180	187, 188, 189, 190	3	0.85 0.76 0.59
4d-8	161-168, 169-174, 175-180	259, 260, 261	Data not available	
10d-8	161-170, 171&173-180, 172	329, 330, 331	1	0.68

With exception of the fractions of 4d-8 that were combined to form only one sample, two to three samples were formed in the other treatments when eluted with dichloromethane and methanol (8:12). Most of these samples produced a relatively small number of compounds (one-two), the highest being 4d clean that demonstrated four compounds (Table 3.11).

When eluted in dichloromethane and methanol in a 2:18 ratio (Table 3.12) the fractions of these treatments were combined to form a similar number of samples. When compared to the separation of other treatments, this solvent system produced a low number of compounds across treatments, notably the 10d infected, 4d-8 and 10d-8 samples that showed only one compound each.

Table 3. 11. Elution of axis extracts with dichloromethane and methanol in a 8:12 ratio

TREATMENT	AXES			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	181-188, 189-200	17, 18	2	0.62 0.41
4d clean	181-190, 191-200	63, 64	4	0.88 0.68 0.50 0.25
10d clean	181-196, 197-200	116, 117	2	0.81 0.65
10d infected	181-189, 190-191, 192-200	191, 192, 193	2	0.76 0.67
4d-8	181-200	262	2	0.81 0.15
10d-8	181-189, 190-197, 198-200	332, 333, 334	1	0.85

Table 3. 12. Elution of axis extracts with dichloromethane and methanol in a 2:18 ratio

TREATMENT	AXES			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	201-220	19	3	0.85 0.62 0.45
4d clean	201-211, 212-220	65, 66	3	0.91 0.82 0.35
10d clean	201-220	118	2	0.82 0.22
10d infected	201-212, 213, 214-216, 217-220	194, 195, 196, 197	1	0.48
4d-8	201-205, 206-211, 212-220	263, 264, 265	1	0.82
10d-8	201-218	335	1	0.79

In 100% methanol, while the fractions of 4d clean, 10d infected and 4d-8 were combined to form three samples per treatment, the fresh, 10d clean and 10d-8 fractions formed only one sample per treatment (Table 3.13). Similar to previous separation with, dichloromethane and methanol (16:4) (Table 3.12), the elution in 100% methanol also produced a low number of compounds across treatments.

Table 3. 13. Elution of axis extracts with 100% methanol

TREATMENT	AXES			
	Fraction combination	**Sample no.**	**Number of compounds**	**R_f value**
Fresh	221-240	20	2	0.62 0.40
4d clean	221-223, 224-226, 227-235, 236-240	67, 68, 69,70	2	0.93 0.79
10d clean	221-240	119	1	0.85
10d infected	222-226, 227-233, 234-240	198, 199, 200	2	0.73 0.57
4d-8	221-225, 226-230, 231-240	266, 267, 268	2	0.88 0.79
10d-8	221-240	336	1	0.70

When cotyledon extracts were eluted with hexane and ethyl acetate (19:1) (Table 3.14), fractions of 10d infected were combined into only one sample that had only evidence of one compound. Similarly, one sample formation and the presence of one possible compound, was also observed in 10d-8 material. The presence of three compounds was demonstrated by cotyledon extracts of fresh and 4d-8 treatments.

Table 3. 14. Elution of cotyledon extracts with hexane and ethyl acetate in a 19:1 ratio

TREATMENT	COTYLEDONS			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	15-17, 18-20	21, 22	3	0.77 0.54 0.38
4d clean	15-20	71a	2	0.57 0.44
10d clean	1-7, 8-11, 12-13, 14-20	120, 121, 122, 123	2	0.64 0.52
10d infected	13-17	201	1	0.75
4d-8	14-16, 17-19	269, 270	3	0.91 0.75 0.58
10d-8	14-20	337	1	0.56

The cotyledon extracts of all analysed treatments separated in a similar way in hexane and ethyl acetate (18:2) solvent system (Table 3.15). While the fractions of 10d-8 were combined to form one sample, the fractions of fresh and 4d clean material formed two samples/treatment. Three samples resulted in each of the 10d infected and 4d-8 materials. Although 10d clean produced five samples, they contained only two compounds. One compound was evident in 10d-8 sample, in contrast to fresh, 4d clean and 10d infected treatments that showed the presence of three compounds.

During elution with hexane and ethyl acetate in a 16:4 ratio (Table 3.16), the fractions across all treatments were combined to form an average of two to three samples per treatment. With exception of 10d-8 and 4d-8 that produced one and two compounds respectively, three compounds were evident across the rest of the treatments.

Table 3. 15. Elution of cotyledon extracts with hexane and ethyl acetate in a 18:2 ratio

TREATMENT	COTYLEDONS			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	21-30, 31-40	23, 24	3	0.96 0.57 0.41
4d clean	21-30, 31-40	71b, 72	3	0.75 0.63 0.46
10d clean	21-26, 27-31, 32-34, 35-38, 39-40	124, 125, 126, 127, 128	2	0.58 0.26
10d infected	21-29, 30-33, 34-40	202, 203, 204	3	0.79 0.58 0.44
4d-8	22-34, 35-36, 37-40	271, 272, 273	3	0.77 0.58 0.44
10d-8	21-40	338	1	0.53

Table 3. 16. Elution of cotyledon extracts with hexane and ethyl acetate in a 16:4 ratio

TREATMENT	COTYLEDONS			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	41-47, 48-52, 53-60	25, 26, 27	3	0.97 0.51 0.41
4d clean	41-50,51-60	73, 74	3	0.85 0.72 0.40
10d clean	41-56, 57-60	129, 130	3	0.94 0.88 0.36
10d infected	46-53, 54-55, 56-60	205, 206, 207	Data not available	
4d-8	46-50, 53-59	274, 275	2	0.51 0.43
10d-8	50-56, 57-59	339, 340	1	0.59

When hexane and ethyl acetate (12:8) was used as the solvent system, it was observed that the fractions of all analysed treatments formed two to three samples per treatment, with the exception of 4d-8 that formed only one sample. The lowest number of possible compounds (two) was evident in 10d clean samples and the highest (four) in fresh and 4d clean material.

Table 3. 17. Elution of cotyledon extracts with hexane and ethyl acetate in a 12:8 ratio

TREATMENT	COTYLEDONS			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	61-67, 68-70	28, 29	4	0.82 0.67 0.56 0.45
4d clean	61-64, 65-77	75, 76	4	0.72 0.61 0.51 0.34
10d clean	61-68, 69-71, 72-80	131, 132, 133	2	0.69 0.36
10d infected	61-67, 68-69, 70-80	208, 209, 210	3	0.72 0.61 0.49
4d-8	69-76	276	3	0.96 0.77 0.69
10d-8	71-72, 73-74, 75-80	34, 342, 343	3	0.93 0.74 0.29

In 100% ethyl acetate (Table 3.18), the sample formation was different across treatments with fractions of 4d clean forming only one sample and 4d-8 and 10d-8 five samples. While some samples contained only three possible compounds (10d clean, 10d infected and 10d-8), the 4d clean and 4d-8 produced four. The highest number of compounds was observed in the fresh material.

Table 3. 18. Elution of cotyledon extracts with 100% ethyl acetate

TREATMENT	COTYLEDONS			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	89-90, 91-100	30, 31	5	0.93 0.84 0.73 0.58 0.49
4d clean	88-100	77	4	0.96 0.87 0.76 0.58
10d clean	81-87, 88-91, 92-96	134, 135, 136	3	0.85 0.74 0.49
10d infected	88-92, 93-94, 95-97, 98-100	211, 212, 213, 214	3	0.92 0.79 0.51
4d-8	90-91, 92-94, 94-96, 97-98, 99-100	277, 278, 279, 280, 281	4	0.85 0.68 0.52 0.42
10d-8	91, 92, 93-95, 96-98, 99-100	344, 345, 346, 347, 348	3	0.87 0.70 0.58

One to four samples were formed through fraction combinations across treatments during elution with dichloromethane and methanol (19:1) (Table 3.19). In addition, the lowest number of compounds was evident in 4d clean (one compound) and in 4d-8 and 10d-8 that had two. The fresh sample produced four compounds.

Table 3. 19. Elution of cotyledon extracts with dichloromethane and methanol in a 19:1 ratio

TREATMENT	COTYLEDONS			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	101-107	32	4	0.52 0.35 0.25 0.13
4d clean	101-107, 108-120	78,79	1	0.40
10d clean	101-103, 104-106, 107-108, 115-120	137, 138, 139, 140	3	0.58 0.43 0.34
10d infected	101-103, 104-108	215, 216	3	0.42 0.26 0.14
4d-8	101-116	282	2	0.16 0.05
10d-8	101-104, 105-109, 110-112, 113-120	349, 350, 351, 352	2	0.45 0.31

During elution of cotyledon extracts with dichloromethane and methanol in 18:2 ratio (Table 3.20), two to four samples were formed in each of the treatments and two to three compounds were produced by extracts eluted in this solvent system.

While the fractions of fresh material were combined to form only two samples, with dicholoromethane:methanol (16:4), the highest number of combinations occurred in 10d clean and 10d-8 treatments. Three possible compounds were generally produced by different treatments, however, while 4d-8 showed evidence of only one compound the 10d-8 samples produced five compounds (Table 3.21).

Table 3. 20. Elution of cotyledon extracts with dichloromethane and methanol in a 18:2 ratio

TREATMENT	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	125-127, 129-130, 131-140	33, 34, 35	3	0.55 0.45 0.36
4d clean	122-127, 128-140	80, 81	3	0.49 0.23 0.14
10d clean	122-124, 125-127, 128-134, 135-139	141, 142, 143, 144	3	0.54 0.43 0.25
10d infected	129-132, 133-140	217, 218	2	0.52 0.40
4d-8	128-132, 133-140	283, 284	2	0.61 0.52
10d-8	121-129, 130-134, 135-137, 138-140	353, 354, 355, 356	2	0.75 0.51

Table 3. 21. Elution of cotyledon extracts with dichloromethane and methanol in a 16:4 ratio

TREATMENT	Fraction combination	COTYLEDONS Sample no.	Number of compounds	R_f value
Fresh	141-147, 148-160	36, 37	3	0.32 0.26 0.10
4d clean	141-149, 150-157, 158-160	82, 83, 84	2	0.96 0.29
10d clean	141-142, 144-147, 148-150, 152-153, 154-160	145, 146, 147, 148, 149	3	0.92 0.77 0.62
10d infected	143-145, 146-148, 149-151, 152-160	219, 220, 221, 222	3	0.73 0.40 0.27
4d-8	141-148, 149-155, 156-160	285, 286, 288	1	0.80
10d-8	141-145, 146-148, 149, 150-154, 155-160	357, 358, 359, 360, 361	5	0.92 0.86 0.72 0.50 0.37

The sample combination varied with dichloromethane and methanol (12:8) as the eluant. The fractions of 4d clean and 10d-8 formed only one sample while the fractions of fresh material were combined to form four samples. The maximum number of compounds produced by each treatment was three, in fresh and 10d clean and one compound was evident in 10d infected and 10d-8 samples (Table 3.22).

Table 3.22. Elution of cotyledon extracts with dichloromethane and methanol in a 12:8 ratio

TREATMENT	COTYLEDONS			
	Fraction combination	**Sample no.**	**Number of compounds**	**R_f value**
Fresh	161-168, 169-170, 171-173, 174-180	38, 39, 40, 41	3	0.64 0.45 0.33
4d clean	161-180	85	2	0.81 0.72
10d clean	161-170, 171-180	150, 151	3	0.94 0.77 0.59
10d infected	161-168, 169-180	223, 224	1	0.73
4d-8	161-165, 166-171, 172-180	289, 290, 291	2	0.77 0.56
10d-8	161-180	362	1	0.74

Most treatments had fractions that were combined in three samples during elution with dichloromethane and methanol (8:12) (Table 3.23). The extract of the fresh material produced the highest number of compounds in this solvent system, in contrast to 10d-8 that produced only one compound.

Table 3. 23. Elution of cotyledon extracts with dichloromethane and methanol in a 8:12 ratio

TREATMENT	COTYLEDONS			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	181-184, 185-200	42, 43	3	0.82 0.68 0.59
4d clean	181-188, 189-191, 192-194, 195-200	86, 87, 88, 89	2	0.82 0.76
10d clean	181-186, 189-194, 195-200	152, 153, 154	2	0.71 0.53
10d infected	181-182, 183-194, 195-200	225, 226, 227	2	0.65 0.54
4d-8	195-200	292	2	0.75 0.38
10d-8	181-189, 190, 191-200	363, 364, 365	1	0.75

When cotyledon extracts of different treatments were eluted with dichloromethane and methanol in a 2:18 ratio (Table 3.24), the highest number of samples (five) was formed in 10d infected. The fresh, 4d clean and 10d-8 had fractions that combined to form only two samples/treatment. The number of present compounds was very similar to previous solvent system (dichloromethane and methanol 8:12) where three compounds were produced by fresh material and only one by 10d-8 sample.

When 100% methanol was used as solvent, fraction combinations were different, with fresh and 10d-8 fractions forming only one sample/treatment, 10d clean forming two samples, 4d clean forming three and 10d infected and 4d-8 forming four samples each. Only 10d infected and 10d-8 samples produced only one compound. The highest number of compounds (four) that was produced using this solvent system was evident in the 4d-8 sample.

Table 3. 24. Elution of cotyledon extracts with dichloromethane and methanol in a 2:18 ratio

TREATMENT	COTYLEDONS			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	201-214, 215-220	44, 45	Data not available	
4d clean	201-211, 212-220	90, 91	3	0.94 0.85 0.29
10d clean	201-214&216, 215, 217-220	155, 156, 157	2	0.87 0.25
10d infected	201-210, 211-212, 213-216, 217, 218	227b, 228, 229, 230, 231	2	0.70 0.60
4d-8	201-205, 206-211, 212-220	293, 294, 295	2	0.72 0.61
10d-8	201-212, 213-220	366, 367	1	0.73

Table 3. 25. Elution of cotyledon extracts with 100% methanol

TREATMENT	COTYLEDONS			
	Fraction combination	Sample no.	Number of compounds	R_f value
Fresh	221-240	46	2	0.67 0.49
4d clean	221-222, 223-236, 237-240	92, 93, 94	3	0.94 0.85 0.29
10d clean	221&228, 222-240	158, 159	2	0.78 0.38
10d infected	221-222&225, 223-224 226-238, 239-240	232, 233, 234, 235	1	0.56
4d-8	221-223, 224&228-230, 225-227, 231-240	296, 297, 298, 299	4	0.73 0.61 0.40 0.31
10d-8	221-240	368	1	0.73

Looking at sample formation, it was observed that when using hexane and ethyl acetate as solvent system, in many instances, the fractions of 4d clean axes formed only one sample. In the more polar solvent system of dichloromethane:methanol, in many cases, only one sample was formed by fresh, 10d clean and 10d-8 axes material. Throughout cotyledon sample

separations it was noticed that in some occasions, only one sample was formed by fresh, 4d clean, 4d-8 and 10d-8 fractions.

In contrast to forming one sample, the highest number of samples formed by fractions of one treatment was the case of 10d-8 axes eluted in hexane and ethyl acetate (12:8) that formed six samples (Table 3.5). Another high number of samples, five, was produced by 4d-8 and 10d-8 fractions of both axis and cotyledon materials when separated in 100% ethyl acetate (Table 3.6 and Table 3.18 respectively). Cotyledons of 10d clean and 10d-8 also produced five samples when dichloromethane and methanol was used in a 16:4 ratio (Table 3.21) and of 10d infected treatment when separation occurred in 2:18 dichloromethane and methanol (Table 3.24).

When analysing the number of compounds produced throughout the present study, it was evident that the highest number of compounds produced by any one treatment was five. This was the case of 10d infected axis samples that were separated with hexane and ethyl acetate (16:4) (Table 3.4). In addition, when dichloromethane and methanol were used in a 16:4 ratio, both axes (4d-8 treatment, Table 3.9) and cotyledon (10d-8 treatment, Table 3.21) samples produced five compounds. The only other instance of five compounds produced by one treatment was demonstrated by fresh cotyledons samples when 100% ethyl acetate was used as solvent system (Table 3.18).

The 10d-8 samples of both axis and cotyledon material exhibited the lowest compound production irrespective of the entire separation process (Tables 3.2-3.25).

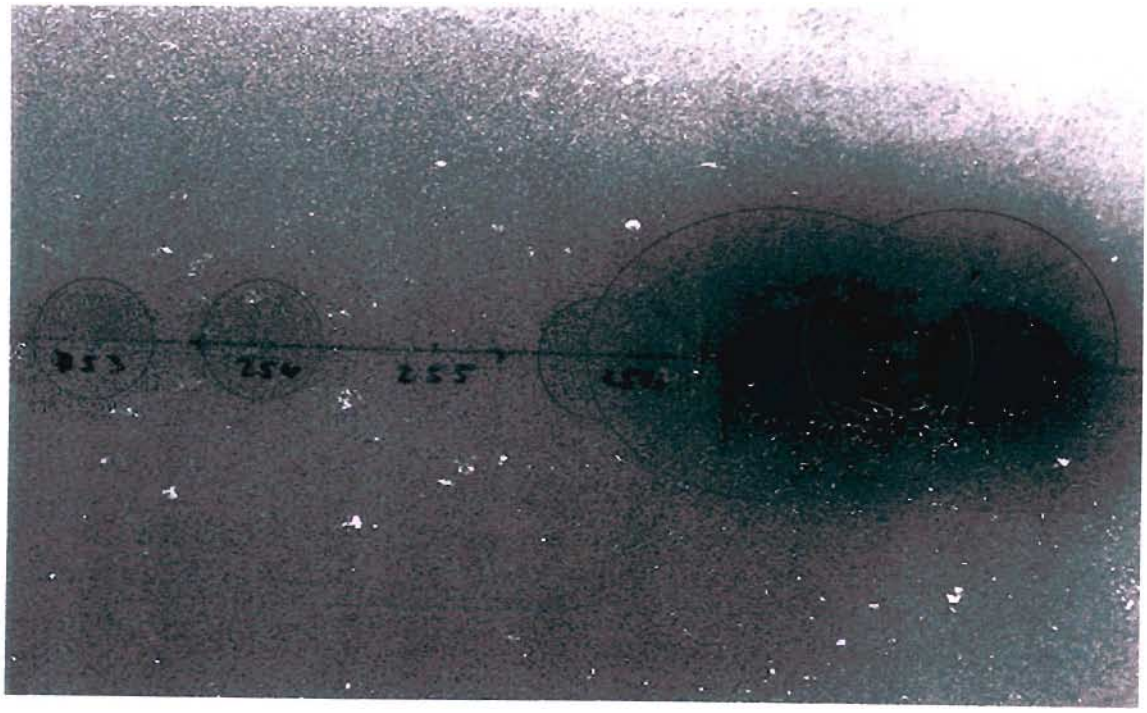
While the highest number of compounds was demonstrated by all cotyledon treatments when 100% ethyl acetate was used as solvent system (Table 3.18), the lowest number was produced by all axes treatments in 100% methanol (Table 3.13). Since methanol is a much more polar solvent than ethyl acetate, it

can be concluded that more compounds with intermediate polarity was produced than compounds like polyphenolic compounds having high polarity.

3c.2 Detection of antifungal activity

A number of samples produced zones of inhibitory activity that were clearly observed as dark areas against the light-coloured fluorescence of the fungal mycelium. The degree of inhibition varied, as was the case of the samples of axes of 4d-8 where samples 253, 254 and 256 produced smaller inhibitory areas in contrast to samples 262 and 263 that exhibited large inhibition zones (Fig. 3.3).

Fig. 3.3. T.L.C. plate of 4d-8 axes samples that demonstrated antifungal activity against *Fusarium moniliforme*.



The axis extracts of fresh and 4d clean treatments had only two and three samples that showed antifungal activity against *F. moniliforme* (Table 3.26). In

contrast, 4d-8 material demonstrated the highest number(17) of combined fractions with antifungal activity. In addition, the fungal growth was significantly inhibited by axes of 10d clean, 10d infected, and 10d-8, which each contained 11 antifungal samples.

The cotyledonary material showed less fungal inhibitory activity across samples, with marked activity being evident in 10d infected cotyledons (14 samples). It was observed that while fresh, 4d clean and 10d clean material had only one sample that produced fungal inhibition zones, each of the 4d-8 and 10d-8 treatments contained eight antifungal samples (Table 3.27).

Step gradient solvent systems were used in detection of possible compounds in different samples, therefore compounds in different samples, especially those samples that were combined in successive gradient systems may be similar because a compound may be detected by several solvent systems. Thus, a particular compound may have different R_f values in different solvent systems (as the polarity of the solvent system increases, so does the R_f of the compound), making it difficult to assess the exact number of compounds across solvents. However, compounds that appeared throughout hexane:ethyl acetate system may be different from those evident during elution with dichloromethane:methanol. For this reason, the lowest and highest numbers of possible compounds are indicated in Tables 3.26 and 3.27.

The embryonic axes of fresh seeds produced the same number of possible compounds as did the axes of 4d clean seeds (Table 3.26). Although 10d clean and 10d-8 samples could have a minimum of four compounds (as for fresh and 4d clean material), it is possible that they could have actually contained as many as 17 and 13 compounds, respectively. An increase in possible compound number was observed in 10d infected and 4d-8 samples that produced between 5-30 and 5-21 compounds, respectively.

Table 3.26. Summary of *in vitro* antifungal activity against *Fusarium moniliforme* demonstrated by axes of *Avicenia marina.*

Treatment	Sample	Total Samples	Number of possible compounds
Fresh	10, 11	2	4-6
4d clean	61, 62, 63	3	4-6
10d clean	95, 96, 102, 104, 105, E, B, C, D, 114, 115	11	4-17
10d infected	160, 162, 176, 177,178, 182, 186, 187, 188, 189, 193	11	5-30
4d-8	241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 256, 262, 263	17	5-21
10d-8	319, 323, 324, 325, 326, 327, 330, 331, 332, 333, 336	11	4-13

The cotyledonary extracts of different treatments produced at least 2-4 compounds (Table 3.27). However, similar to axis material, there is a probability that 10d infected and 4d-8 sample could actually show an increase in compound production, 20 possible compounds being evident in 10d infected and 11 in 4d-8 sample.

Table 3.27. Summary of *in vitro* antifungal activity against *Fusarium moniliforme* demonstrated by cotyledons of *Avicenia marina.*

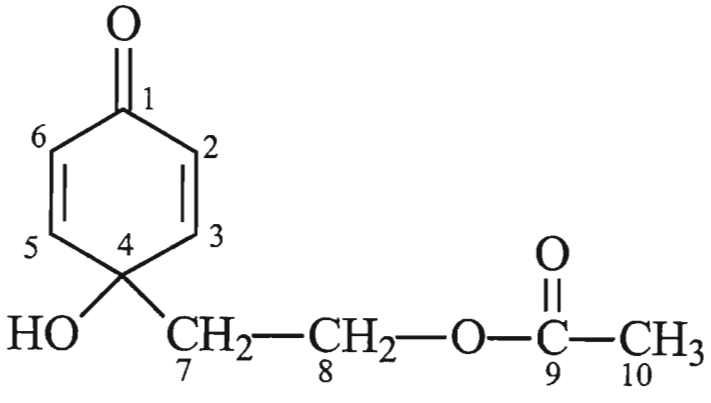
Treatment	Sample	Total Samples	Number of possible compounds
Fresh	27	1	3
4d clean	71	1	3
10d clean	155	1	2
10d infected	201, 202, 203, 204, 208, 209, 210, 212, 213, 216, 219, 225, 231, 233	14	3-20
4d-8	276, 278, 279, 289, 280, 281, 282, 293	8	4-11
10d-8	337, 338, 339, 341, 432, 343, 352, 354	8	3-10

3c.3 Identification of possible antifungal compounds[1]

The establishment of antifungal activity in all analysed samples was followed by an attempt to identify possible compounds contained in these samples. However, after taking into consideration the large number of samples (a total of 88 of which 55 were axis samples and 33 were cotyledon samples), compound identification was carried out only for fresh, 4d clean and 4d-8 embryonic axes samples as they were treatments that were related the most to seed survival.

All samples contained more than one compound (Table 3.26). Some compounds were present in concentration higher than others, their spectrum being dominant, thus making structural elucidation possible with NMR analysis. Four such compounds namely A, B, C and D have been identified. However, although in lower concentrations, NMR data analysis also demonstrated the possible presence of other compounds (E-G) in the analysed samples. Due to low yield, it was not possible to test each of these identified compounds for antifungal activity. The literature, however, suggests their involvement in plant defence mechanisms (detailed in Discussion).

- **Structural elucidation of compound A**



A

[1] The assistance of Dr. Peter Cheplogoi from the School of Pure and Applied Chemistry is gratefully acknowledged.

Compound A was the major component in sample 10 of the fresh embryonic axis extract.

The ^{1}H NMR spectrum of compound A (Fig. 3.4) showed the presence of five proton resonances, two of which were equivalent olefinic methine group resonances at δ 6.18 (d, 10.2 Hz) and δ 6.85 (d, 10.2 Hz), a deshielded methylene group resonance at δ 4.15 (t, 6.2 Hz), indicating an OCH_2 group, a methylene group resonance at δ 2.09 (t, 6.2 Hz) and a methyl group resonance at δ 2.03, characteristic of an acetyl methyl group. The ^{13}C NMR spectrum (Fig. 3.5) showed eight carbon resonances, two of which were the equivalent olefinic methine resonances at δ 127.4 and δ 151.4, indicating a compound with ten carbon atoms. The carbon resonances at δ 185.5 and δ 170.7 were carbonyl carbon resonances, the latter being characteristic of an acetyl carbonyl carbon. A quaternary deshielded carbon atom was seen at δ 68.0 and a deshielded methylene group carbon resonance was seen at δ 59.6, indicating C-O- and -OCH_2- groups respectively. A further methylene group carbon resonance was seen at δ 38.8 and the methyl carbon resonance of the acetyl group was seen at δ 20.8.

COSY (correlated spectroscopy) coupling (coupling between protons on adjacent carbon atoms) was seen between the equivalent olefinic methine groups at δ 6.18 and δ 6.85 (Fig. 3.6). Further coupling was also seen between the two methylene group proton resonances at δ 4.15, the deshielded resonance and δ 2.09. Since both resonances were triplets, this indicated that they were next to each other and not adjacent to any other protonated carbon atoms. The deshielded methylene group was thus bonded to oxygen and the other methylene group.

The carbon resonance at δ 185.0 showed HMBC (heteronuclear multiple bond correlation spectroscopy) (Fig. 3.7) correlations to the olefinic methine proton resonance at δ 6.85 and the corresponding carbon resonance of this olefinic proton resonance at δ 150.1, determined by the HSQC (heternuclear single quantum coherence) spectrum (Fig. 3.8) showed HMBC correlations to the non deshielded methylene group at δ 2.09. The carbon resonance at δ 185.0 was therefore assigned to C-1, the equivalent carbon resonance at δ 150.1 and the proton resonance at δ 6.85 were assigned to the carbon and hydrogen atoms at positions 3 and 5 and the methylene group at δ 2.09 was assigned to the hydrogen atoms at position 7. Since the methylene group at position 7 was coupled to the other deshielded methylene group at δ 4.15 in the COSY spectrum, this resonance can be attributed to the protons on the methylene group at position 8. The resonance at δ 6.18 was ascribed to H-2/6 because of the COSY coupling with H-3/5.

The deshielded quaternary carbon resonance at δ 68.4 must therefore be assigned to the remaining position on the ring at C-4 and since this resonance is deshielded, a hydroxy group must be present C-4. C-4 also showed HMBC correlations with H-2/6, H-7 and H-8, which support this assignment. The acetyl group with carbon resonances at δ 170.7 for the carbonyl carbon and δ 20.8 for the methyl carbon must be attached to the deshielded methylene group at C-8. These carbon resonances were therefore assigned to C-9 and C-10 respectively. Since C-9 showed HMBC correlations with both CH_2-8 and the methyl resonance at the 10- position, this confirmed these assignments.

NOESY (nuclear Overhauser effect spectroscopy) that shows interactions between protons through space, confirmed the assignments made above by showing interactions between H-2/6 and H-3/5 and between H-3/5 and H-7 and H-8 (Fig. 3.9).

Compound **A** was therefore identified as hallerone, $C_9H_{12}O_4$. The observed NMR data compare favourably with that from literature (Nishino *et al.*, 1988) (Table 3.28).

Table 3.28. NMR data for hallerone (1) in $CDCl_3$, 400 MHz.

Pos.	^{13}C	$^{13}C^{lit}$	^{1}H	$^{1}H^{lit}$	COSY	HMBC	NOESY
1	185.0	185.5				3/5	
2	128.3	127.4	6.18, d (10.2)	6.17, d (10)	3/5		3/5
3	150.1	151.4	6.85, d (10.2)	6.97, d (10)	2/6	7	2/6, 7, 8
4	68.4	68.0				2/6, 7, 8	
5	150.1	151.4	6.85, d (10.2)	6.97, d (10)	2/6	7	2/6, 7, 8
6	128.3	127.4	6.18, d (10.2)	6.17, d (10)	3/5		3/5
7	38.7	38.8	2.09, t (6.2)	2.14, t (6)	8	8	3/5, 8
8	59.6	59.6	4.15, t (6.2)	4.17, t (6)	7	7	3/5, 7
9	170.7	170.7				8, 10	
10	20.9	20.8	2.01, s	2.03, s			
-OH				4.25, s (br)			

3	2745.589	6.865	19.0
4	2745.040	6.864	19.6
5	2735.334	6.839	21.2
6	2734.967	6.838	21.1
7	2476.206	6.191	19.3
8	2466.500	6.167	17.6
9	2465.950	6.166	17.4
10	1667.323	4.169	15.6
11	1660.731	4.152	32.1
12	1654.504	4.137	16.2
13	841.776	2.105	17.4
14	841.227	2.103	17.4
15	835.183	2.088	34.5
16	834.634	2.087	34.3
17	828.591	2.072	17.1
18	828.224	2.071	17.1
19	806.432	2.016	9.4
20	802.769	2.007	100.0
21	802.037	2.005	98.1
22	491.450	1.229	18.8

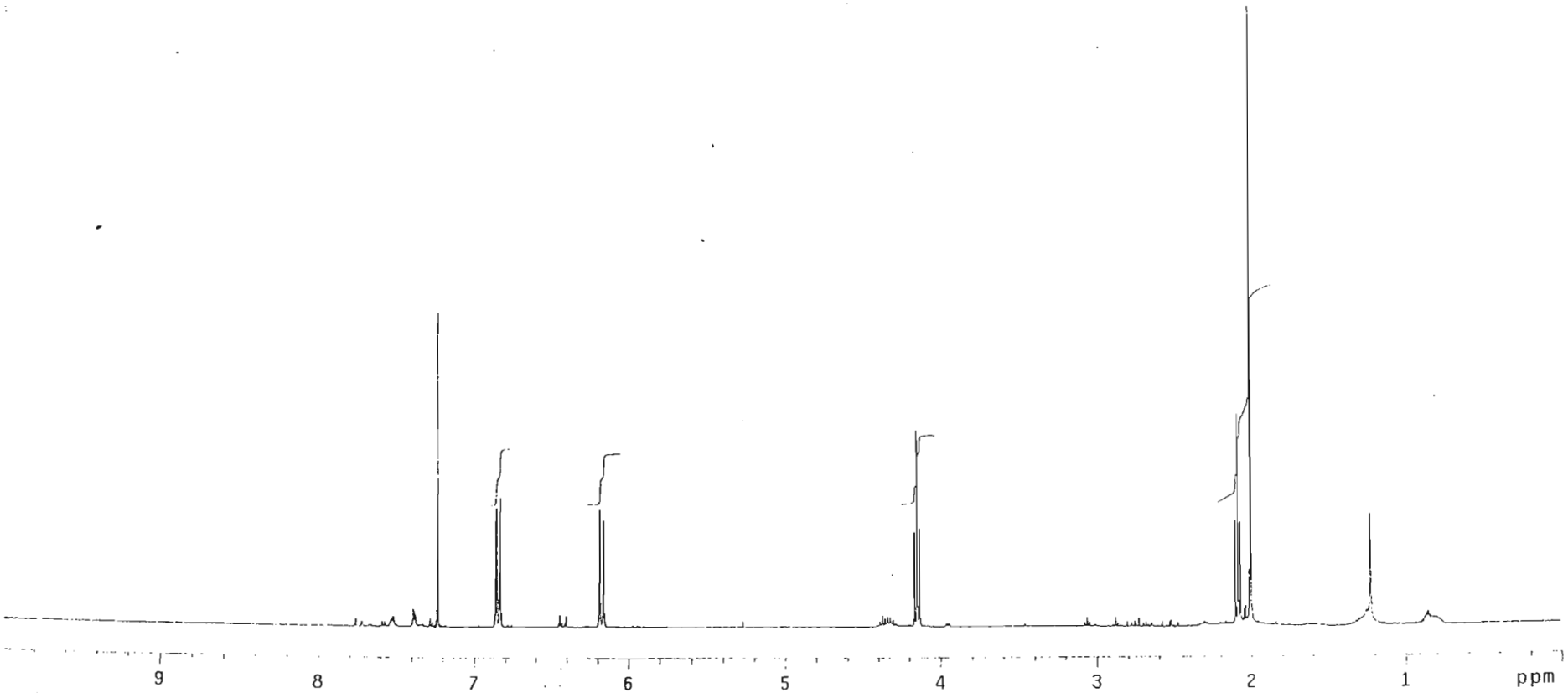


Fig. 3.4. ^{1}H NMR spectrum of compound **A**

3	15093.507	150.087	36.5
4	12967.614	128.947	5.3
5	12902.519	128.300	36.9
6	7774.833	77.311	100.0
7	7763.297	77.197	8.3
8	7742.697	76.992	98.2
9	7711.386	76.680	99.6
10	6879.980	68.413	8.7
11	5991.720	59.580	23.8
12	3888.074	38.662	25.1
13	2984.158	29.674	6.2
14	2098.369	20.866	10.7

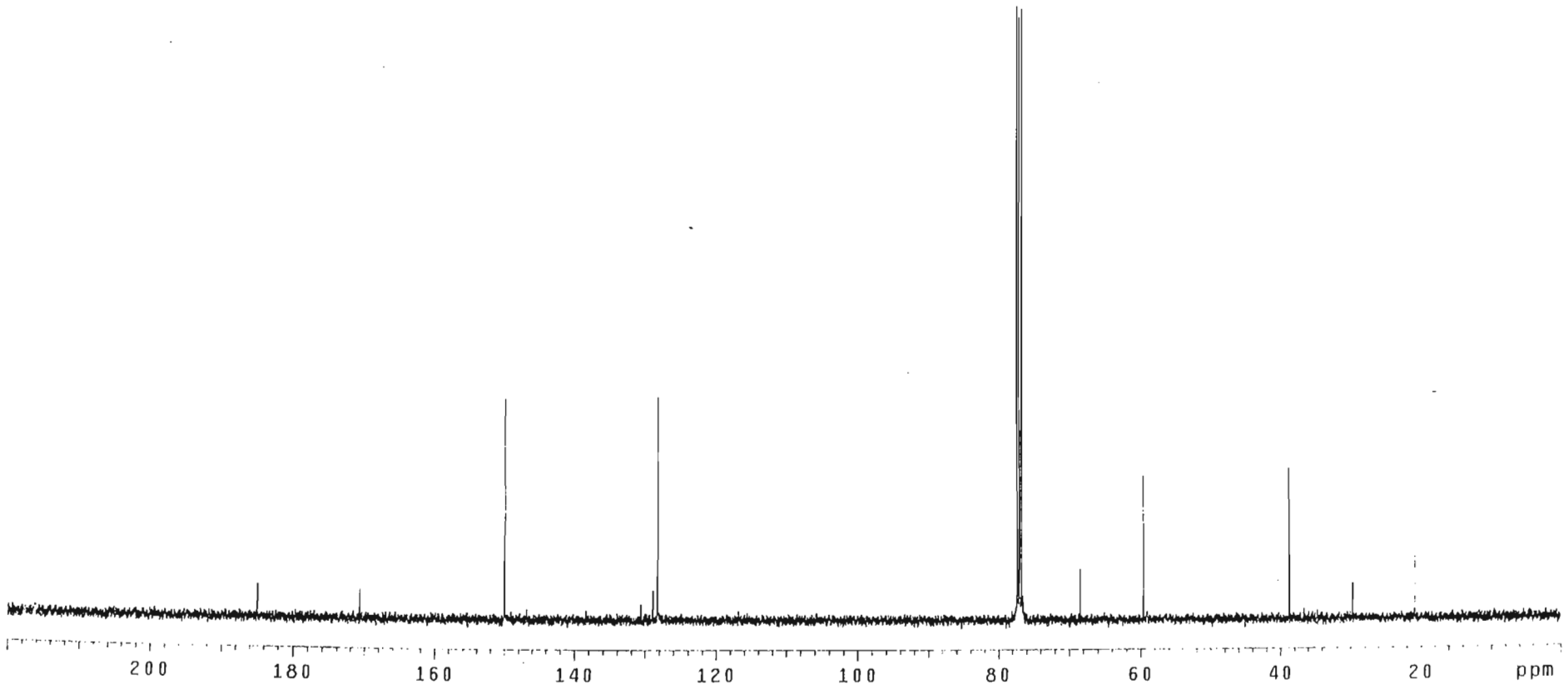


Fig. 3.5. ^{13}C NMR spectrum of compound **A**

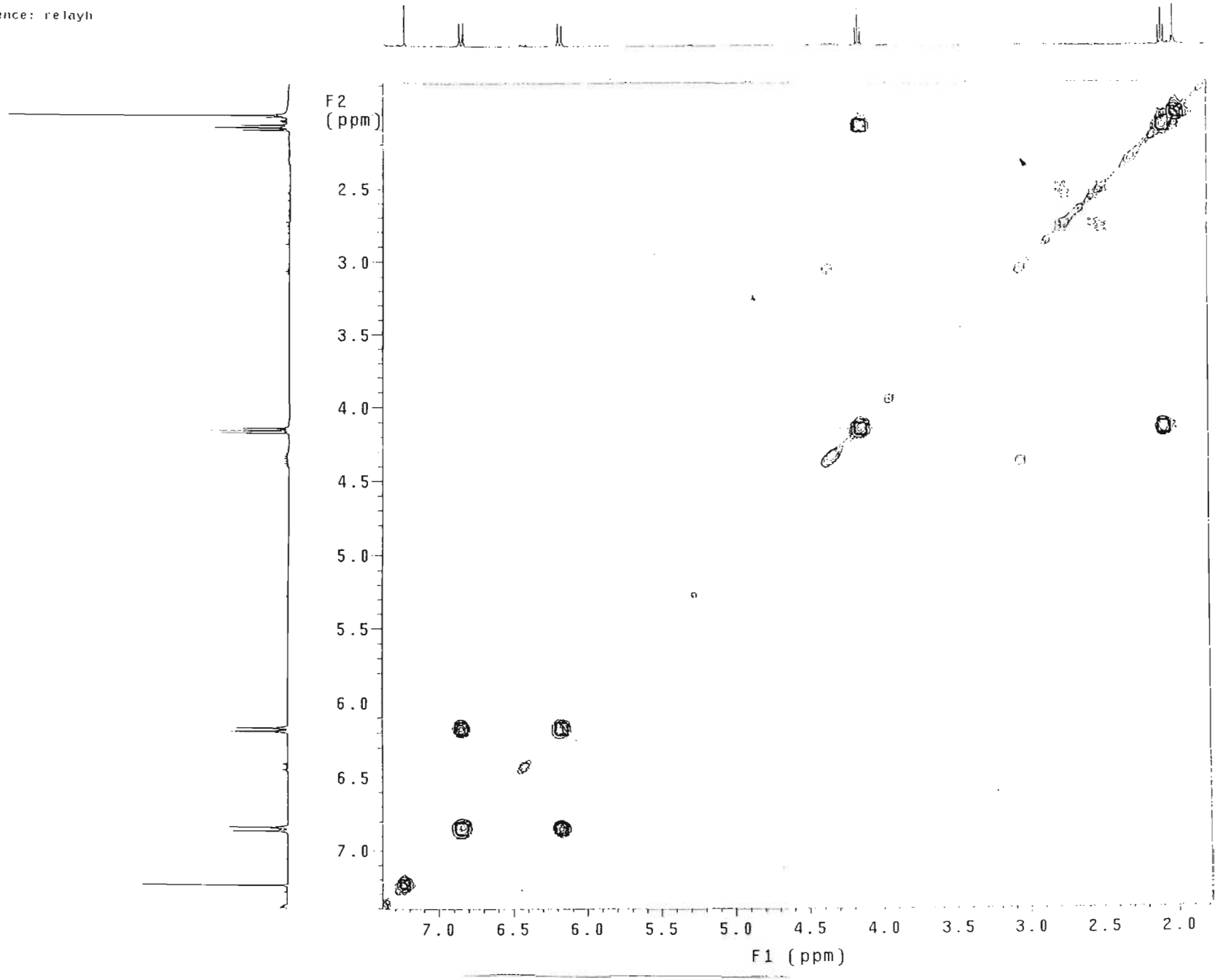


Fig. 3.6. COSY spectrum of compound **A**

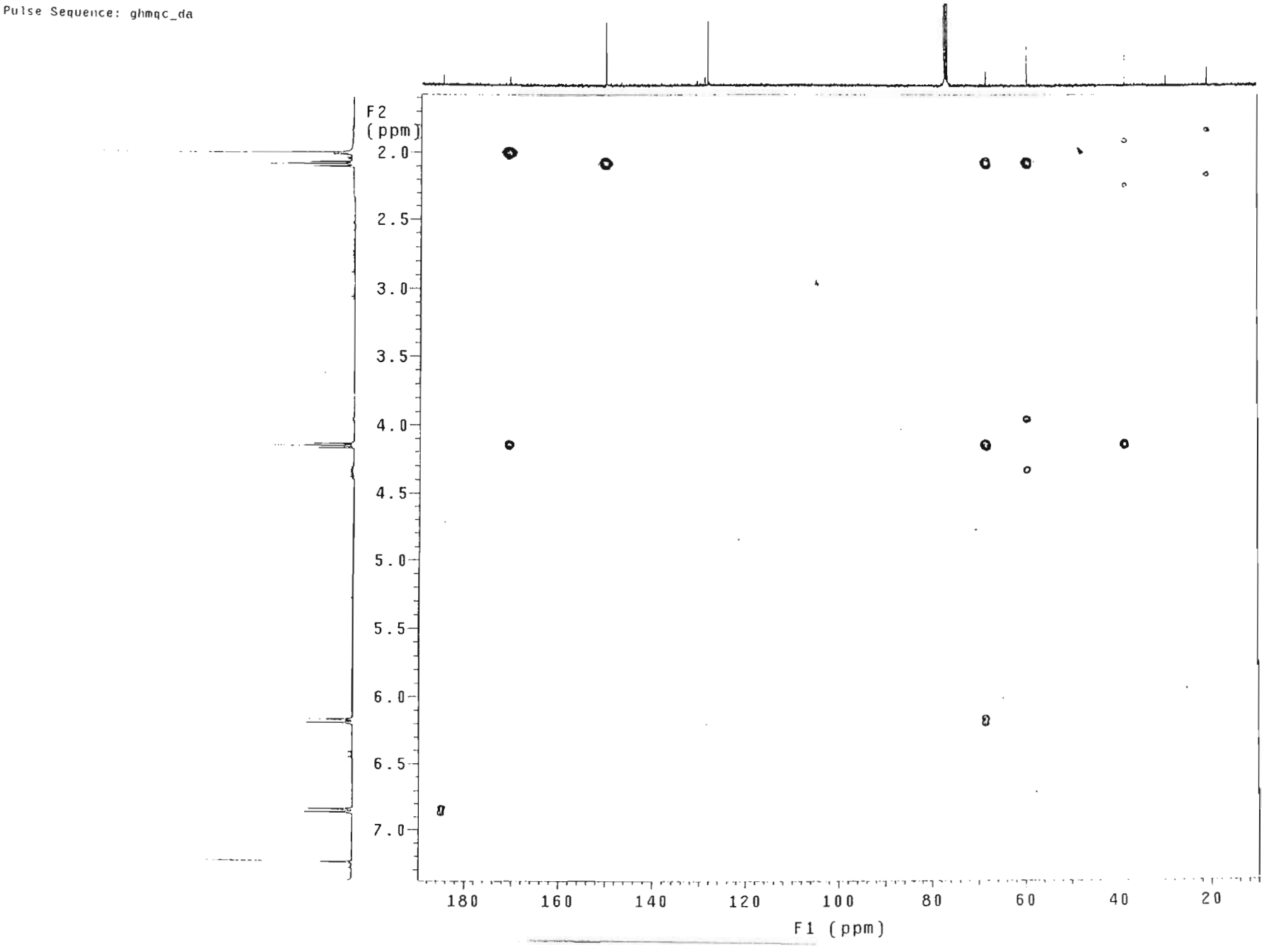


Fig. 3.7. HMBC spectrum of compound **A**

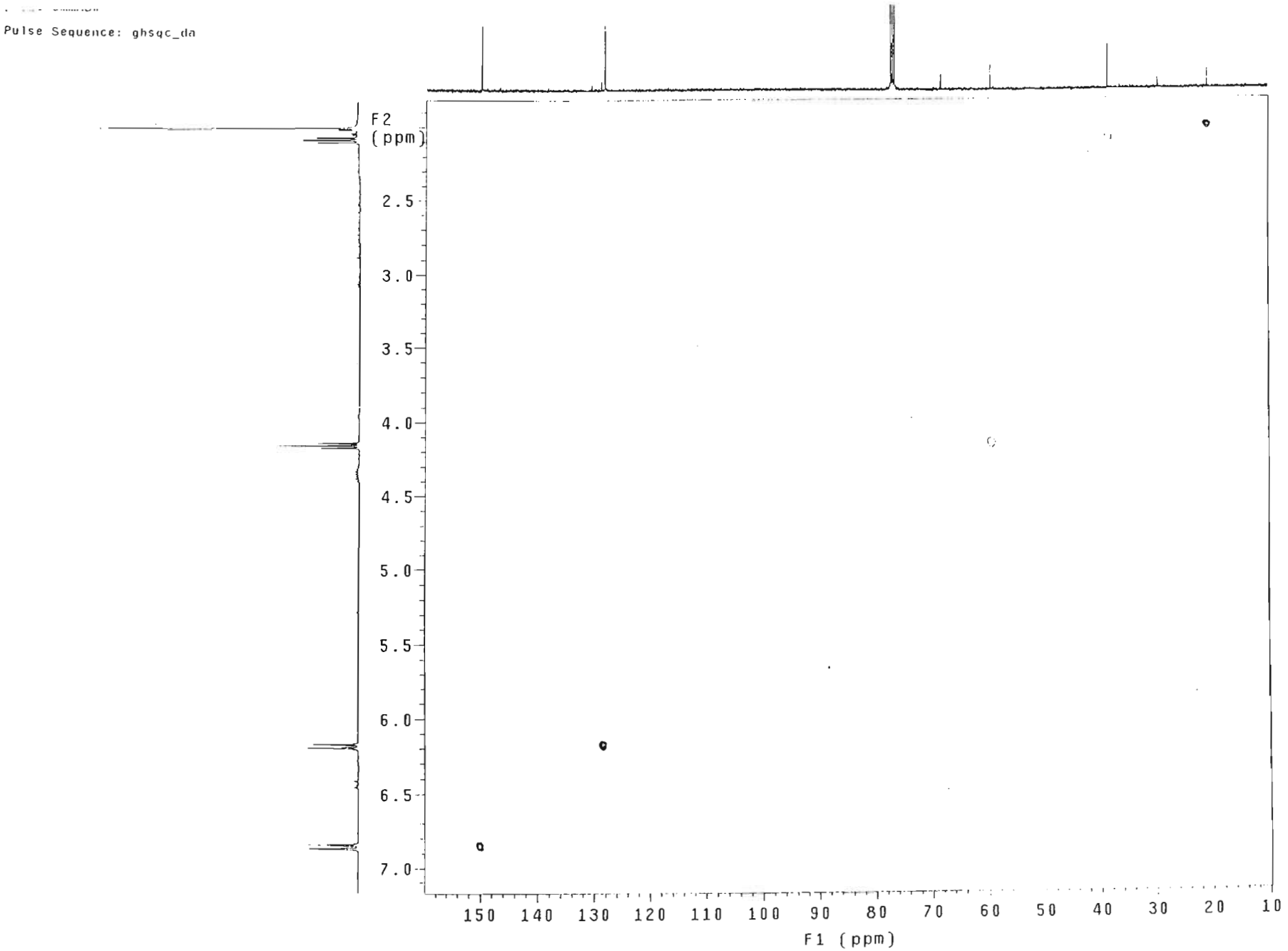


Fig. 3.8 HSQC spectrum of compound **A**

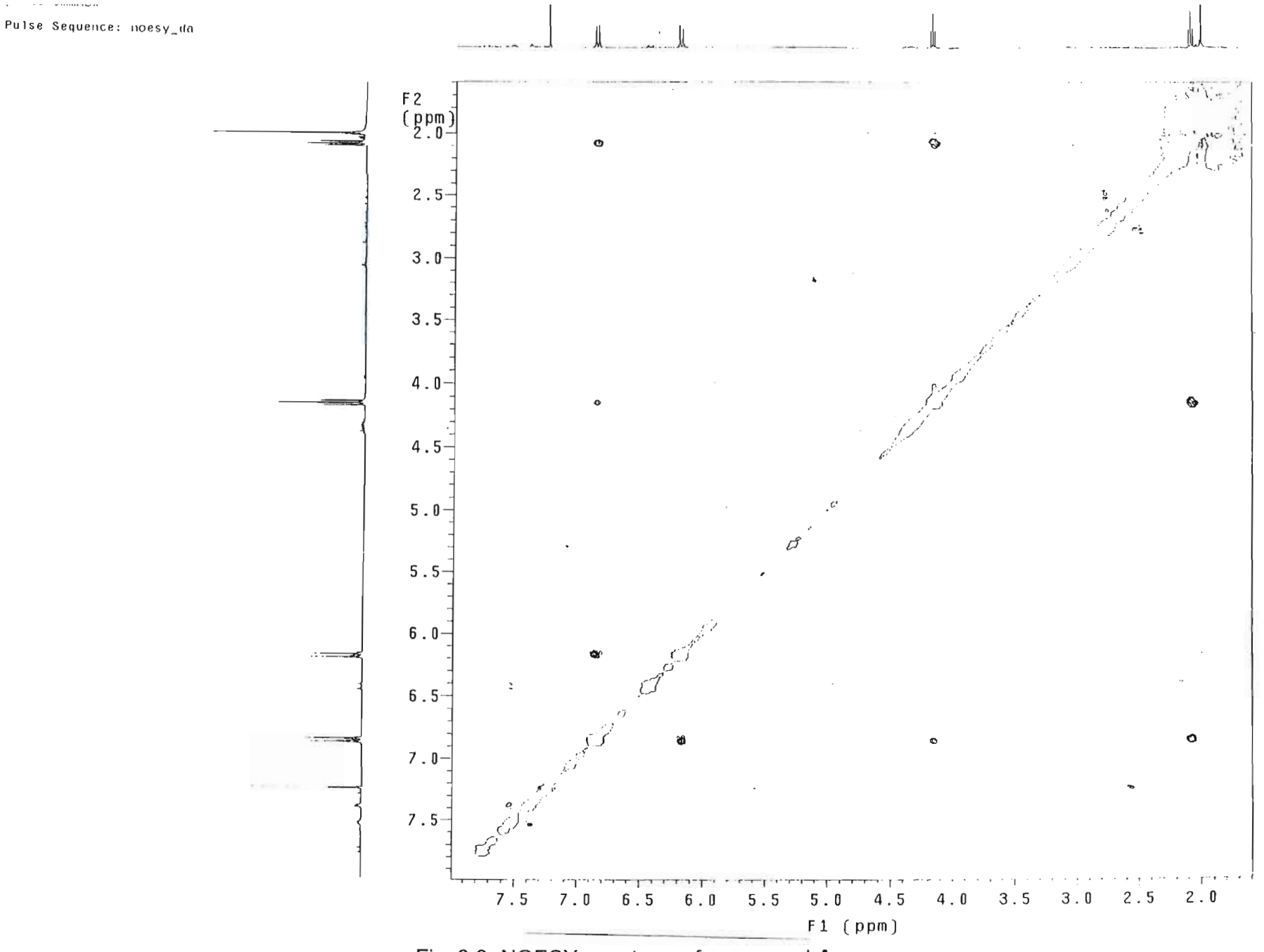
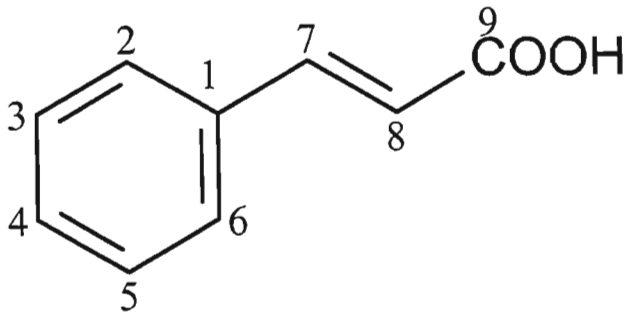


Fig. 3.9. NOESY spectrum of compound **A**

- **Structural elucidation for compound B**



B

Compound **B** was a major component in samples 62 and 63 of 4d clean axes extract.

The ^{1}H NMR spectrum of compound **B** showed the presence of a monosubstituted aromatic ring with two-proton and three-proton multiplet resonances at δ 7.38 and δ 7.53 respectively (Fig. 3.10). Also evident in this spectrum by their large coupling constants (16.1 Hz) were two trans olefinic proton resonances at δ 6.44 and δ 7.77. The ^{13}C NMR spectrum (Fig. 3.11) showed the presence of seven carbon atoms, two of which were equivalent carbon atoms at δ 128.4 and δ 129.0, indicated by the HSQC spectrum (3.12) because correlations were seen between the aromatic proton resonances and these carbon resonances. The third carbon resonance, to which the three-proton multiplet resonance correlated to, was the carbon resonance at δ 130.73. Thus, this resonance must be assigned to C-4.

A carbonyl resonance at δ 171.4 was present and since no methyl groups were present for an acetyl group and no other alkyl groups present for an ester carbonyl, this must be the carbonyl of an acid group. This acid group was bonded to the trans double bond, indicated by HMBC correlations between the carbonyl peak and both the proton resonances of the trans olefinic bond (Fig.

3.13) . The resonance at δ 134.0 showed HMBC correlations to the multiplet at δ 7.38 and the olefinic proton resonance at δ 6.44. The corresponding carbon resonance of the other olefinic resonance at δ 7.77 showed HMBC correlations to the aromatic proton multiplet at δ 7.53. These correlations allowed the resonance at δ 134.0 to be assigned to C-1, the olefinic proton resonances at δ 7.38 and δ 7.53 to be assigned to H-3/5 and H-2/6 respectively, and the olefinic proton resonances at δ 7.77 and δ 6.44 to be assigned to H-7 and H-8 respectively. The NMR data are tabulated in Table 3.29 (additional data for COSY and NOESY correlations can be found in Figs. 3.14 and 3.15).

Thus, compound **B** was identified as cinnamic acid (3-phenyl-2-propenoic acid). The structure was confirmed by comparing the proton and ^{13}C NMR spectra with a standard spectrum in the Aldrich Library of FT NMR spectra.

Table 3.29. NMR data for cinnamic acid (B) in $CDCl_3$, 400 MHz.

Position	^{13}C	^{1}H	COSY	HMBC	NOESY
1	134.0			3/5, 8	
2/6	128.4	7.53, m	3/5, 4	7	3/5, 7, 8
3/5	129.0	7.38, m	2/6, 4	4	2/6, 4
4	130.7	7.38, m	3/5	2/6	3/5
7	147.0	7.77, d (16.1)	8	2/6	2/6, 8
8	117.2	6.44, d (16.1)	7	7	2/6, 7
9	171.4			7, 8	

3	3019.550	7.550	11.2
4	3017.536	7.545	13.3
5	3013.507	7.535	16.0
6	3010.943	7.528	10.0
7	3010.211	7.527	14.7
8	3009.845	7.526	14.6
9	2959.301	7.399	41.9
10	2956.737	7.393	32.5
11	2954.723	7.388	13.5
12	2953.624	7.385	17.1
13	2952.708	7.383	21.0
14	2895.572	7.240	100.0
15	2583.336	6.459	31.9
16	2567.221	6.419	28.4
17	492.365	1.231	85.9
18	343.115	0.858	10.0

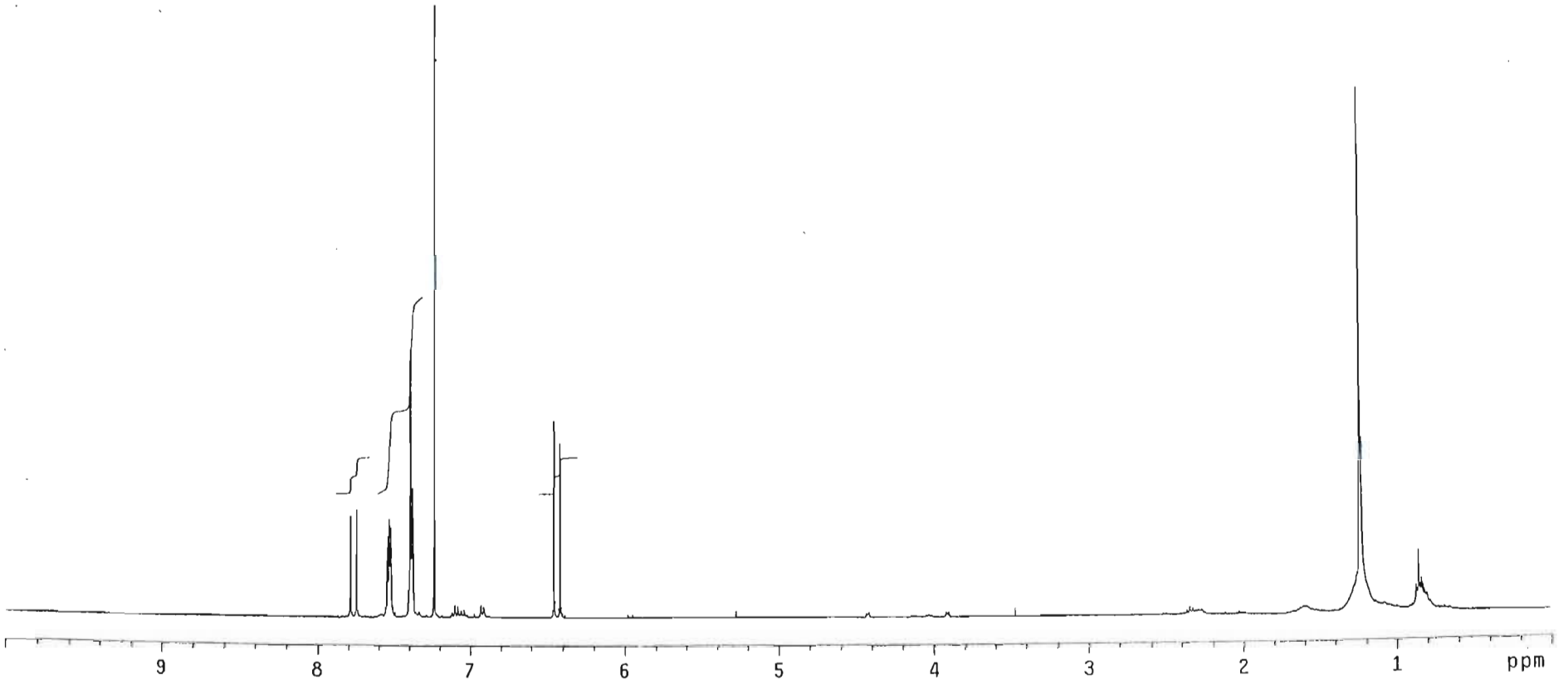


Fig. 3.10. ^{1}H NMR spectrum of compound **B**

4	13147.244	130.734	20.6
5	12969.262	128.964	39.2
6	12907.463	128.349	40.1
7	11777.773	117.116	15.2
8	7775.657	77.320	78.3
9	7743.521	77.000	76.9
10	7712.209	76.689	80.0
11	2985.806	29.690	23.3

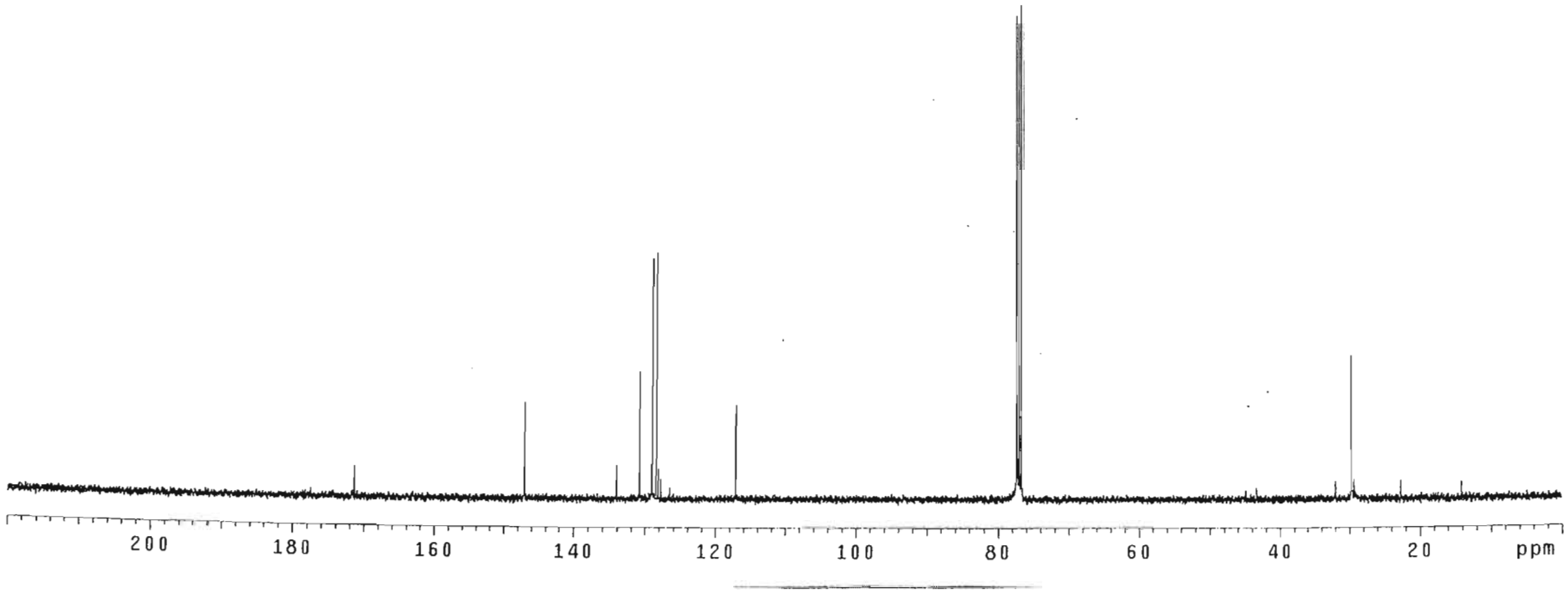


Fig. 3.11. ^{13}C NMR spectrum of compound **B**

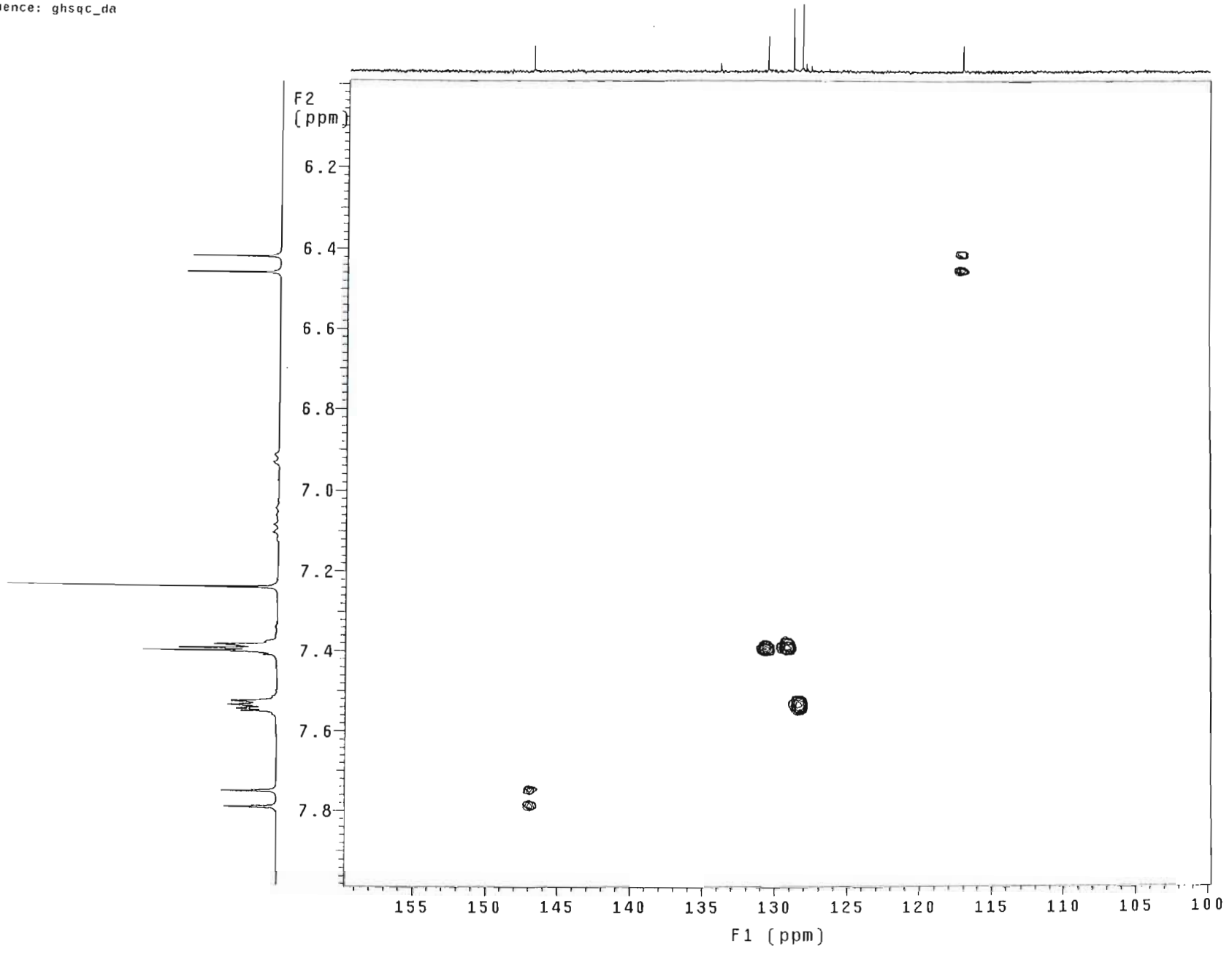
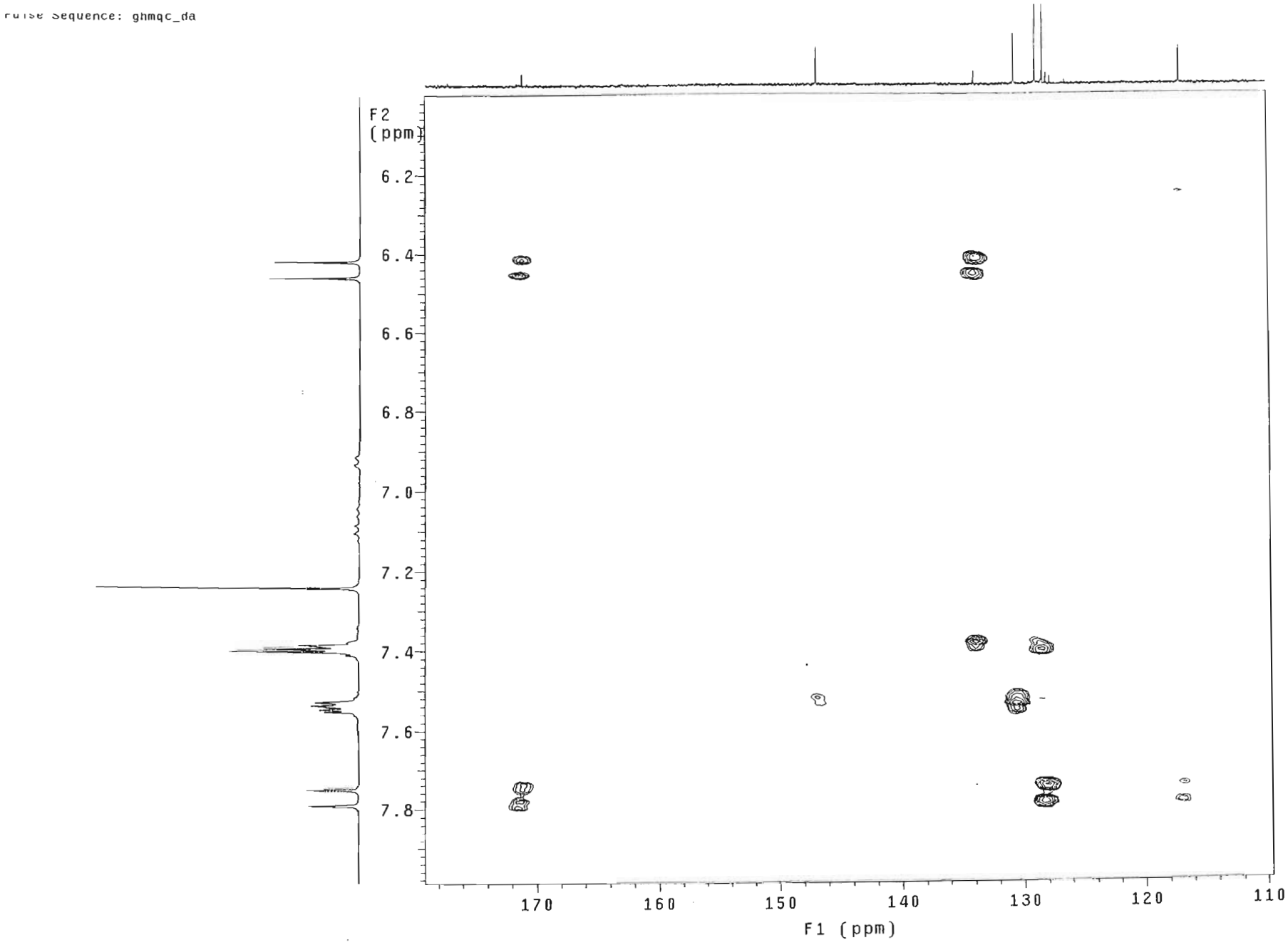


Fig. 3.12. HSQC spectrum of compound **B**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6.4
6.6
6.8
7.0
7.2
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150
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130
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F1 (ppm)

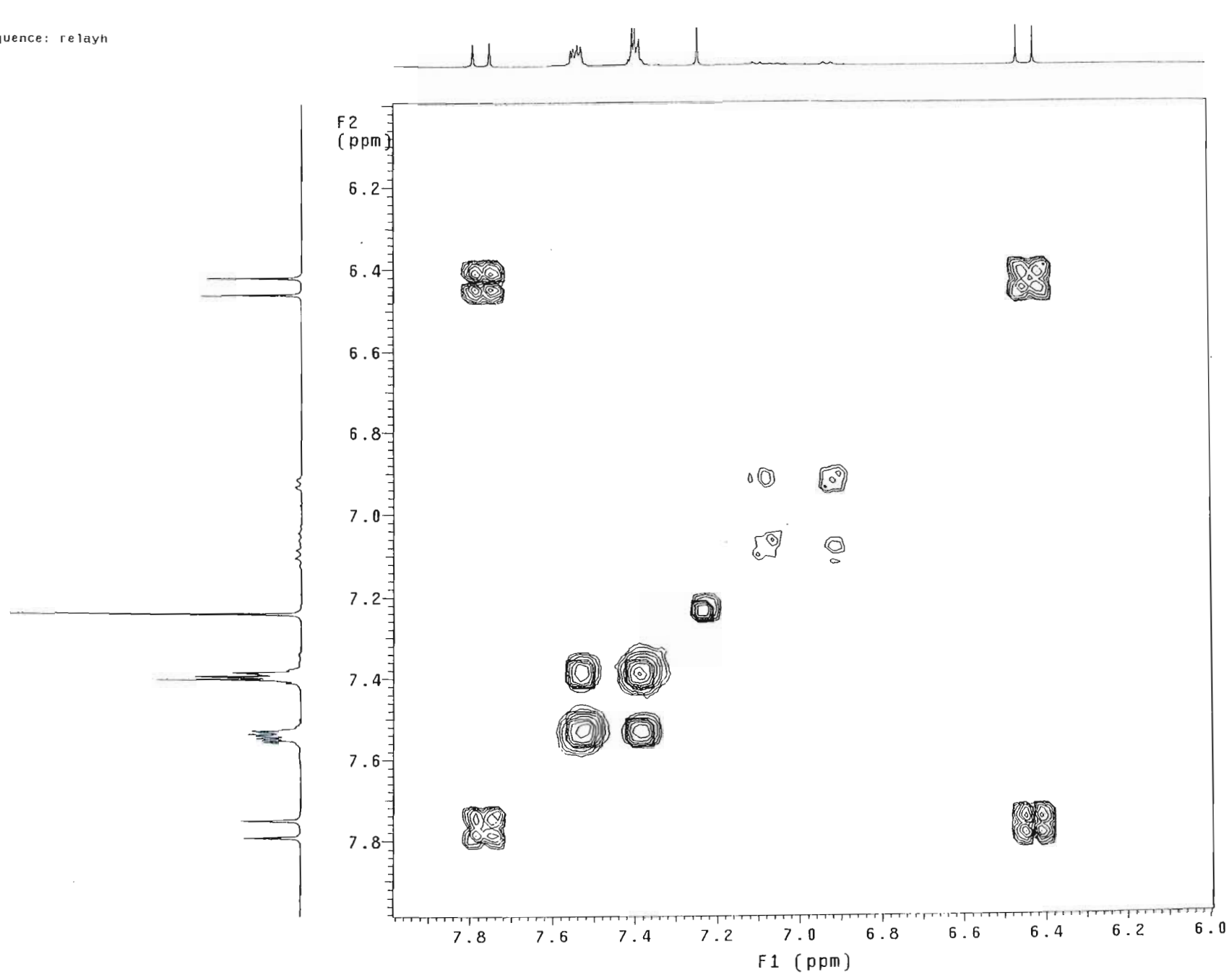


Fig. 3.14 COSY spectrum of compound **B**

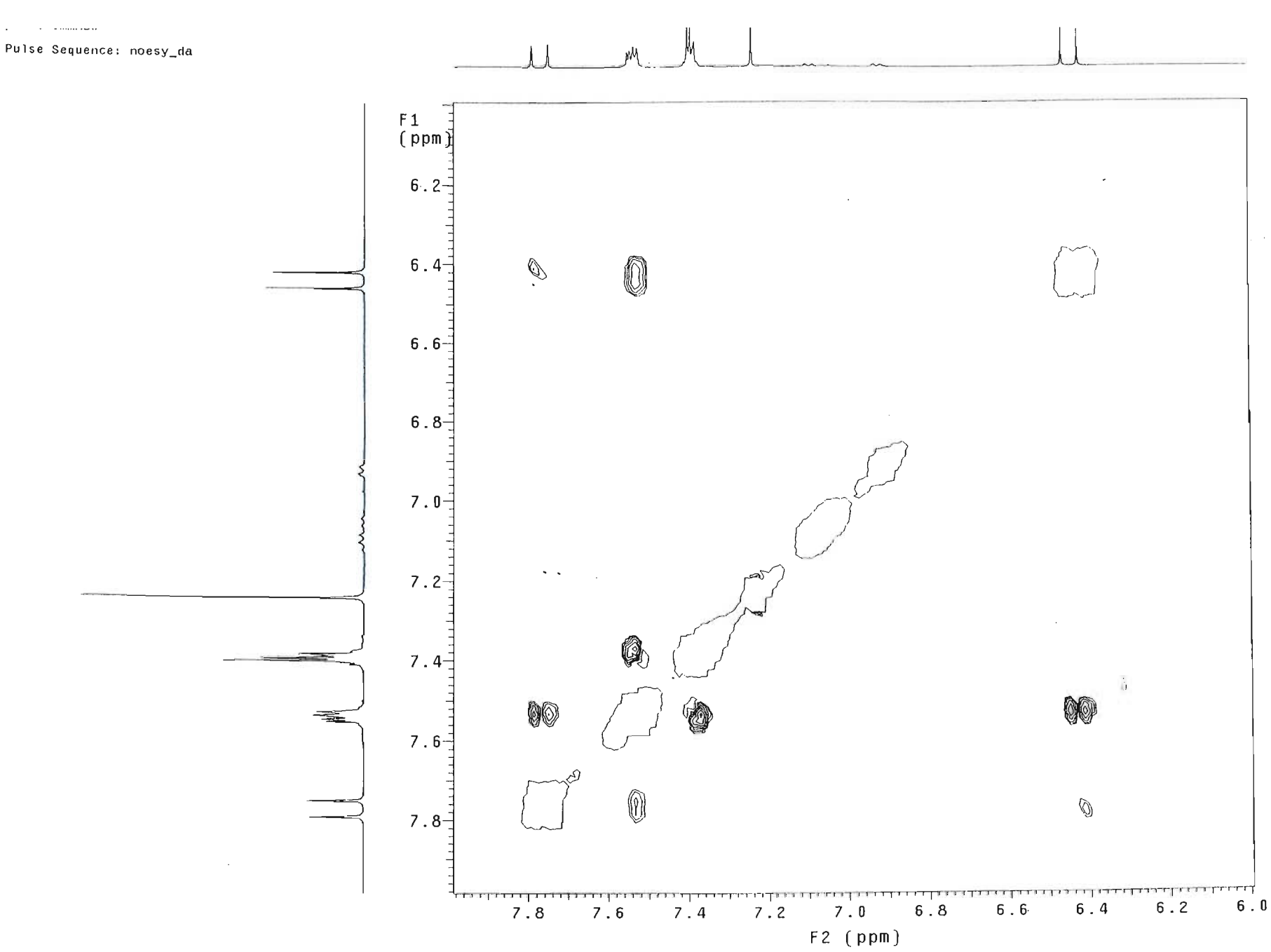
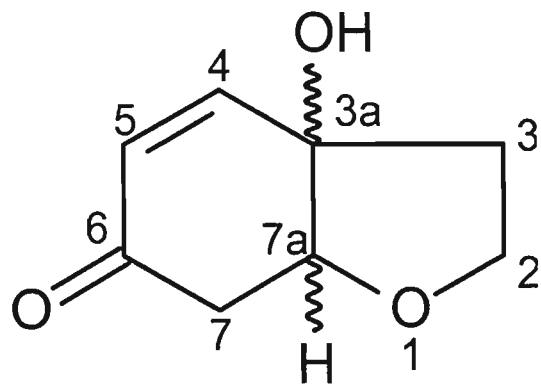


Fig. 3.15 NOESY spectrum of compound **B**

- **Structural elucidation of compound C**



c

Compound **C** was a major component in samples 62 and 63 of 4d clean axes extract.

The ^{1}H NMR spectrum of compound **C** (Fig. 3.16) showed the presence of nine proton resonances, each integrating to one proton at δ 2.17 (m), 2.30 (m), 2.55 (dd, 17.03, 5.86 Hz), 2.72 (dd, 17.03, 5.86 Hz), 3.90 (m), 4.03 (m), 4.20 (m), 5.97 (d, 10.26), 6.73 (d, 10.26). The multiplet resonances at δ 3.90 (m), 4.03 (m) and 4.20 (m) were deshielded and therefore were attached to carbon atoms, which were in turn attached to oxygen atoms. The HSQC spectrum showed that the proton resonances at δ 3.90 and δ 4.03 were protons on a methylene carbon and that δ 4.20 was a proton on a methine carbon (Fig. 3.17). This therefore suggested CH_2O and CHO groups respectively in the molecule. The resonances at δ 5.97 (d, 10.26) and δ 6.73 (d, 10.26) indicated a cis double bond by their coupling constant of 10.26 Hz.

The ^{13}C NMR spectrum (Fig. 3.18) showed the presence of eight carbon resonances, methylene carbon resonances, indicated by the DEPT spectrum - which shows the number of protons attached to each protonated carbon - at δ 39.5 and δ 40.1, a deshielded methylene carbon resonance at δ 66.2, with

corresponding proton resonances at δ 3.90 and δ 4.03, characteristic of a CH_2O group, a deshielded methine resonance at δ 81.5, with corresponding proton resonance at δ 4.20 indicating a CHO group, and two olefinic methine resonances at δ 128.6 and δ 148.1. The HSQC spectrum indicated that the corresponding proton resonances for the methylene carbon resonance at δ 39.5 was δ 2.17 (m) and δ 2.30 (m), and for the resonance at δ 40.1 was δ 2.55 (dd, 17.03, 5.86 Hz) and δ 2.72 (dd, 17.03, 5.86 Hz). The other two remaining carbon resonances was a carbonyl resonance at δ 197.0 and a deshielded quaternary carbon resonance at δ 75.5, indicated a C-O group, possibly C-OH.

COSY coupling between the two olefinic proton resonances indicated that they were adjacent to each other (Fig. 3.19 a,b). COSY coupling between the resonance at δ 4.20 (CH group) and the methylene protons at δ 2.55 and δ 2.72 (CH_2 group) indicated that these groups were adjacent to each other and COSY coupling between the deshielded methylene group at δ 3.90 and δ 4.03 (CH_2O) and the methylene group at δ 2.17 and δ 2.30 (CH_2) indicated that they were adjacent to each other.

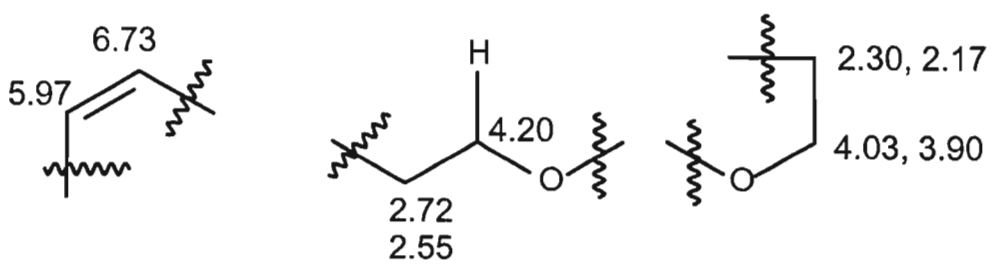


Fig. 3.19b. COSY couplings of compound **C**

NDEX	FREQUENCY	PPM	HEIGHT	INDEX	FREQUENCY	PPM	HEIGHT
1	2895.938	7.241	32.0	40	1038.274	2.596	84.9
2	2895.572	7.240	55.2	41	1032.414	2.581	82.9
3	2697.609	6.745	73.4	42	1026.554	2.567	9.0
4	2696.144	6.741	67.6	43	1021.243	2.553	56.7
5	2687.354	6.719	79.4	44	1018.130	2.546	12.4
6	2685.889	6.716	76.6	45	1015.566	2.539	54.6
7	2394.530	5.987	96.8	46	932.974	2.333	30.3
8	2384.275	5.962	89.3	47	926.565	2.317	32.1
9	1687.651	4.220	31.5	48	924.550	2.312	32.1
10	1686.186	4.216	31.4	49	919.972	2.300	52.0
11	1681.790	4.205	57.6	50	918.141	2.296	33.1
12	1677.212	4.194	32.8	51	913.563	2.284	53.6
13	1675.930	4.190	30.5	52	911.548	2.279	52.0
14	1627.035	4.068	28.0	53	905.139	2.263	48.1
15	1620.442	4.052	34.4	54	885.910	2.215	44.1
16	1618.428	4.047	63.8	55	879.501	2.199	48.5
17	1612.018	4.031	66.9	56	877.669	2.194	48.3
18	1609.821	4.025	42.4	57	872.725	2.182	33.6
19	1603.411	4.009	40.3	58	871.260	2.178	46.0
20	1575.209	3.939	44.6	59	866.498	2.167	30.2
21	1573.012	3.933	11.7	60	864.667	2.162	30.5
22	1568.800	3.923	50.3	61	858.258	2.146	27.2
23	1566.968	3.918	71.5	62	487.421	1.219	22.7
24	1560.559	3.902	73.0				
25	1558.727	3.897	35.8				
26	1558.361	3.896	38.7				
27	1555.797	3.890	15.1				
28	1551.769	3.880	36.3				
29	1486.025	3.716	14.8				
30	1358.201	3.396	18.9				
31	1355.454	3.389	17.8				
32	1355.088	3.388	23.4				
33	1115.738	2.790	10.3				
34	1108.229	2.771	65.5				
35	1103.468	2.759	62.8				
36	1091.381	2.729	100.0				
37	1086.620	2.717	94.7				
38	1059.883	2.650	9.5				
39	1056.953	2.643	8.1				

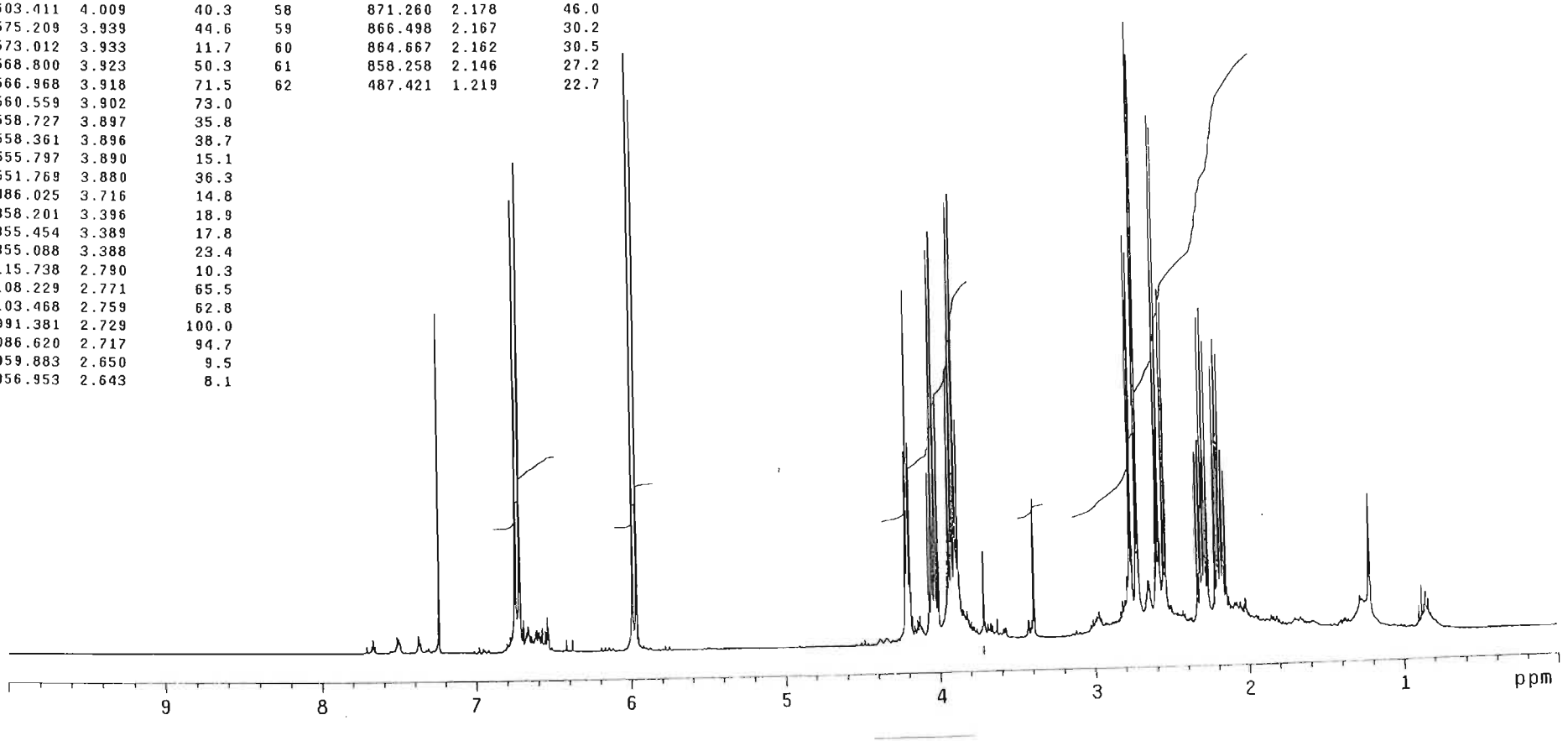


Fig. 3.16 ^{1}H NMR spectrum of compound C

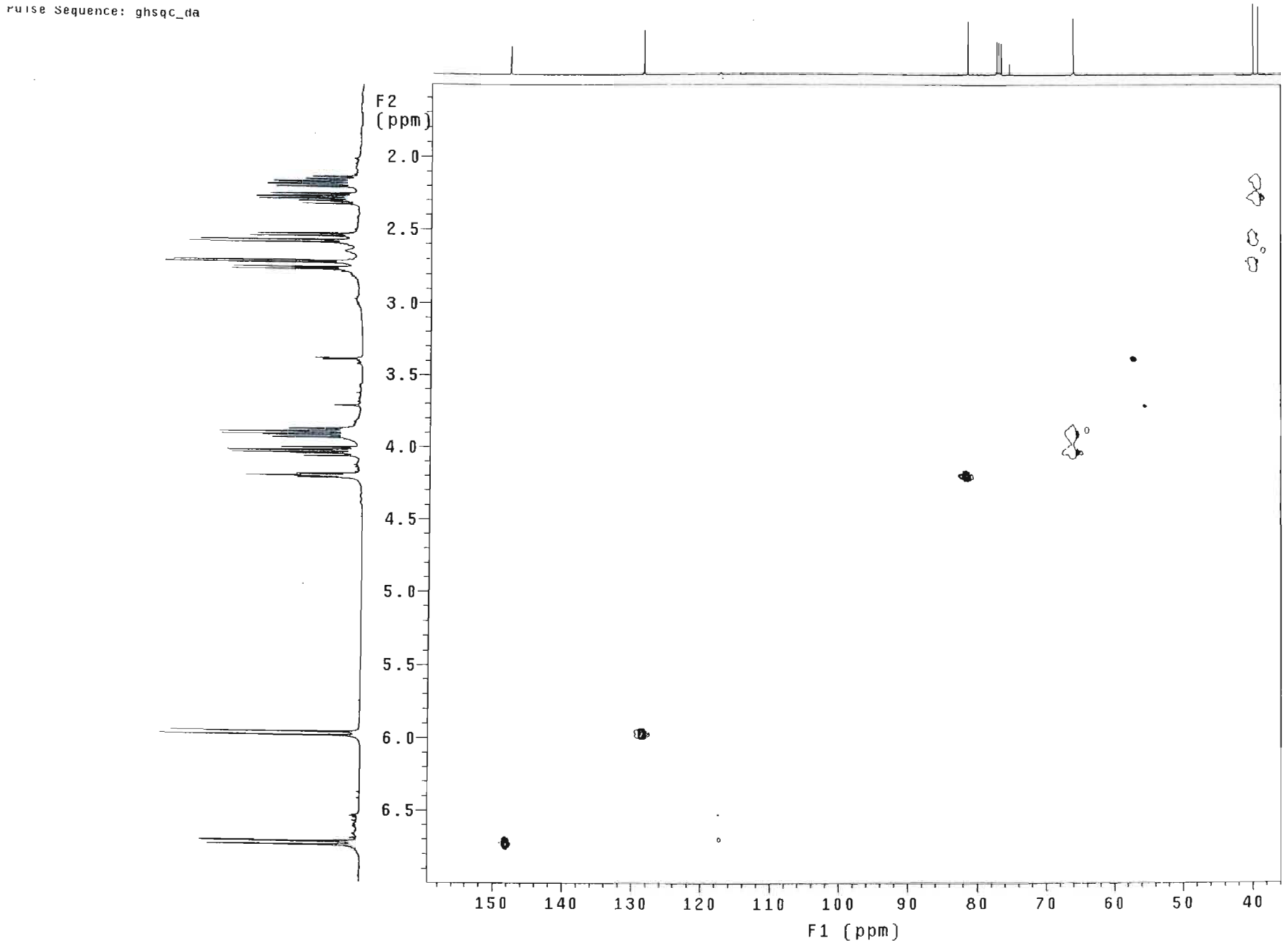


Fig. 3.17. HSQC spectrum of compound **C**

3	12937.127	128.644	45.9
4	8196.716	81.506	53.7
5	7775.657	77.320	33.3
6	7743.521	77.000	33.1
7	7712.210	76.689	32.1
8	7592.731	75.501	11.8
9	6659.975	66.225	57.1
10	4034.745	40.121	80.0
11	3968.002	39.457	69.1

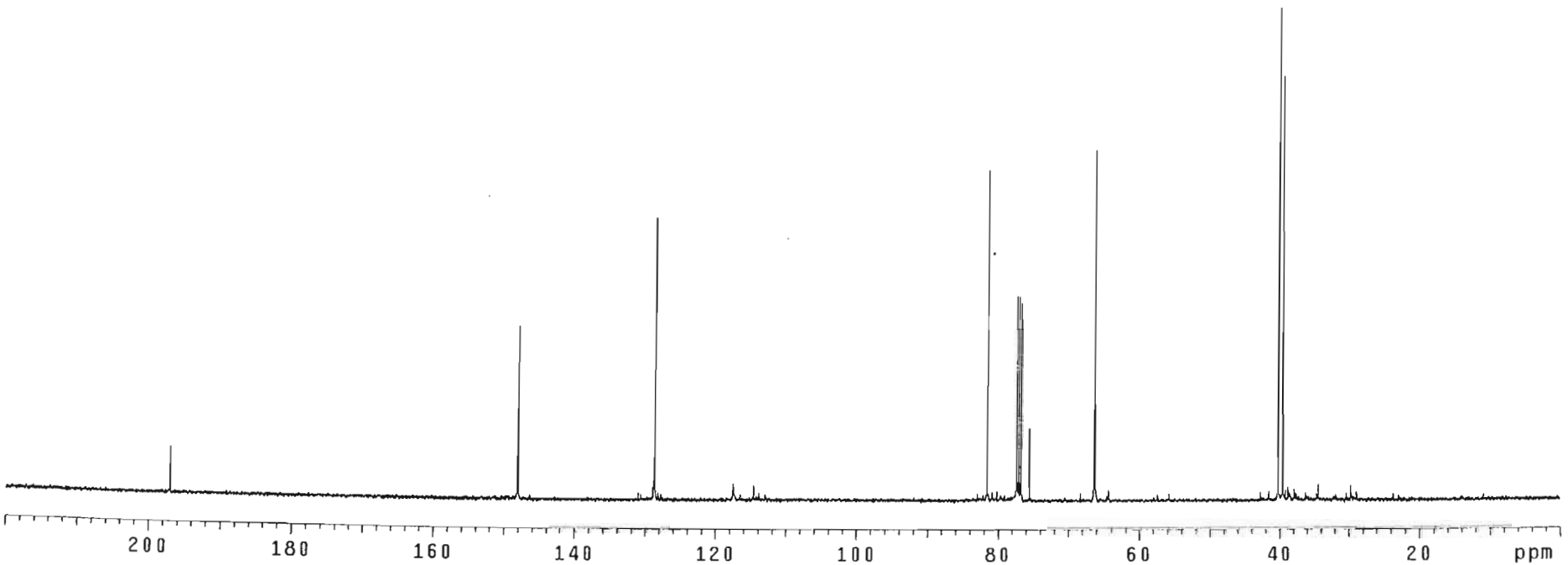


Fig. 3.18. ^{13}C NMR spectrum of compound **C**

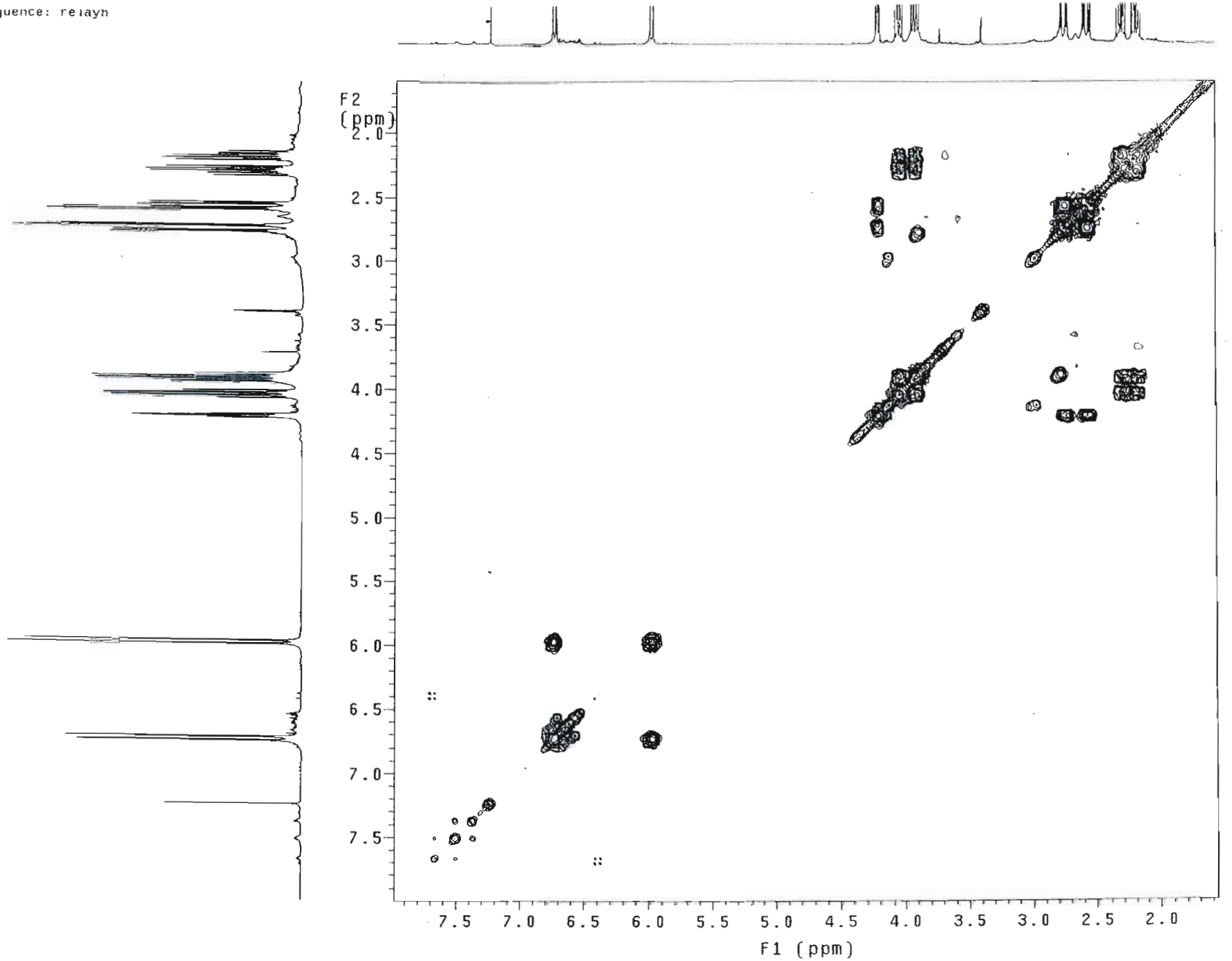


Fig. 3.19 COSY spectrum of compound 2

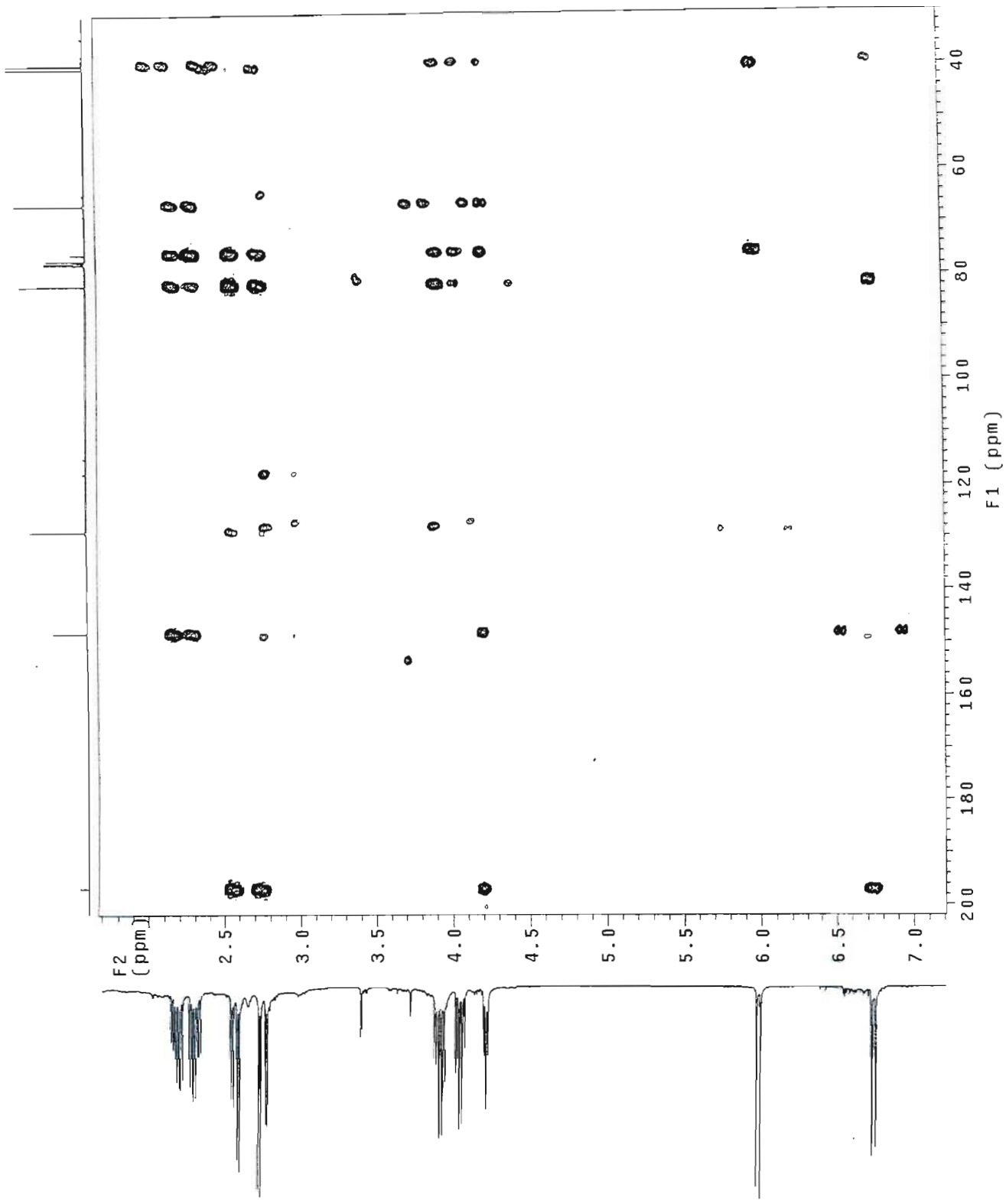


Fig.3.20A.HMBC spectrum of compound **C**

HMBC correlations between the carbonyl resonance at δ 197.0 and the resonances at δ 6.73 (olefinic), δ 2.55 and δ 2.72 (methylene) and δ 4.20 (oxygenated methine), as well as HMBC correlations between the olefinic carbon at δ 127.9 (with corresponding proton resonance at δ 5.97) and the oxygenated methine at δ 4.20 allowed fragments to be joined together (Fig. 3.20 a, b, c) as follows:

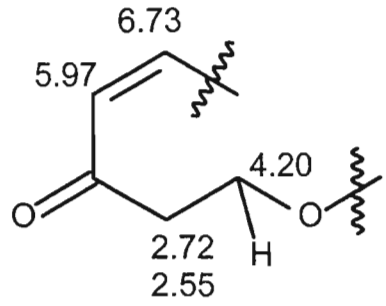


Fig. 3.20b. HMBC correlations of compound **C**

The corresponding carbon resonance of the oxygenated methine at δ 4.20 at δ 81.9 showed HMBC correlations to four proton resonances, at δ 6.73 and δ 2.77 and 2.55, which confirms the structure of the fragment above and also to δ 4.03 and 3.90 (oxygenated methylene) and δ 2.30 and 2.17 (methylene). Since the oxygenated methine resonance showed an HMBC correlation to the oxygenated methylene resonance, there must be only one oxygen atom joining the methine and methylene groups. This allowed the third fragment to be joined to the working structure as follows:

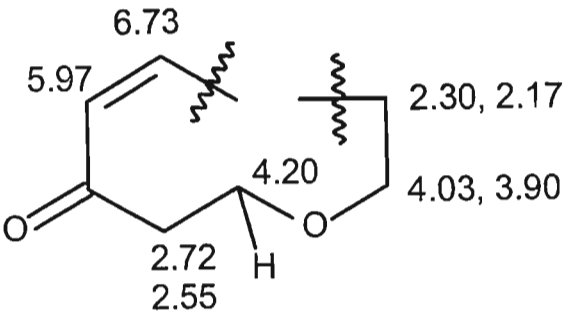
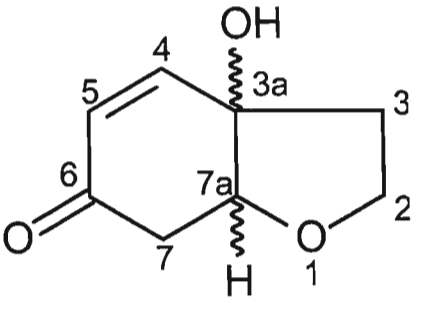


Fig. 3.20c. HMBC correlations of compound **C**

HMBC correlations between the oxygenated quaternary carbon atom and δ 2.30, 2.17, 4.03, 3.90, 5.97, 2.72, 2.55 and 4.20 resulted in the final structure, 3,3a,7,7a-tetrahydro-3a-hydroxy-6(2H)-benzofuranone with the common name of rengyolone.



C

Further confirmation of this structure was given by HMBC correlations between C-3 and H-2 and H-4, by C-7 and H-5 and H-7a and by C-2 and H-3 and H-7a. The absolute stereochemistry cannot be determined from the available spectra, however by the use of NOESY correlations (Fig. 3.21), some relative stereochemistries can be determined. Since the 7a proton resonance shows a NOESY interaction with the H-3 resonance at δ 2.17, the proton of this resonance must be on the same side as that of H-7a. The H-7a proton

additionally shows NOESY interactions with both H-7 proton resonances therefore H-7a must be in the middle of these protons. The H-2 resonance at 3.90 shows a NOESY interaction with the proton at δ 2.55, therefore these two protons must be on the same side. NMR data of compound **C** was similar to that of literature (Endo and Hikino, 1984; Hase *et al.*, 1995; Tian *et al.*, 1997) and is given in Table 3.30.

Table 3.30. NMR data of compound C (rengyolone), 3,3a,7,7a-tetrahydro-3a-hydroxy-6(2H)-benzofuranone in CDC^{l3}, 400 MHz.

Pos.	^{13}C	$^{13}C^{lit}$	^{1}H	$^{1}H^{lit}$	COSY	HMBC	NOESY
2x	66.2	66.3	4.03, m	4.07, ddd (5.6, 8.5, 8.7)	3x, 3y	3, 7a	3x, 3y, 7a
2y			3.90, m	3.89, ddd (7.2, 7.8, 8.5)	3x, 3y		3x, 3y, 7a
3x	39.5	40.2	2.30, m	2.46, ddd (7.2, 8.7, 12.7)	2x, 2y	2,4	2x, 2y, 7a, 4
3y			2.17, m	2.21, ddd (5.6, 7.8, 12.7)	2x, 2y		2x, 2y, 4
3a	75.5	74.8				2,3,5,7,7a	
4	148.1	150.2	6.73, d (10.26)	6.96, dd (1.8, 10.1)	5	3, 7a	3x, 3y, 5
5	128.6	127.9	5.97, d (10.26)	6.16, dd (0.6, 10.1)	4	7	4
6	197	196.9				4, 7, 7a	
7x	40.1	40.4	2.72, dd (5.86, 17.03)	2.87, ddd (0.6, 4.8, 16.6)	7a	5, 7a	7a
7y			2.55, dd (5.86, 17.03)	3.00, dd (4.3, 16.6)	7a		7a, 2x, 2y
7a	81.5	81.9	4.20, m	4.51, ddd (1.8, 4.3, 4.8)	7x, 7y	2,3,4,7	3x, 3y, 7x, 7y

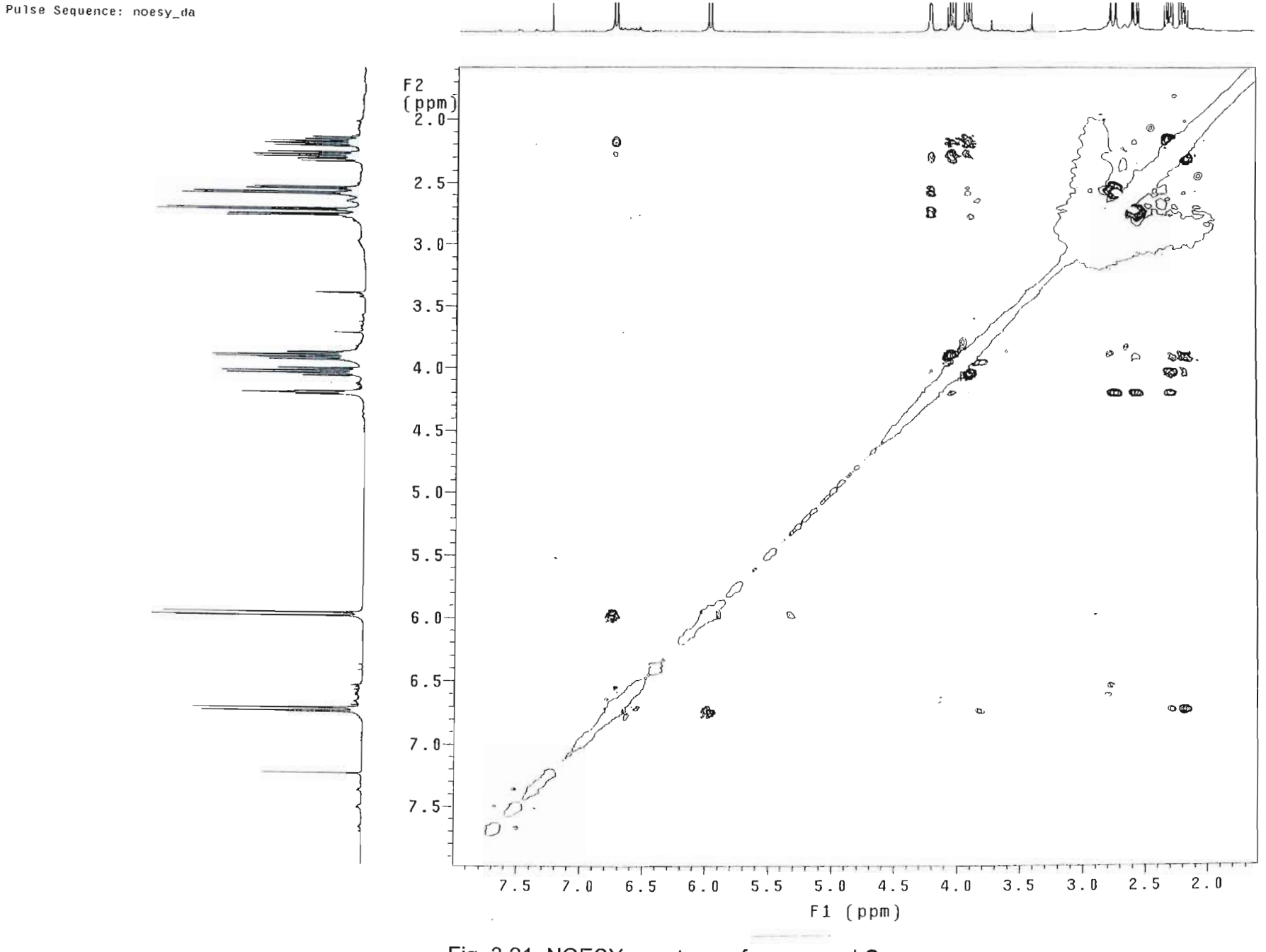
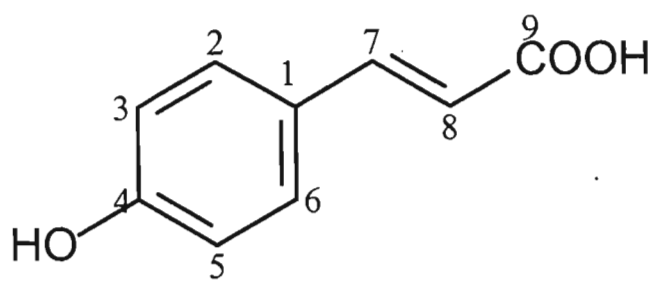


Fig. 3.21. NOESY spectrum of compound **C**

Further analysis of the proton NMR spectra of the antifungal samples obtained during fractionation revealed that sample 10 of fresh axes demonstrated minor presence of a possible cinnamic acid type compound (Fig. 3.22) indicated by the presence of doublets with large coupling constants (~16 Hz) at δ 6.3 and δ 7.7. This cinnamic acid compound (compound **D**) may have a p-subsituted benzene ring, indicated by the coupling constants (~8-10 Hz) of the pair of doublets at δ 6.8 and δ 7.4.

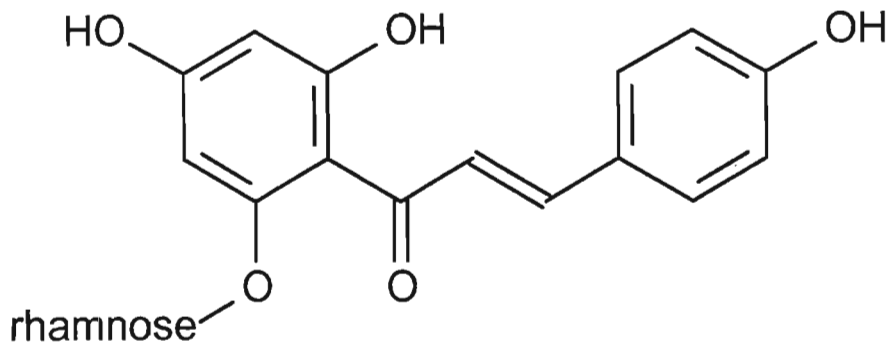


D

Compound **D** was present in minor amounts in the sample 10 of extracts of fresh axes, but was the major component in sample 11 of fresh axes (Fig. 3.23).

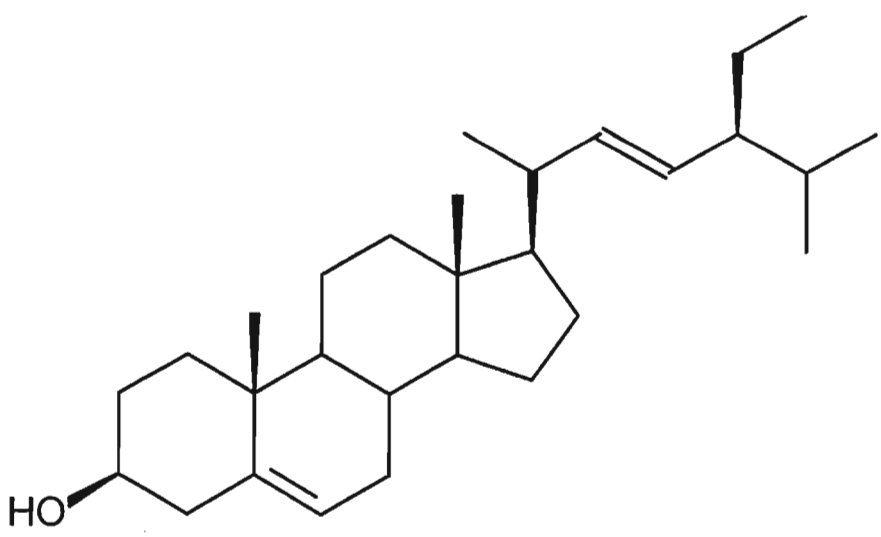
Samples 61 (Fig. 3.24), 62 (Fig. 3.25) and 63 (Fig. 3.26) of 4d clean embryonic axes extracts, all show the same mixture of compounds with varying degrees of purity. This mixture probably contains compound **A**, indicated by the presence of doublets at δ 6.2 and δ 6.8 with coupling constants of approximately eight to ten hertz and a triplet at δ 2.1 as is present in compound **A**. A chalcone glycoside may also be present in the mixture, indicated by the trans olefinic methine peaks at δ 6.3 and δ 7.6 (J = ~16 Hz), aromatic proton resonances between δ 6.6 and δ 7.1 and proton resonances of the sugar group between δ 3.5 and δ 4.1. The resonance at δ 5.2 indicates the glycosidic proton resonance

and the resonance at δ 1.1 (δ) indicates that the sugar may be rhamnose as indicated in compound **E**.



E

Samples 241 to 244 (Figs. 3.27-3.30) of 4d-8 axes indicated the presence of stigmasterol, with the largest concentration being present in sample 243 and minor quantities in samples 241, 242 and 244. This spectrum of sample 243 was successfully compared to the spectrum of an authentic sample of stigmasterol (**F**).

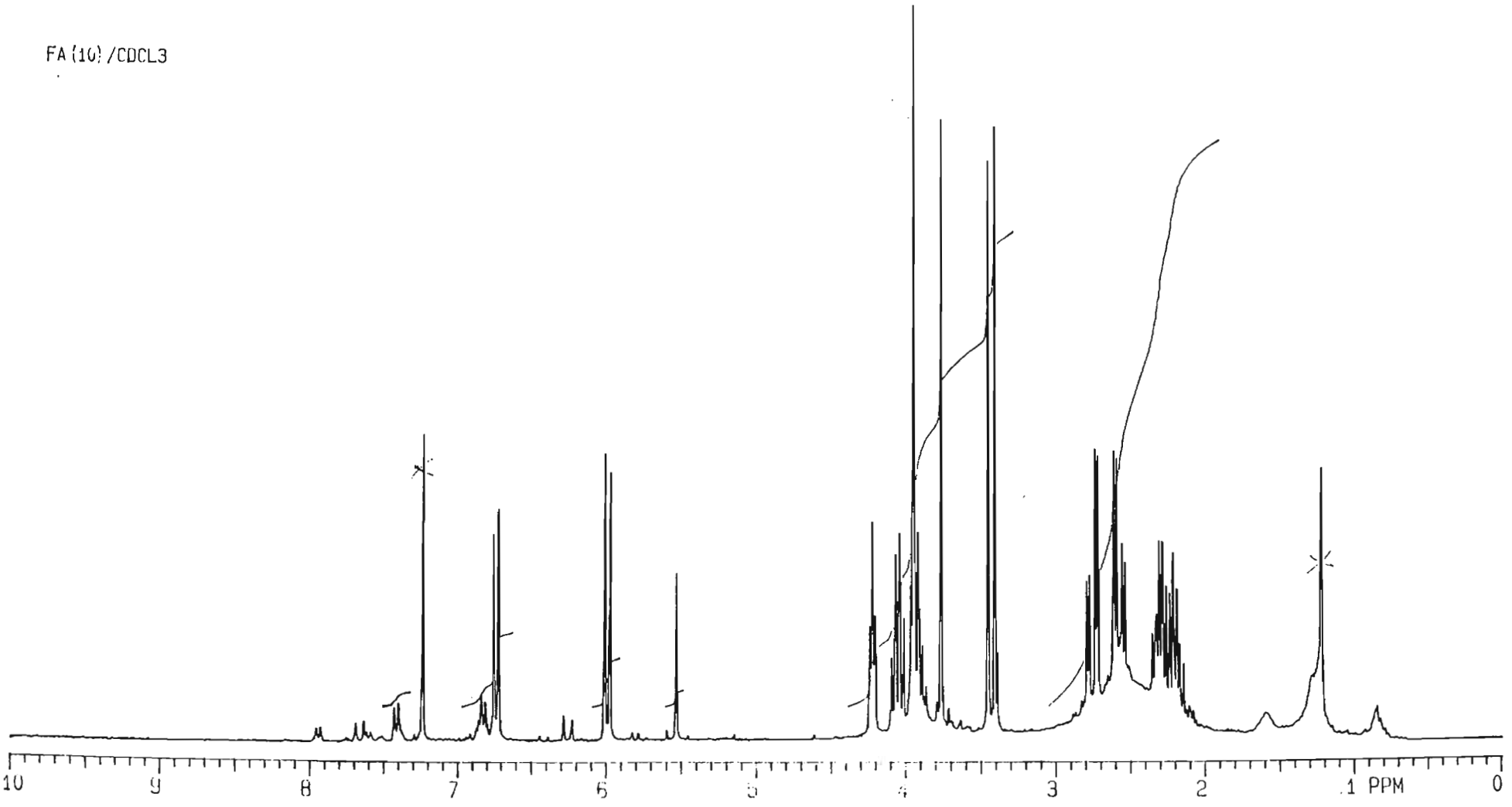


F

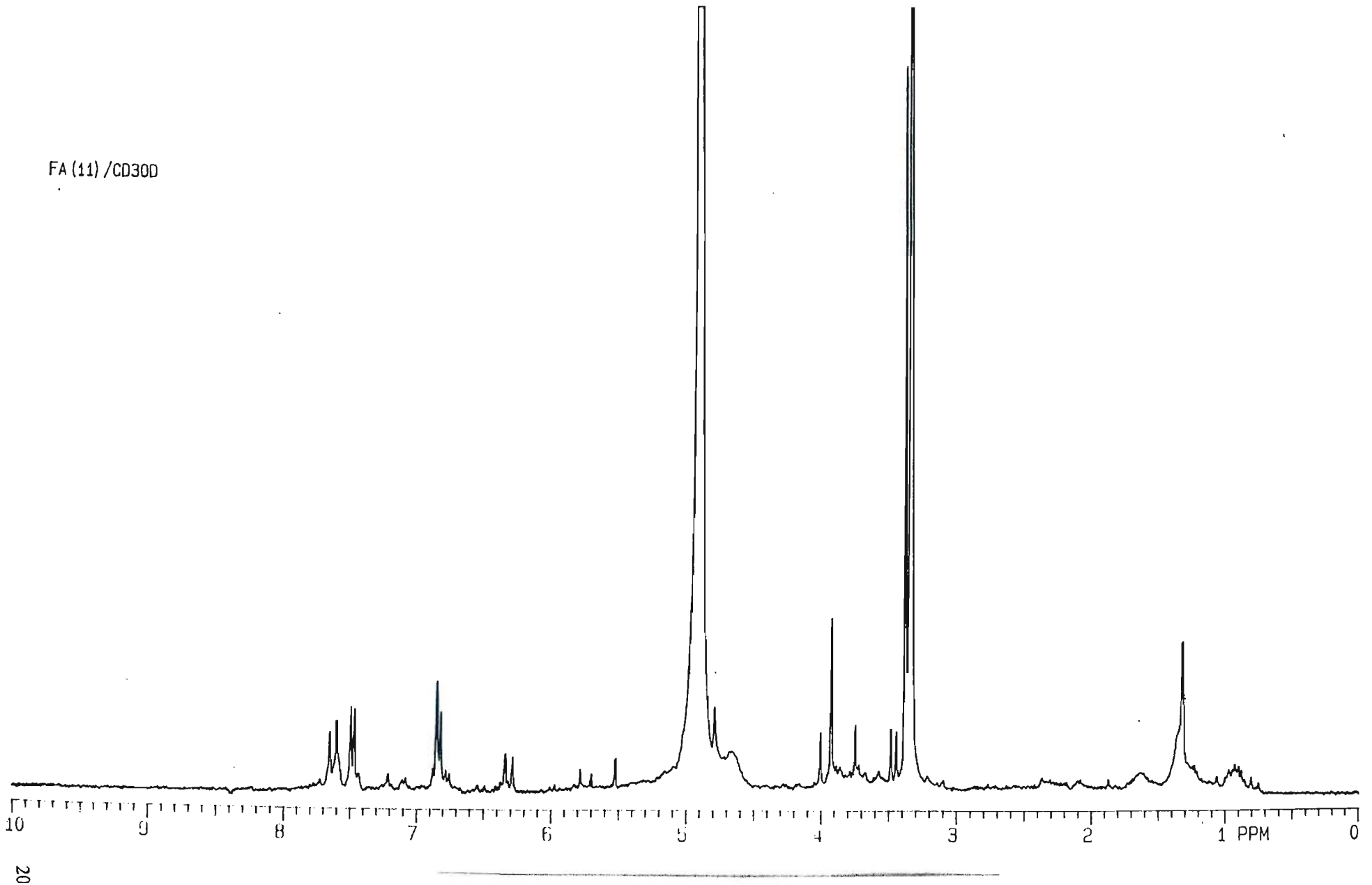
The proton NMR spectra of 4d-8 axes samples 245 (Fig. 3.31), 246 (Fig. 3.32) and 248 (Fig. 3.33) indicated the presence of a possible fatty acid (compound **G**), by peaks at δ 5.3, δ 4.2, δ 3.9, 3.5-3.7 and δ 2.4. The strongest concentration was contained in sample 245 with the concentration decreasing in samples 246 and 248.

Samples 251-253 (Figs. 3.34-3.36) of 4d-8 exbryonic axes show minor quantities of compound **C**, with the strongest concentration being present in sample 251, but not as concentrated as that found in sample 10 of fresh axes (Fig. 3.22).

The 4d-8 embryonic axes samples 261-263 (Figs. 3.37-3.39) show the same proton NMR pattern as was found in samples 61-63 (Figs. 3.24-3.26), but were of a smaller concentration as found in the previous samples and therefore could contain small amounts of compound **E**.



201


FA (11) /CD3OD
10
9
8
7
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5
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3
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1 PPM
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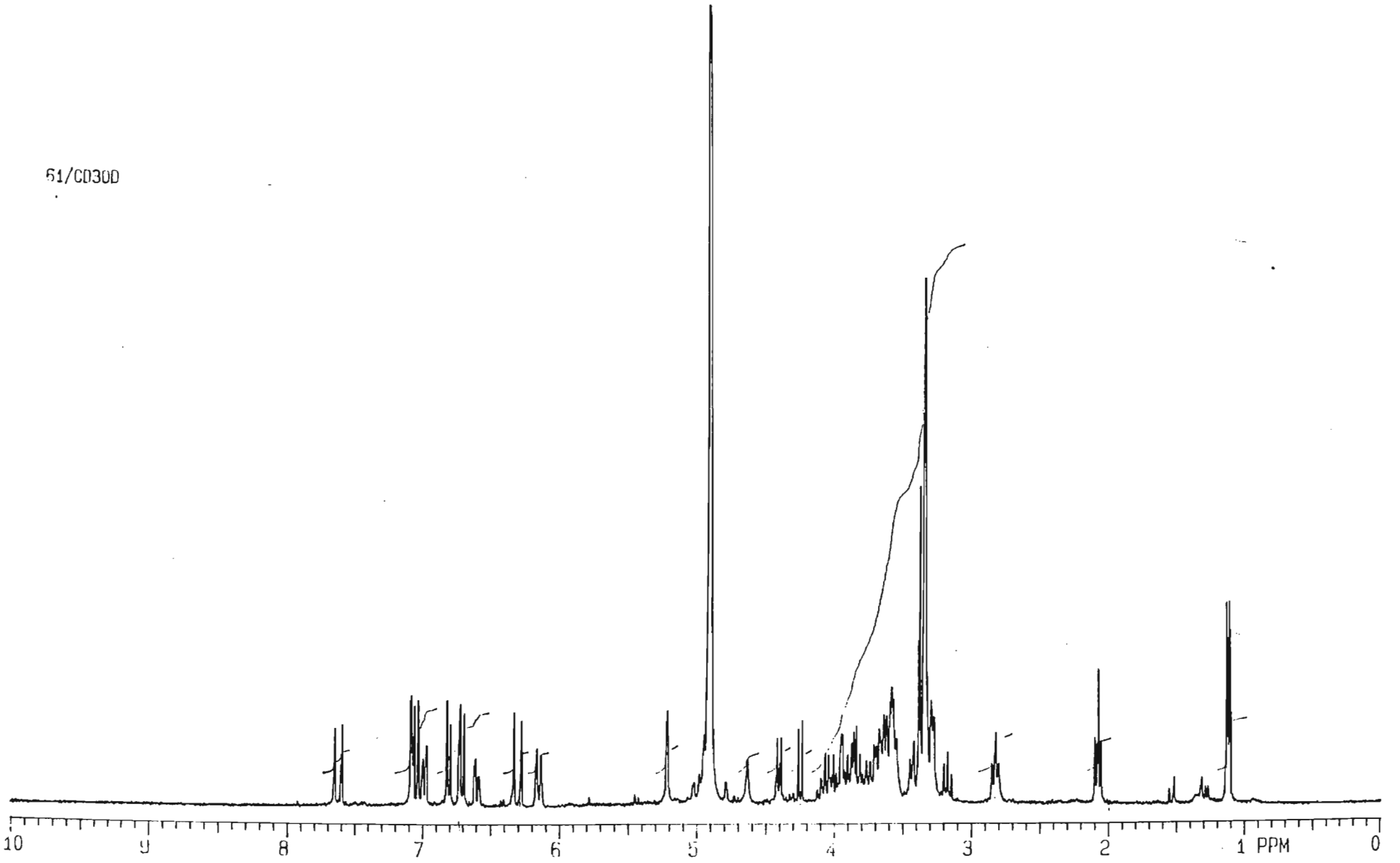
61/CD30D
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9
8
7
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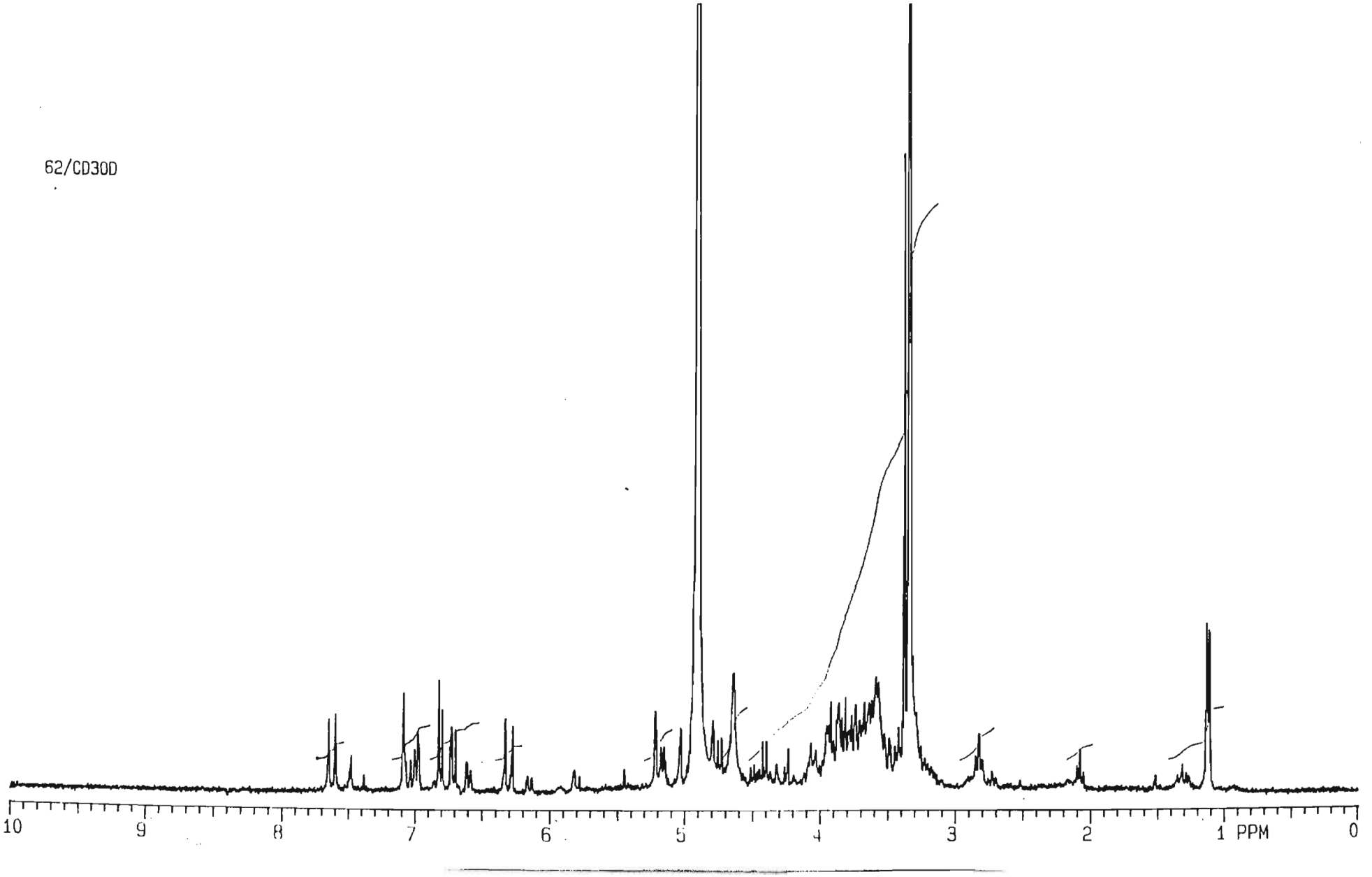
62/CD30D



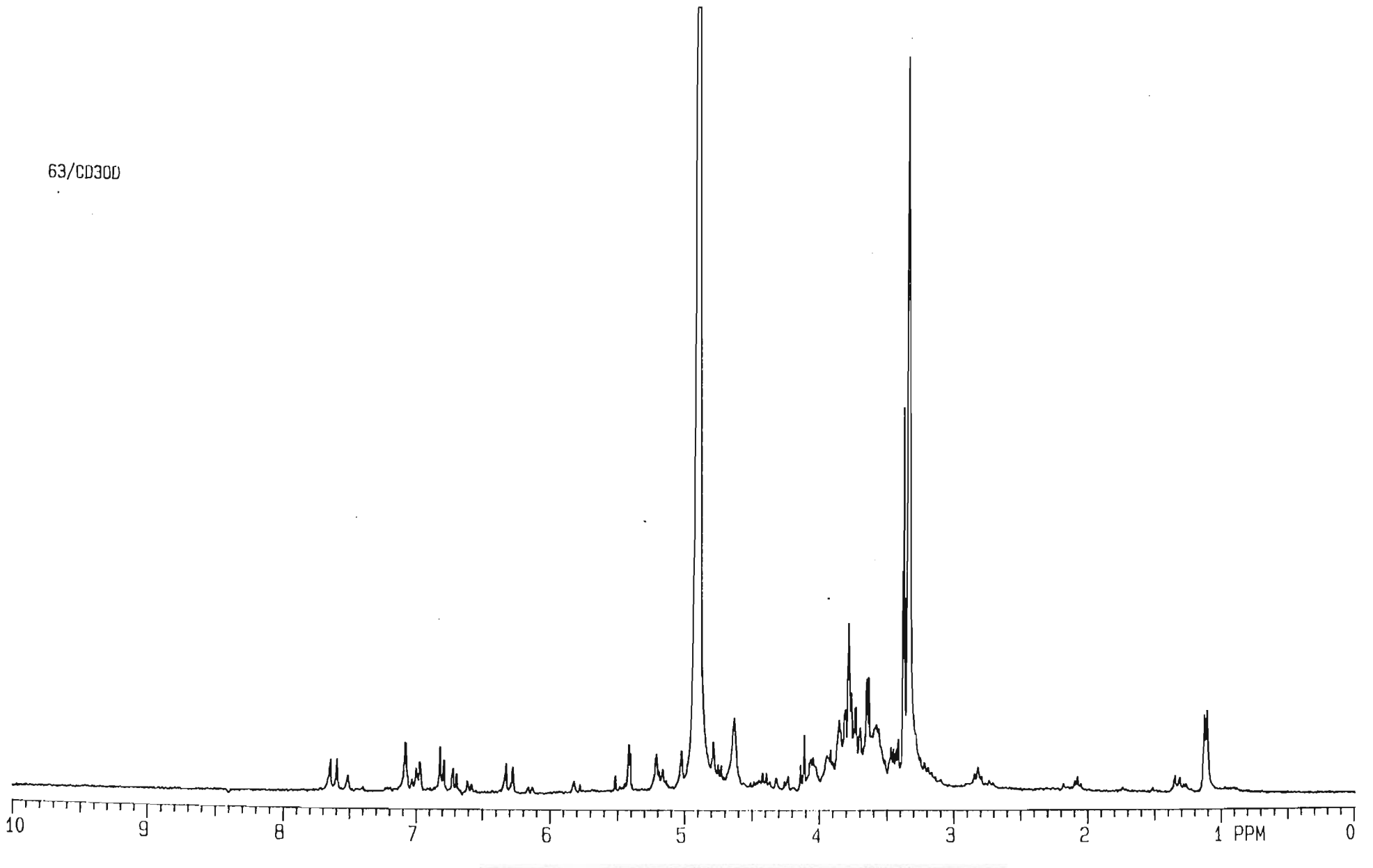


Fig. 3.26. ^{1}H NMR spectrum of sample 63 of 4d clean embryonic axes

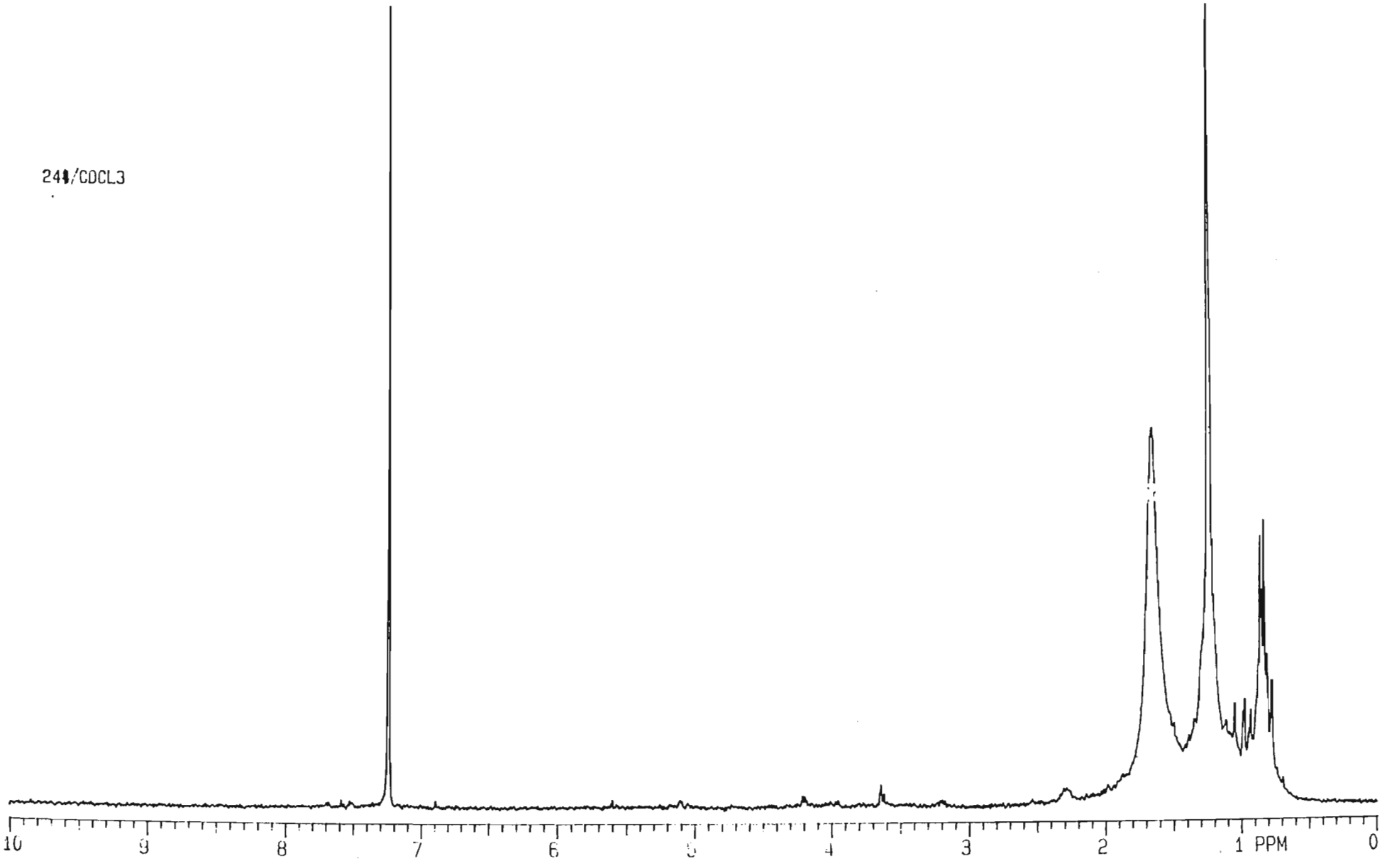
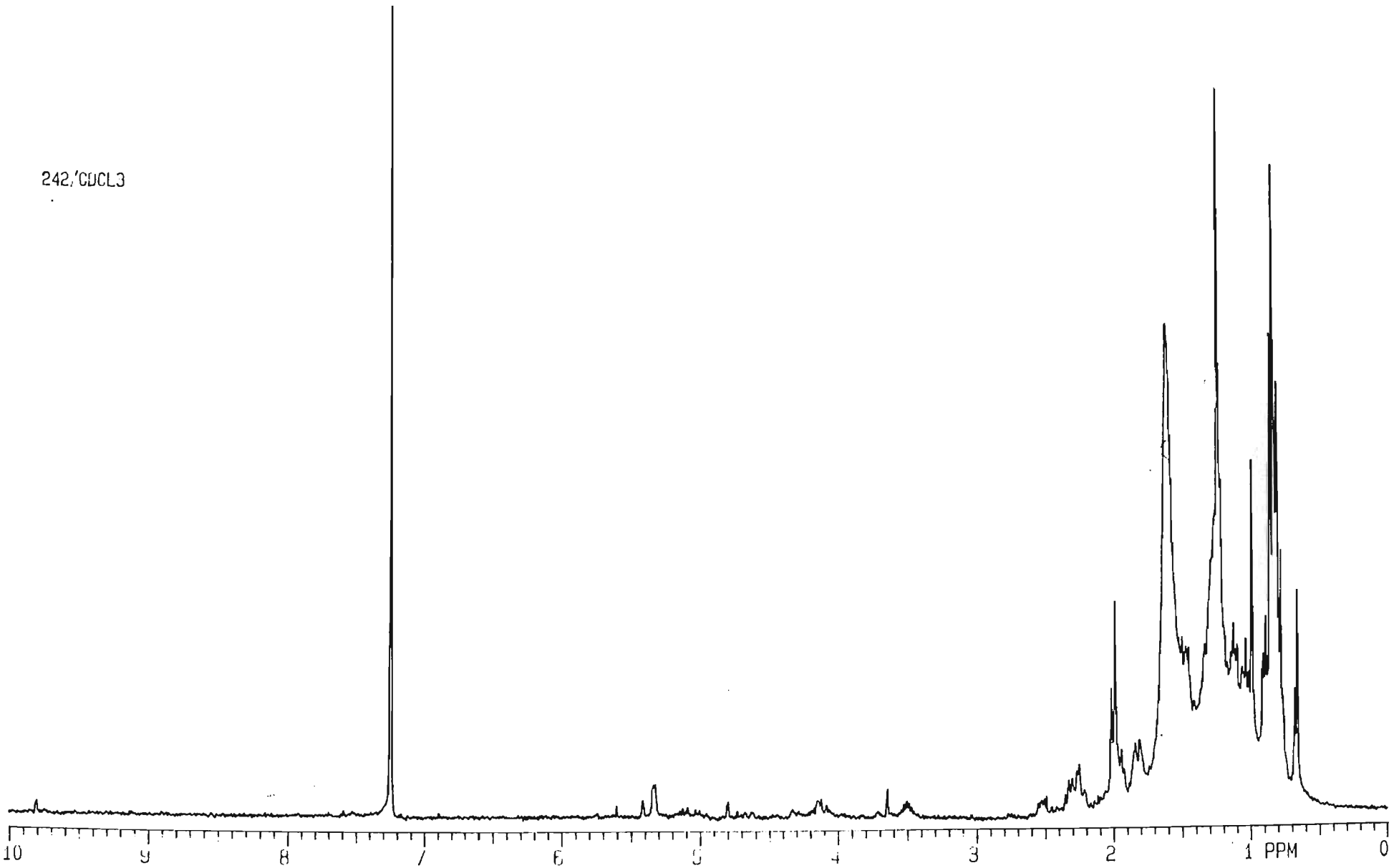


Fig. 3.27 ^{1}H NMR spectrum of sample 241 of 4d-8 embryonic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9
8
7
6
5
4
3
2
1 PPM
0

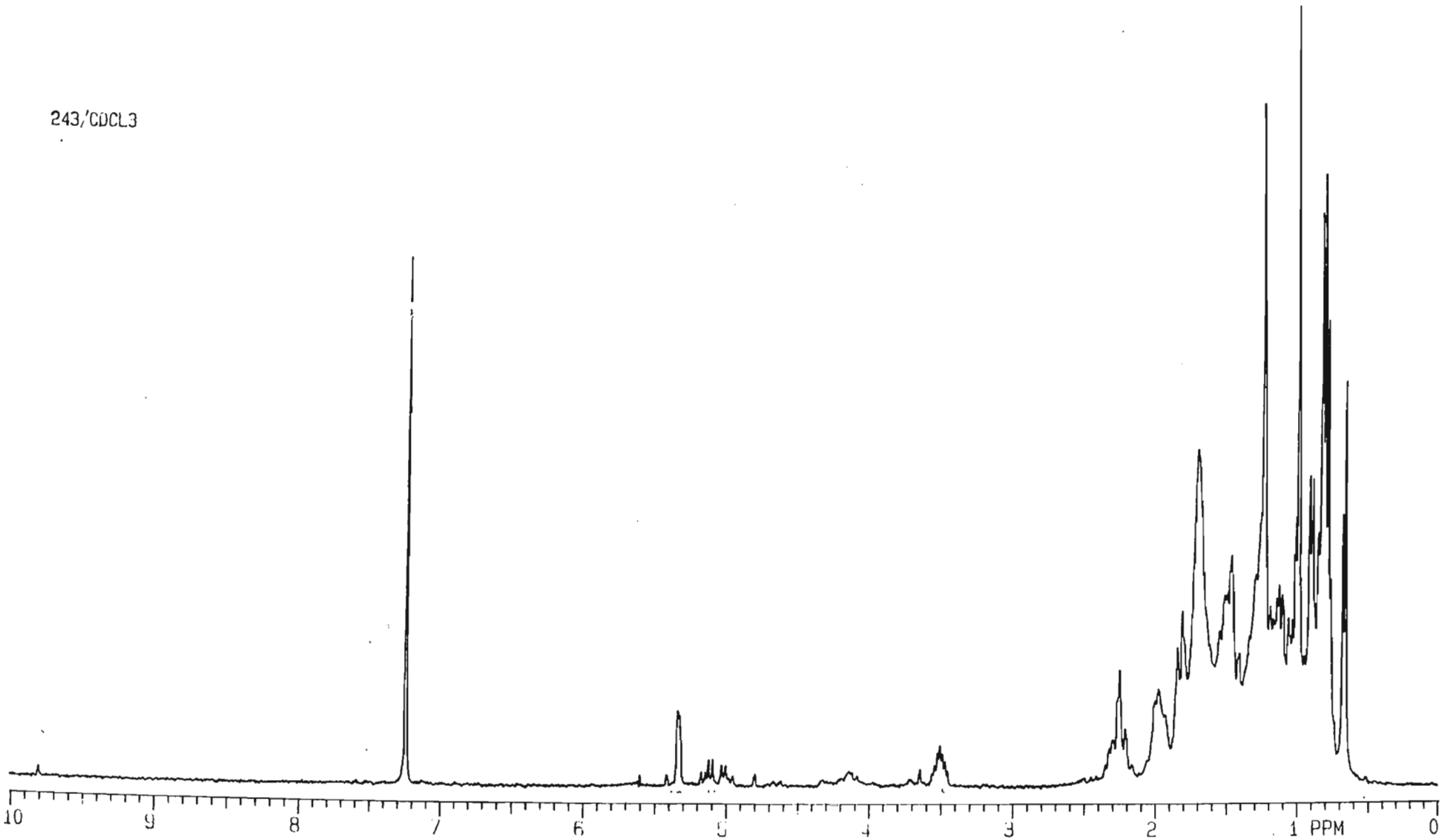


Fig. 3.29. ^{1}H NMR spectrum of sample 243 of 4d-8 embryonic axes

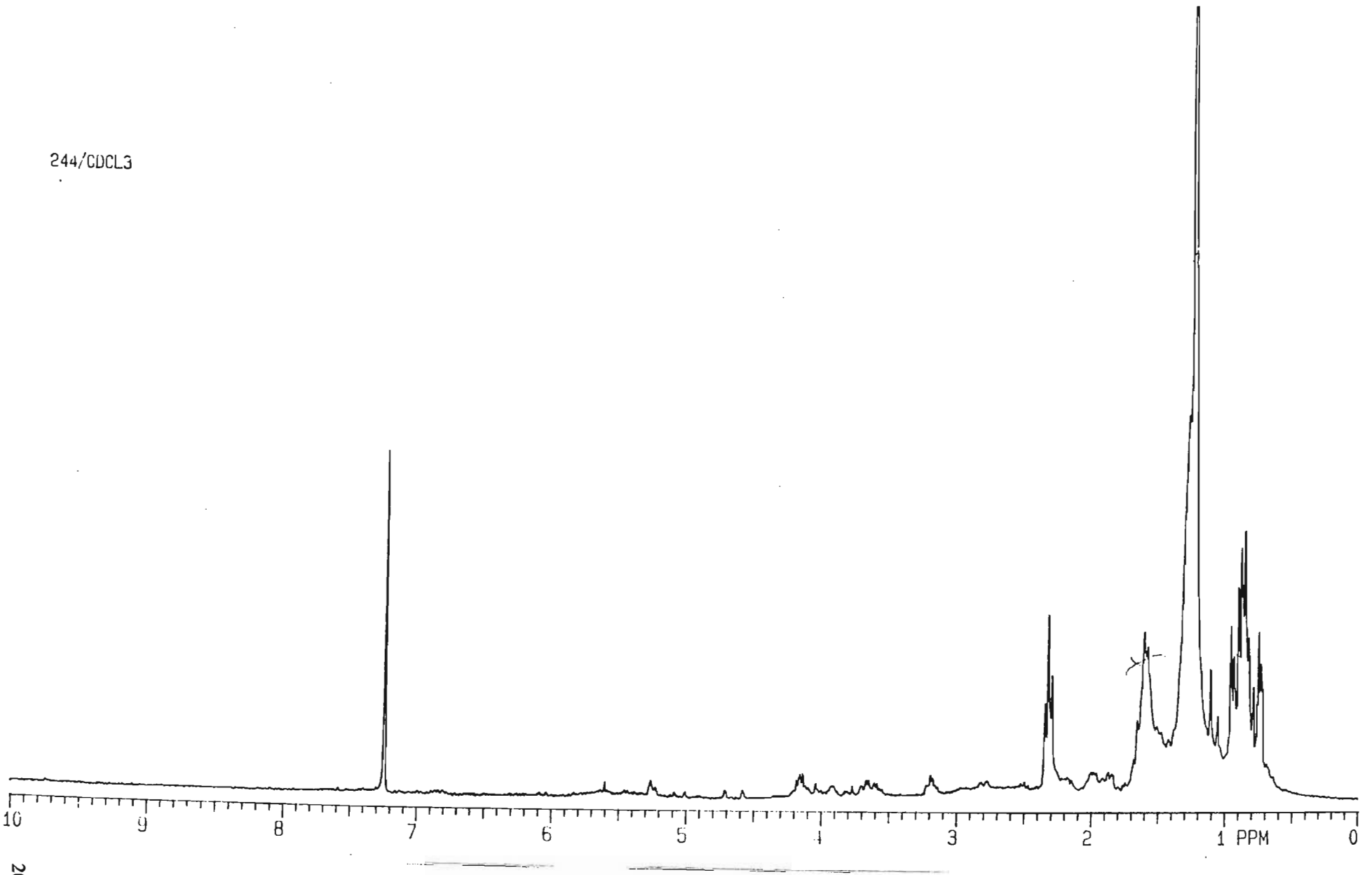
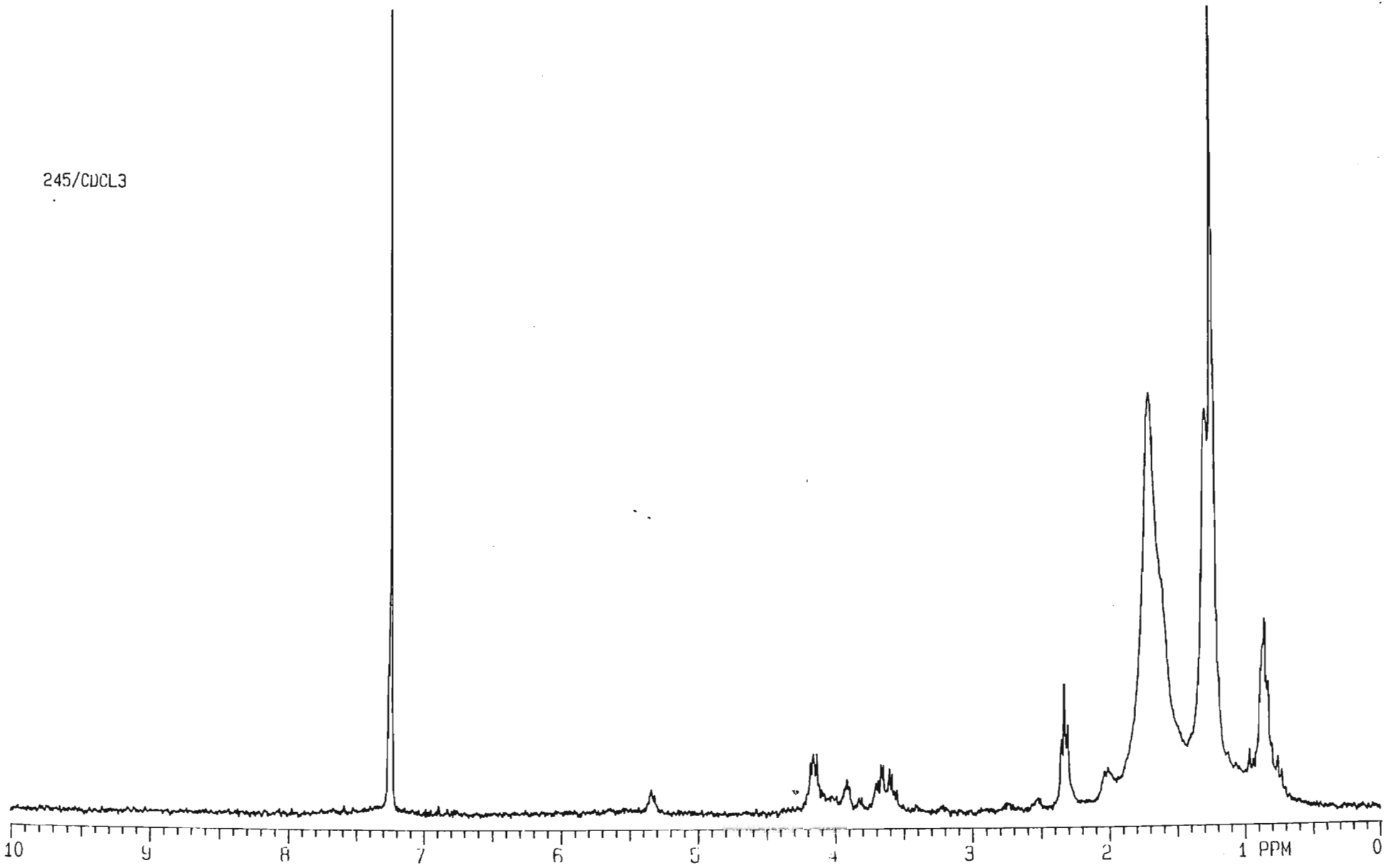


Fig. 3.30. ^{1}H NMR spectrum of sample 244 of 4d-8 embryonic axes


245/CDCL3
10
9
8
7
6
5
4
3
2
1 PPM
0

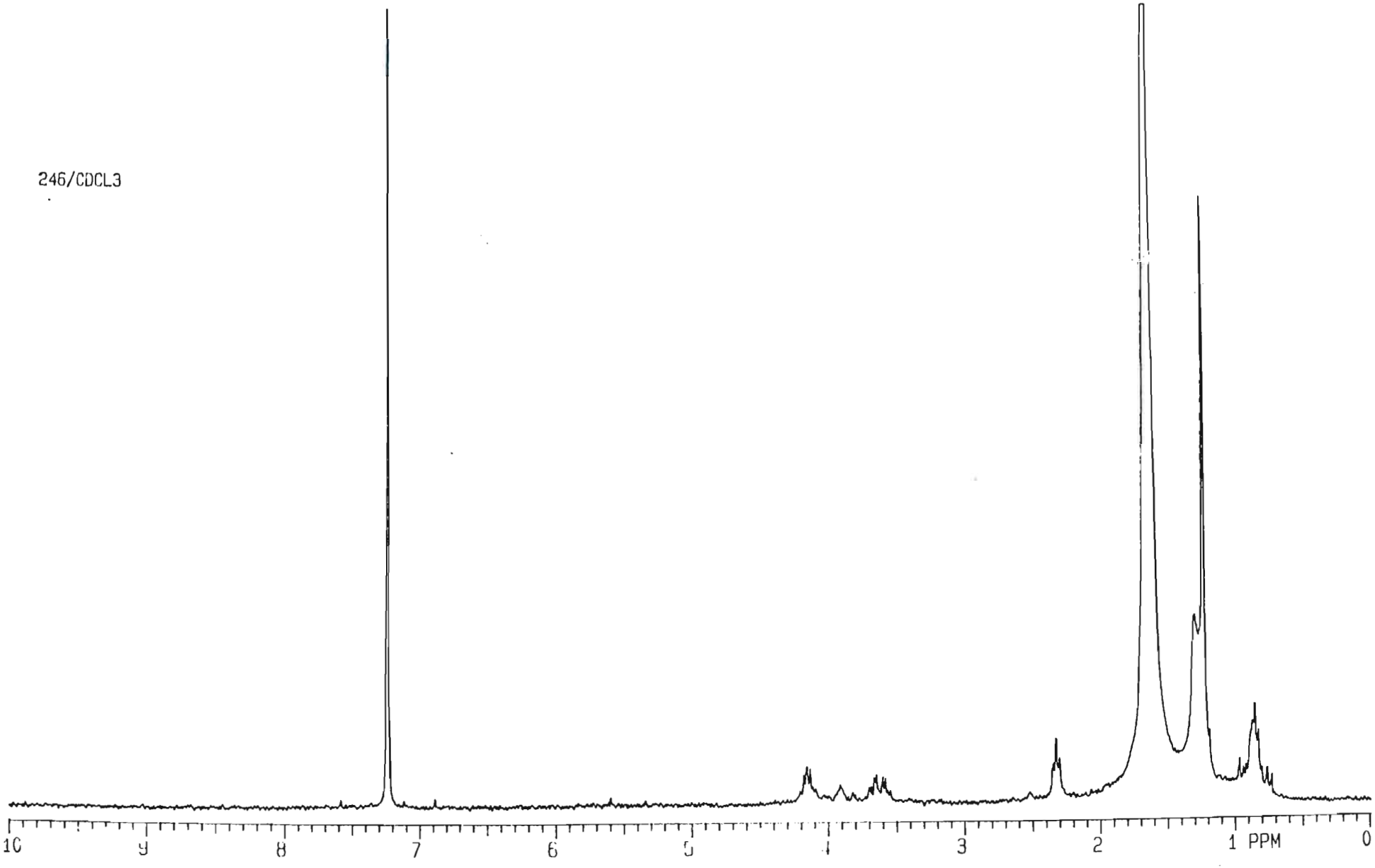
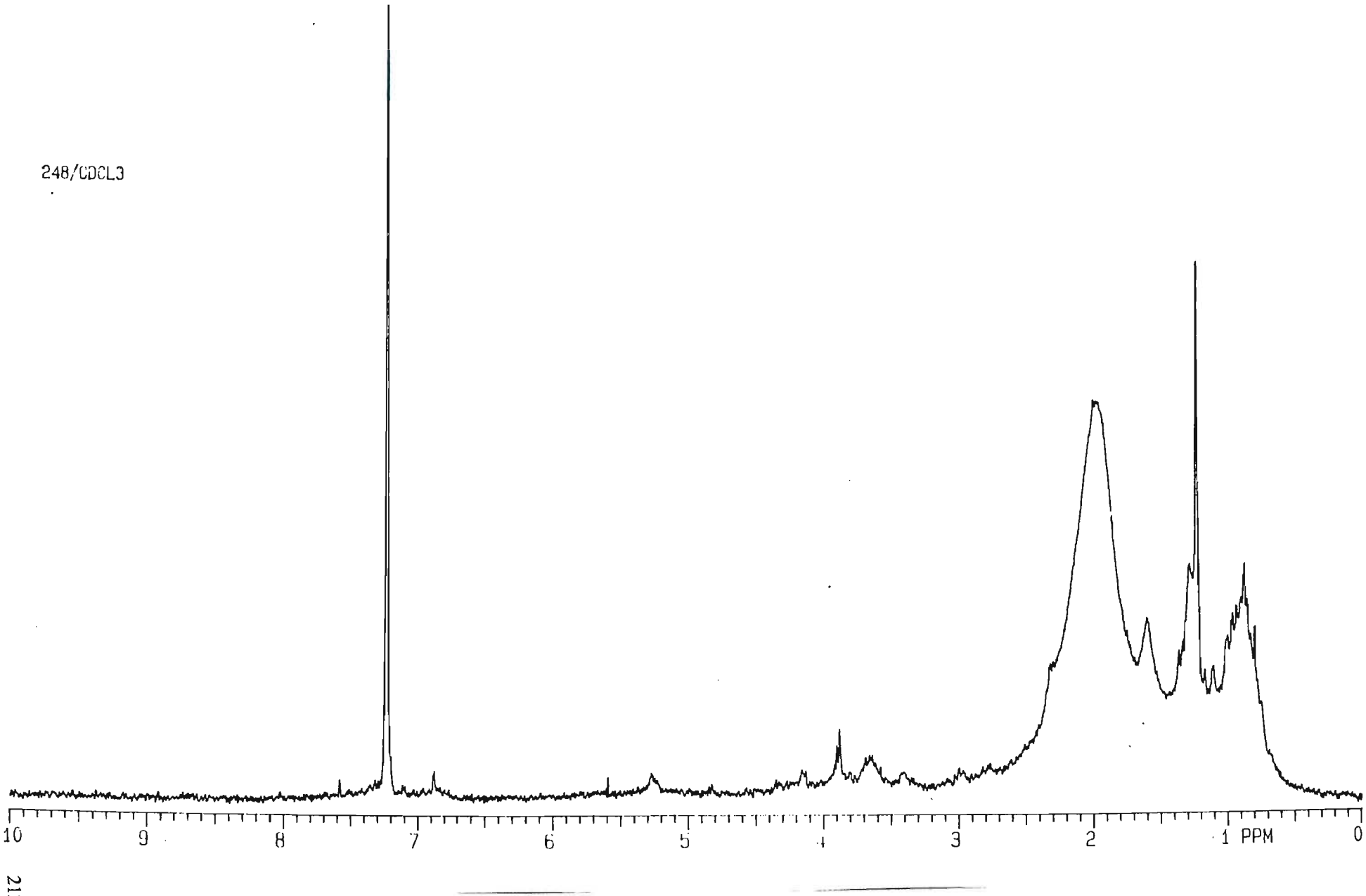
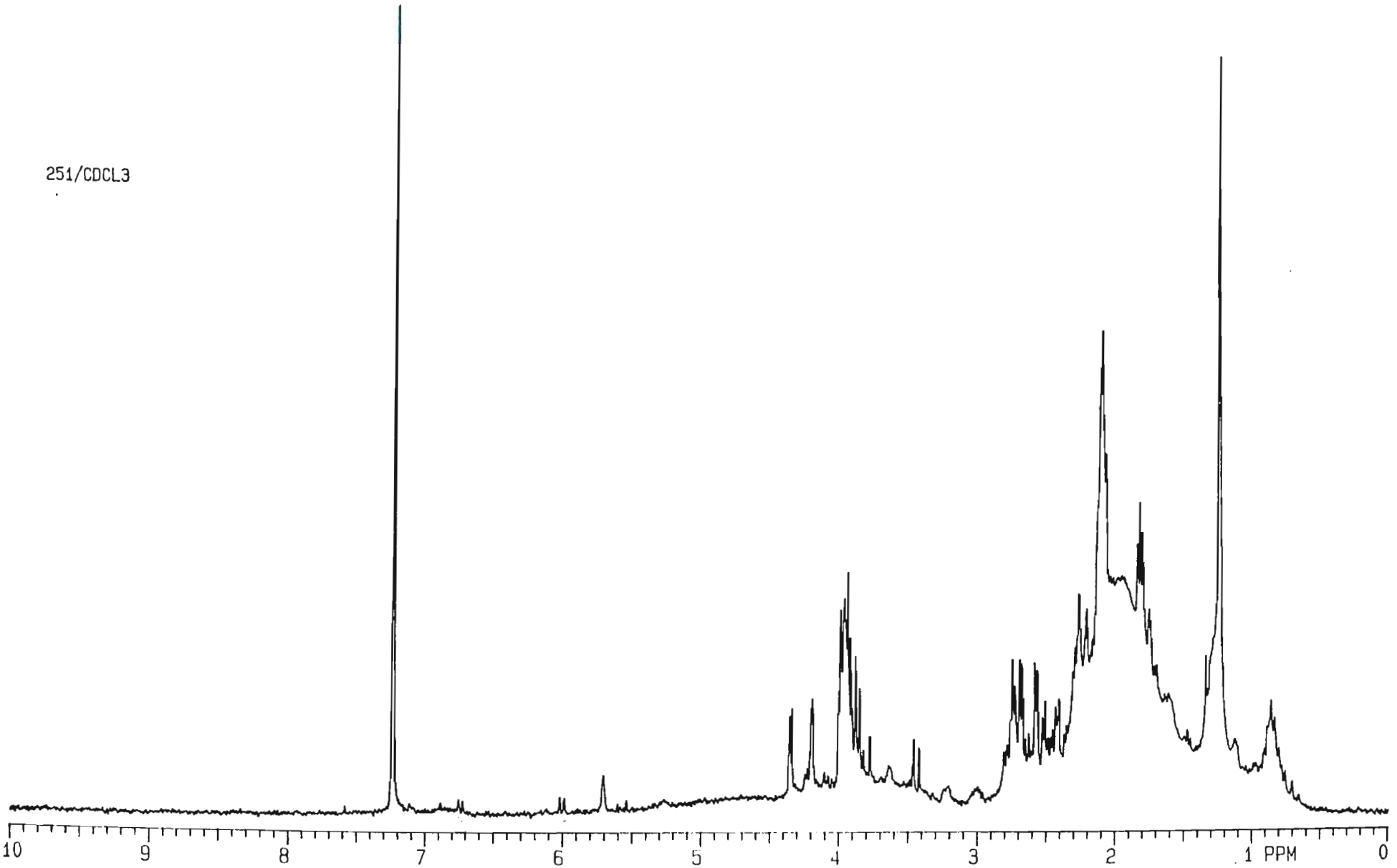


Fig. 3.32. ^{1}H NMR spectrum of sample 246 of 4d-8 embryonic axes


248/CDCL3
10
9
8
7
6
5
4
3
2
1 PPM
0


251/CDCL3
10
9
8
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1 PPM
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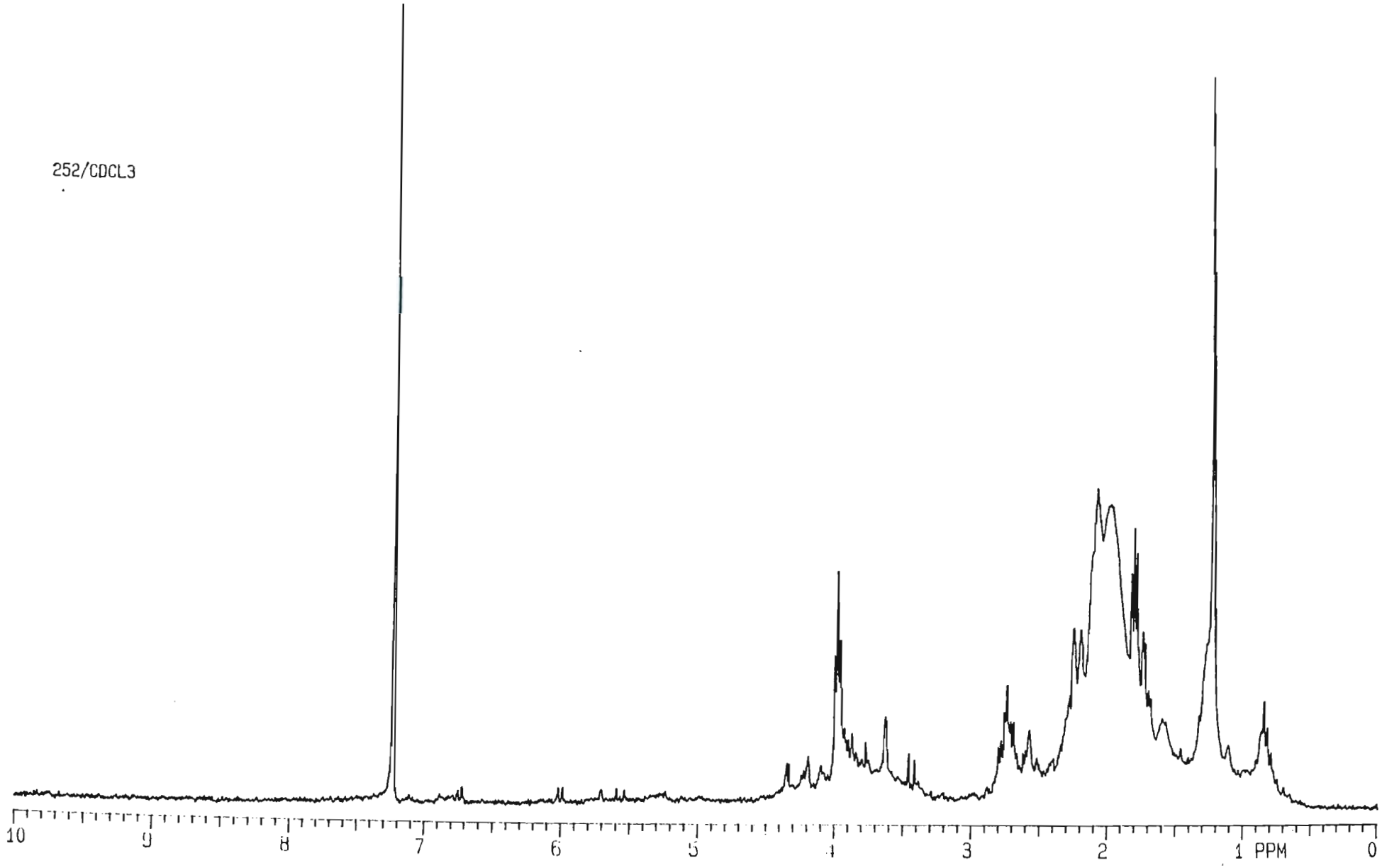


Fig. 3.35. ^{1}H NMR spectrum of sample 252 of 4d-8 embryonic axes

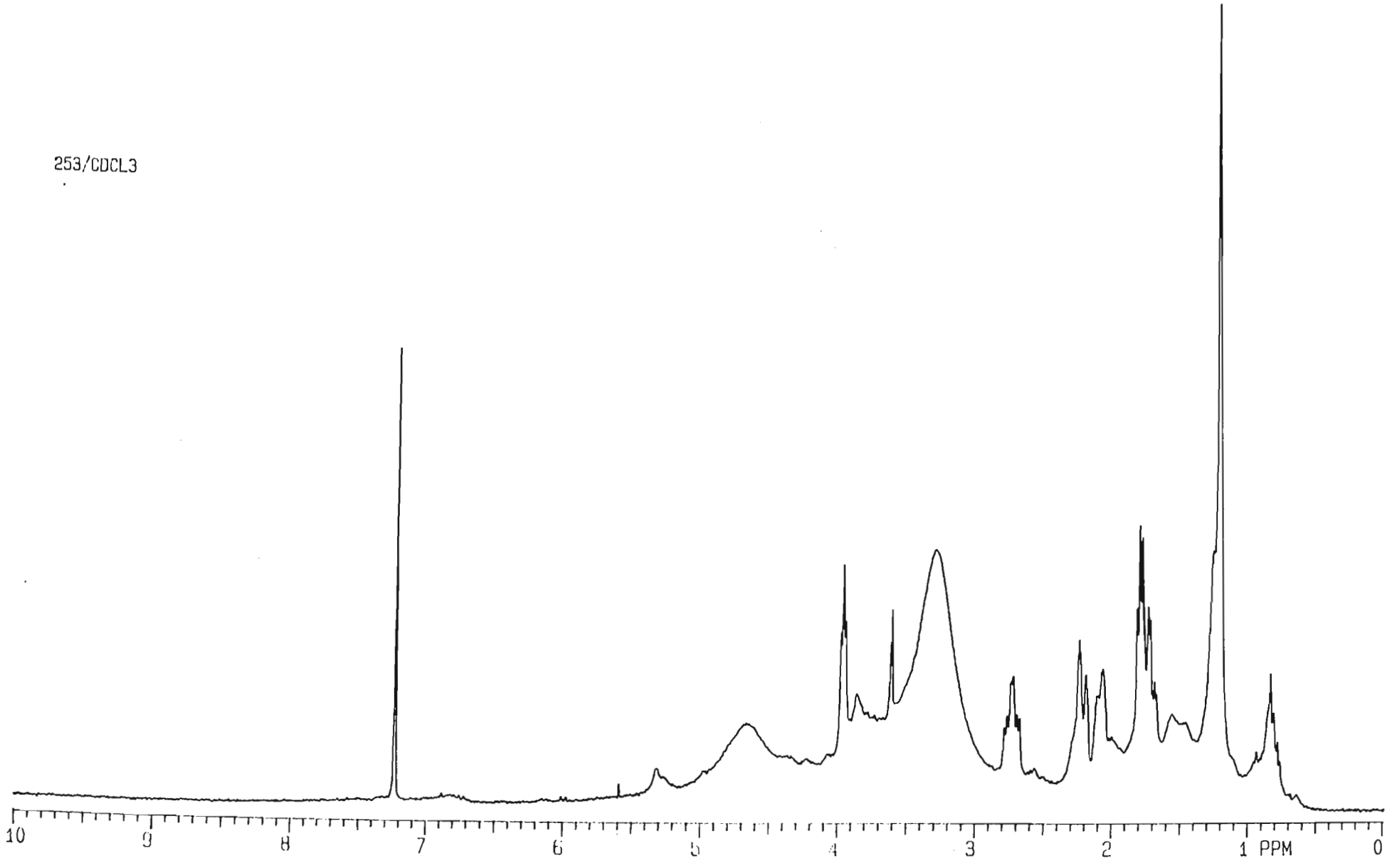


Fig. 3.36. ^{1}H NMR spectrum of sample 253 of 4d-8 embryonic axes

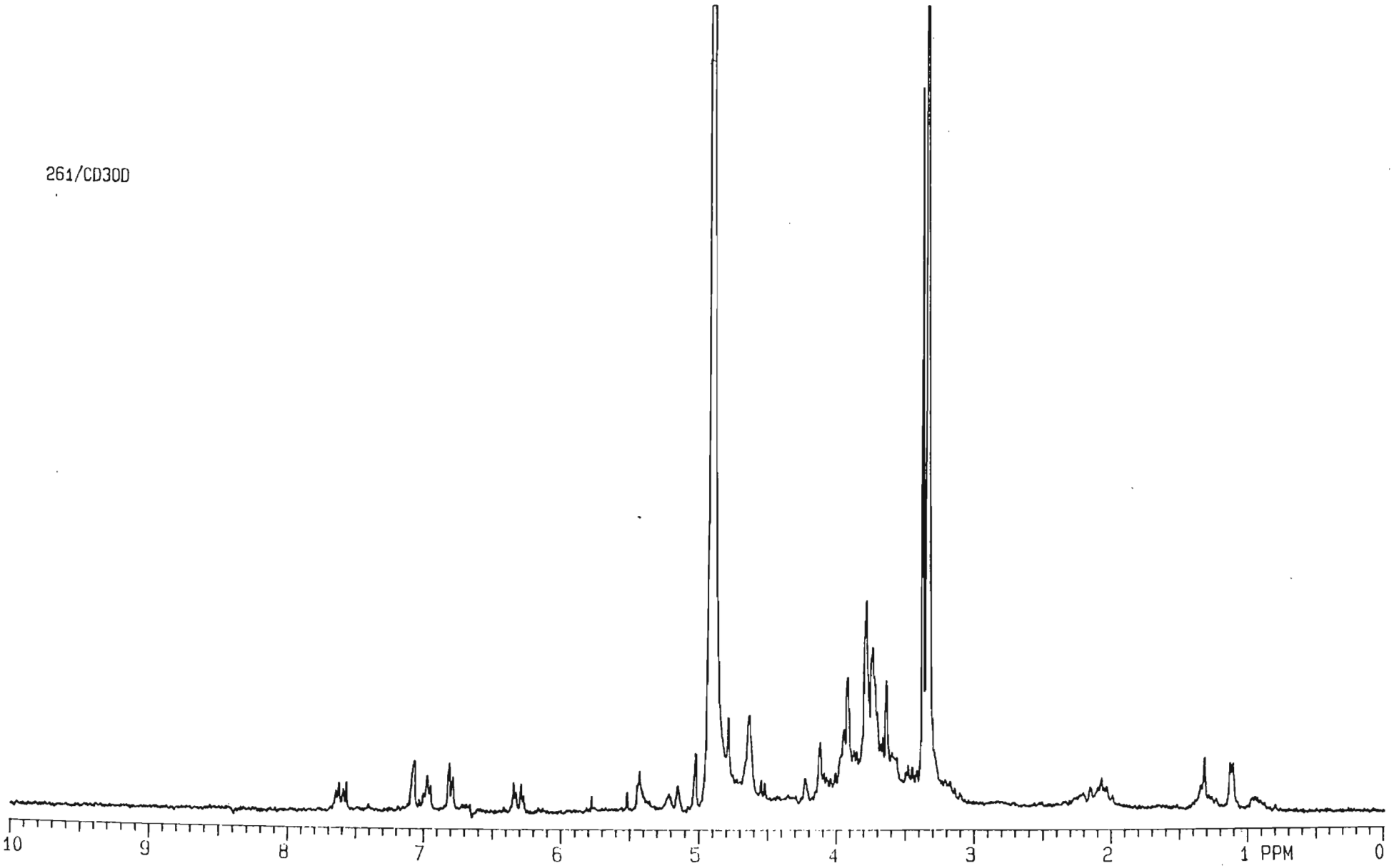


Fig. 3.27. ^{1}H NMR spectrum of [illegible] 261 of 1d-2 embryonic axes

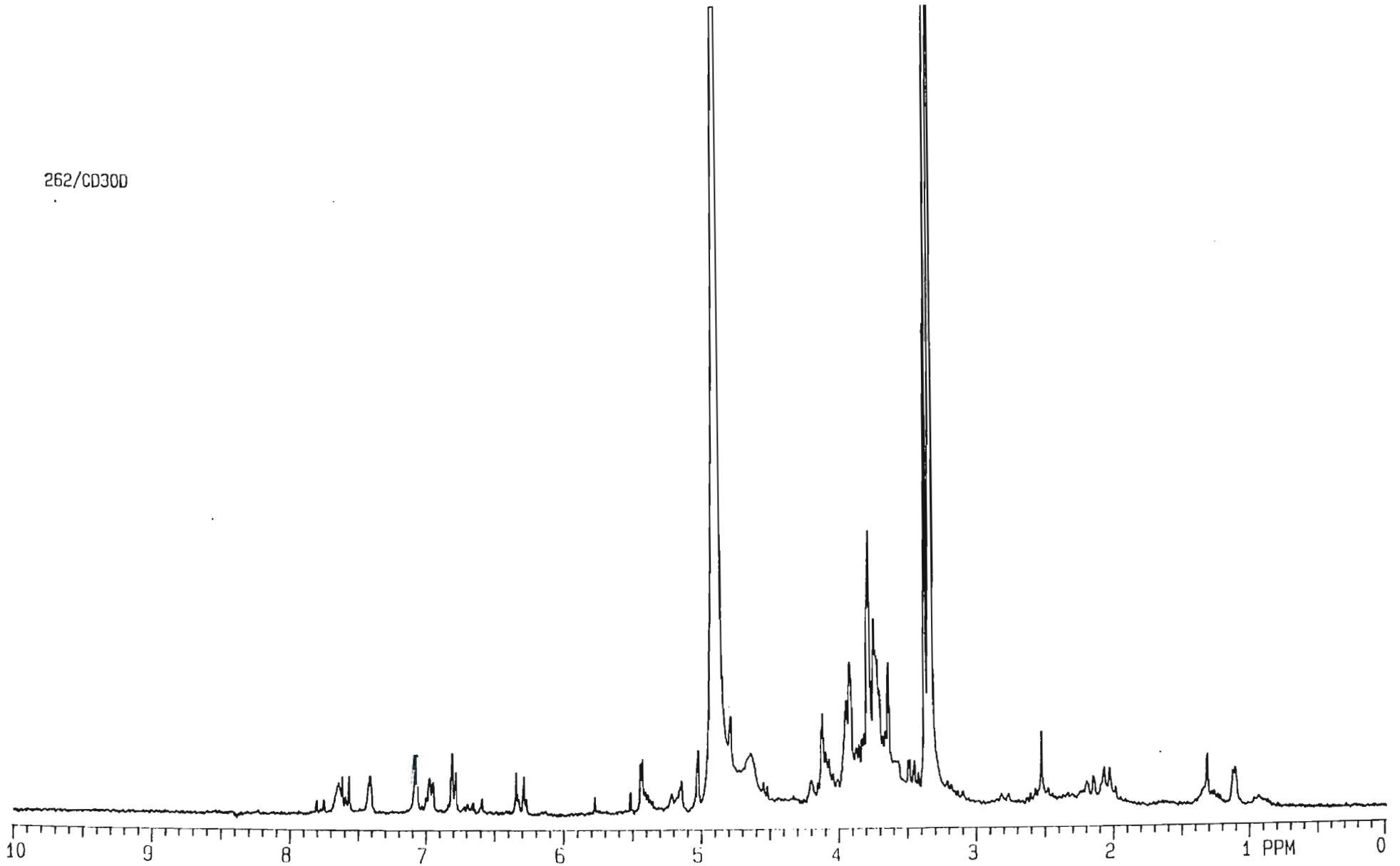


Fig. 3.38. ^{1}H NMR spectrum of sample 262 of 4d-8 embryonic axes

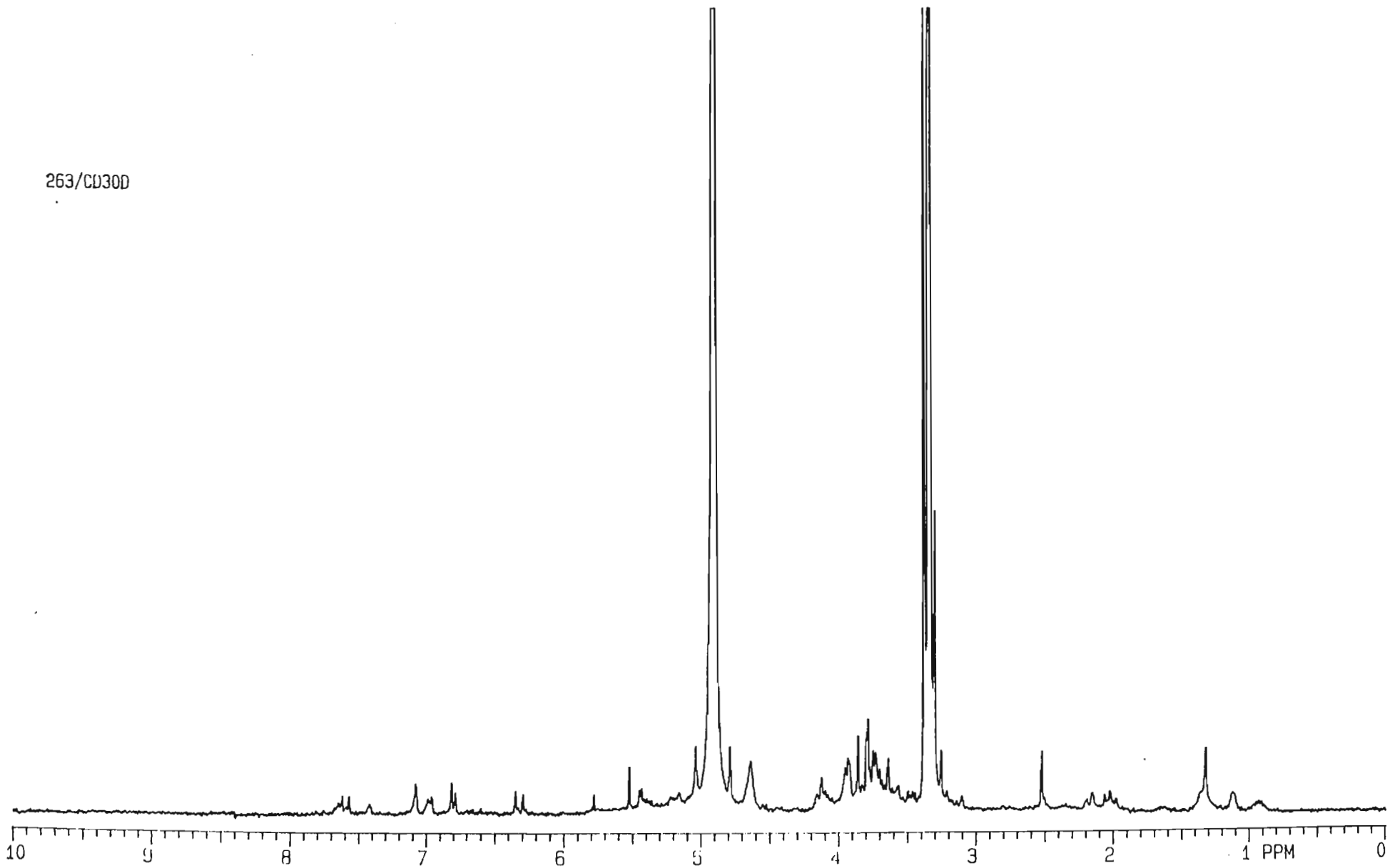


Fig. 3.39 ^{1}H NMR spectrum of sample 263 of 4d-8 embryonic axes

3c.4 Assessment of β-1,3-glucanase and chitinase activity[2]

Although activity of β-1,3-glucanase (Fig. 3.27) was observed in fresh seeds, after four days of clean wet-storage the activity had increased more than two-fold. This heightened level activity was still apparent in seeds for 10d clean. However, significant decrease in β-1,3-glucanase level activity was observed in seeds that were stored longer than 10 days. This activity was two times lower in seeds stored for 12 days and three times lower in 18 days when compared to 4d clean material. High levels of β-1,3-glucanase were measured in infected seeds (8d infected) and in seeds of 4d-8 and 10d-8 treatments.

Fig. 3.28 represents chitinase activity demonstrated by different treatments. The trend in levels of chitinase activity in seeds stored cleaned for up to 10 days were similar to those of β-1,3-glucanases. However, in contrast to β-1,3-glucanases, the levels of chitinase activity appeared to remain higher even after 12 and up to 18 days of hydrated clean storage. In addition, it was observed that there was no difference between the levels of chitinase activity in fresh seeds and in 8d infected seeds. Similar to β-1,3-glucanases, the chitin levels remain high in 4d-8 and 10d-8 seeds.

[2] The assistance of Dr. Vesselina Mehar from the School of Life and Environmental Sciences is gratefully acknowledged.

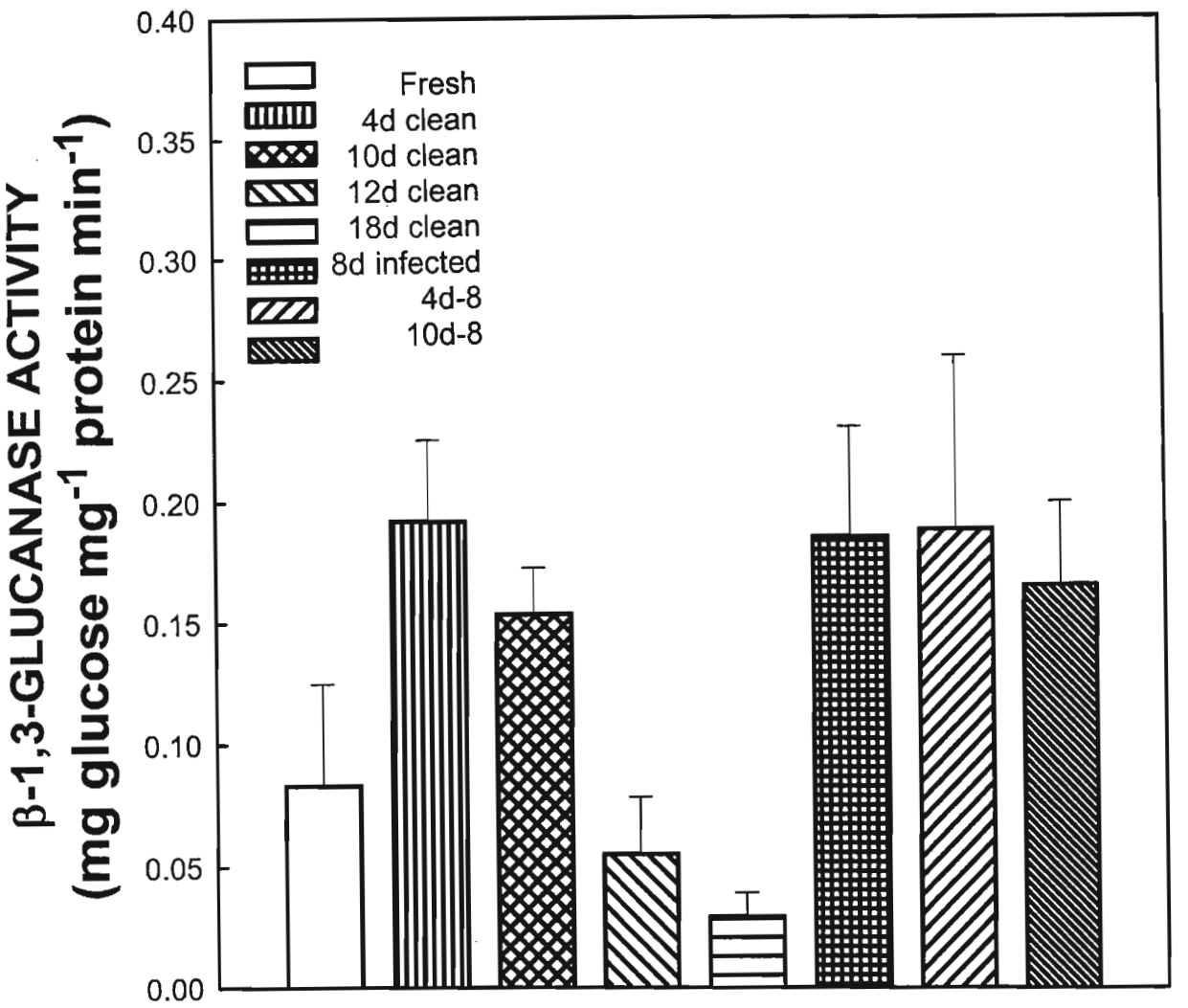


Fig. 3.27. Total β-1,3-glucanase activity in the axes of recalcitrant seeds of *A. marina* subjected to different storage/treatments. Data presented are the means of three replicates. Error bars represent positive SD. Data from the first experiment only are presented but similar trends were observed in replicate studies.

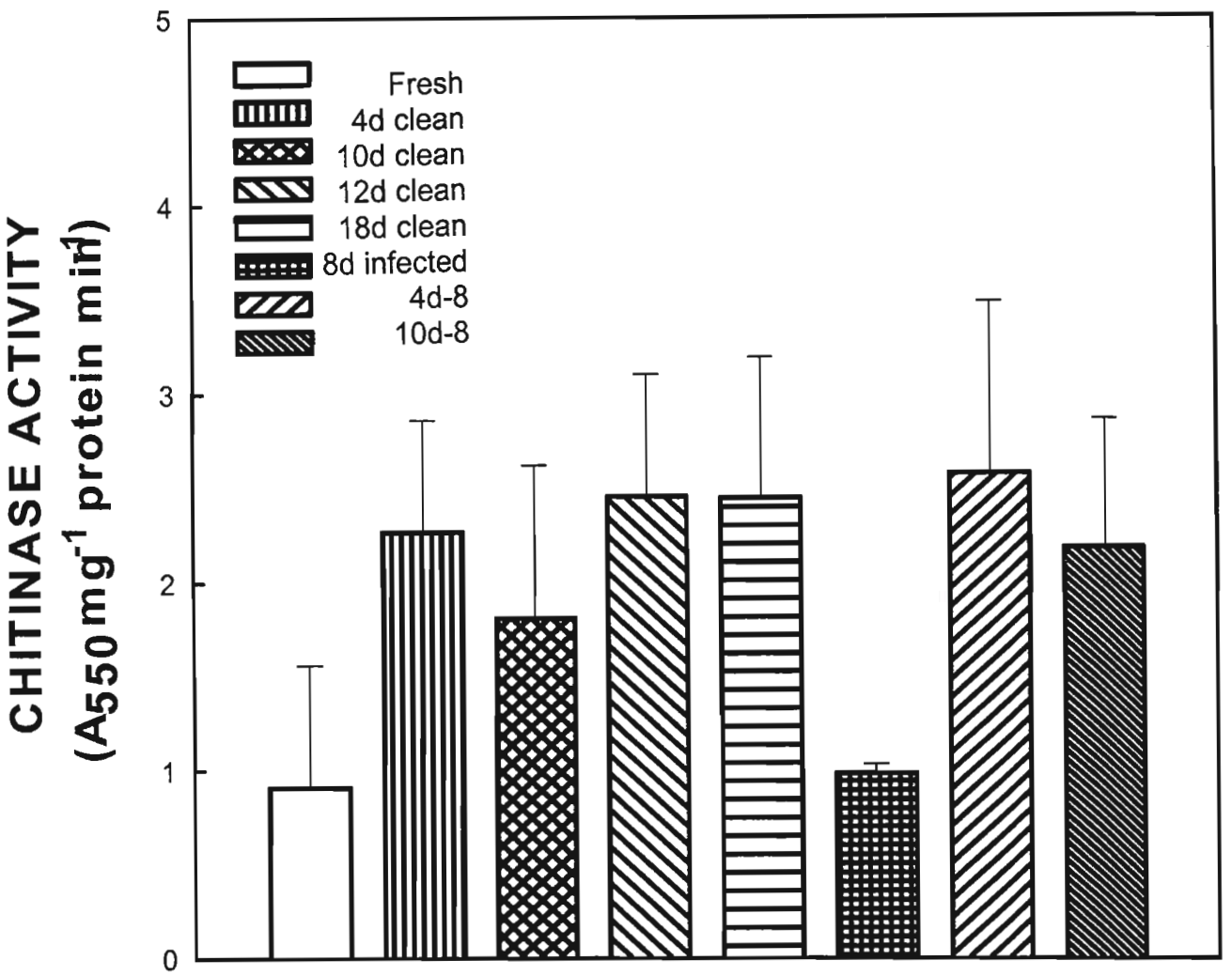


Fig. 3.28. Total chitinase activity in axes of recalcitrant seeds of *A.marina* subjected to different storage/treatments. Data presented are the means of three replicates. Error bars represent positive SD. Data from first experiment only are presented but similar trends were obtained in replicate studies.

3d. DISCUSSION

The role of mycoflora is emerging as an important factor in determining seed longevity in hydrated storage. While there is evidence that fungal proliferation is almost invariably associated with recalcitrant seeds during wet-storage (Murray, 1974; Delatour, 1978; Dalbir-Singh and Singh, 1990; Mycock and Berjak, 1990), there are no data on the responses of recalcitrant seeds to fungal infection during hydrated storage. The current study investigates possible defence mechanisms of highly recalcitrant seeds of *Avicennia marina* to *Fusarium moniliforme* infection during short-term hydrated storage.

Defence mechanisms against pathogens fall into two categories: those that are present constitutively (pre-formed defences) and those that are induced upon exposure to a pathogen. The passive or pre-existing defence mechanisms involve structural barriers, such as a waxy cuticle, secondary thickening, leaf hairs, or strategically placed reservoirs of antimicrobial compounds that function to prevent plant tissue colonisation by invading pathogens (Jackson and Taylor, 1996; Osborne, 1996). Preformed antifungal compounds within plants are generally tissue specific (Poulton and Li, 1994), but they have a tendency to be concentrated in the outer layer of plant organs, suggesting that they could act as deterrents to pathogens (Osbourn, 1996). Plants also have induced cellular defences that prevent further pathogen invasion of tissue once host structural barriers have been breached. At least three types of induced/active defence responses may be identified in tissues of infected plants: primary responses that are localised in cells which are in direct contact with the invading pathogen, secondary responses that are induced in the surrounding cells adjacent to the infection site in response to diffusible elicitors (signal molecules) and systemically acquired responses that are hormonally induced throughout the plant (Hutcheson, 1998).

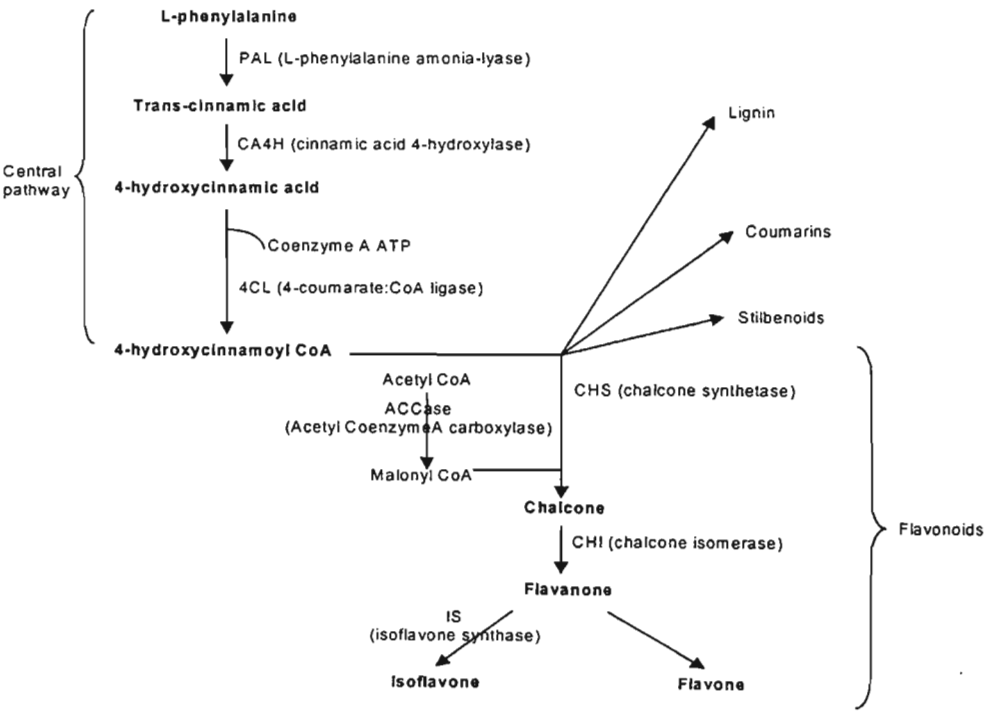
From the results of the present study, the defence mechanisms of *A. marina* seeds appear to include both constitutive and induced mechanisms. The same number of possible preformed antifungal compounds was present in apparently fungal-free axes (between four to six compounds) and cotyledons (three compounds) of freshly harvested seeds and of seeds stored clean for four days (4d clean) (Table 2.1, Tables 3.26-3.27). Fresh seeds contained hallerone (compound **A**) and a cinnamic acid-type compound (compound **D**). Four days further into clean storage (4d clean) the hallerone concentration was low but three more compounds were produced: cinamic acid (compound **B**), rengyolone (compound **C**) and a type of chalcone glycoside (compound **E**).

These are aromatic compounds that are part of general phenylpropanoid metabolism: the sequence of reactions converting L-phenylalanine into substituted cinnamic acid CoA esters has been termed general phenylpropanoid metabolism (Jackson, 1986). This metabolism is important in higher plants, providing a number of precursors through several specific pathways into which the central pathway branches (Ebel, 1986) (Fig. 3.29). The enzymes catalysing the individual steps of this central pathway are phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CA4H) and 4-coumarate:CoA ligase (4CL). Several other enzymes of this biosynthetic pathway have been studied at the molecular level and they include 6-hydroxychalcone synthetase and chalcone isomerase. All these enzymes are involved in the biosynthesis of a large class of secondary metabolites that have important roles in plant development and protection against pathogenic fungi (Chakravorty and Scott, 1991). These secondary metabolites can be pre-formed in plant cells or induced by pathogenic attack and they include simple and alkylated phenols, phenolic acids, phenylpropanoids, coumarins, flavonoids, isoflavonoids, stilbenoids, quinines and xanthones (Grayer and Harborne, 1994). Sometimes, as is the case of isoflavonoids and stilbenoids, the same aromatic compound could be a constitutive antifungal compound in

one species and an induced constituent in another plant (Grayer and Harborne, 1994).

Compounds **B**, **D** and **E** could be part of a flavonoid synthesis pathway (Fig. 3.29), and their presence is an indication that fresh and 4d clean stored seeds have a constitutive mechanism that prepares them for fungal infection. Many flavonoids and especially isoflavonoids have been reported to play a role in plant protection against pathogens, both as preformed antifungal compounds and as phytoalexins. Flavonoid classes most often associated with antifungal activity are flavones and flavans, but additionally, lipophilic flavones and flavonols, certain biflavones, chalcones and dihydrochalcones are known to be active (Grayer and Harborne, 1994).

Fig. 3.29. Pathway for biosynthesis of phenylpropanoid natural products elaborated from phenylalanine (flavonoid pathway is detailed after Bowles, 1990; Kuc, 1995)



In addition to the production of possible preformed antifungal compounds, *A. marina* seeds have been shown to contain β-1,3- glucanases and chitinases.

Although these enzymes were present in seeds when they were shed, the activity levels significantly increased when fresh seeds were subjected to further 4d of clean storage (4d clean) (Figs. 3.27 and 3.28). As these seeds contained no internal fungal contaminants, it appears that the β-1,3-glucanases and chitinases activities in these seeds were not induced during storage by fungal infection. It has been shown that the induction of PR proteins is not specifically restricted to pathogen attack but they are also induced during various physiological and developmental processes such as seed germination (Leubner-Metzer and Meins, 1999; Neuhaus, 1999).

Seeds of *A. marina* are known to initiate germinative metabolism and become more metabolically active during four day wet-storage (Farrant *et al.*, 1986; Berjak *et al.*, 1989; observations in this study). It is apparent that both secondary antifungal compounds and antifungal enzyme productions are stimulated during hydrated seed storage in association with ongoing germinative metabolism.

It is important to understand why uninfected *A. marina* seeds exhibit production of both possible antifungal compounds and antifungal enzymes. In addition to their role in pathogen defence, secondary (antifungal) metabolites may be involved in storage, protection from UV and protection against osmotic and other environmental stresses (Bennett and Wallsgrove, 1994). Their distribution within a plant, both between tissues and during growth and development is seldom uniform, many being synthesised by, and accumulated in, young developing tissues or in reproductive tissues such as flowers and seeds. It is believed that their presence provides protection to young tissues (Bennett and Wallsgrove, 1994). Class I β-1,3-glucanases are also present in seeds where it has been suggested that they promote endosperm rupture and radicle protrusion during the germination process (Leubner-Metzer *et al.*, 1996). This is not the case for *A. marina* seeds that are exendospermous and have no obvious mechanical barrier to root protrusion (Farrant *et al.*, 1993a). Thus, the

high level of β-1,3-glucanase and chitinase activity in *A. marina* seeds during short-term hydrated storage could be part of a strategy to create an antifungal environment prior to pathogen infection, rather than a requirement for protrusion of roots developing from the primordia during wet-storage. β-1,3-glucanase and chitinase could have a similar antifungal role in germinating pea (Petruzzelli *et. al.*, 1999) and tomato (Wu *et al.*, 2001) seeds.

Chitinase and β-1,3-glucanase activities remained high even when *A. marina* seeds were infected and subjected to further storage for 8d (4d and 10d-8) (Figs. 3.27-3.28), therefore induction of new isoenzymes cannot be precluded. In addition to this, these seeds produced an increased number of new compounds (5 to 21 in 4d-8 and 4 to13 in 10d-8) (Tables 3.24-3.25). Although rengyolone (compound **C**) and chalcone glycoside (compound **E**) occurred as preformed compounds in 4d clean and were present in 4d-8 in low concentrations, two new compounds were possibly induced in the later sample, stigmasterol (compound **F**) and some type of fatty acid (compound **G**). Terpenes and related plant secondary metabolites (sesquiterpenoids and sterols) have been shown to be important factors in resistance to several pathogens (Bennet and Wallsgrove, 1994). Stigmasterol is known to be active in defence mechanisms in most plants (Saubhnik *et al.*, 1999) including soyabean (*Glycine max*) (Hadjiakhondi *et al.*, 2003), pepper (*Piper nigrum* Linn) (Siddiqui *et al.*, 2003) and oil palm fruit (*Elaeis guineensis*) (Wattanapenpaiboon and Wahlqvist, 2003). Long-chain fatty acids are known to also be a major group of antifungal plant compounds (Grayer and Harborne, 1994). Several fatty acids have been found to be able to induce systemic resistance of potato plants to infection by *Phytophthora infestans*, including arachidonic, linoleic, linolenic and oleic acids (Cohen *et al.*, 1991). Four of the antifungal fatty acids found in rice act as endogenous elicitors of the diterpenoid phytoalexins and their concentration increases rapidly in leaves inoculated with *Pyricularia oryzae* (Grayer and Harborne, 1994).

The fact that 4d-8 seeds apparently significantly increased the production of antifungal enzymes and antifungal compounds, some of which could have antifungal activity (up to 21 in axes and 11 in cotyledons), of which some are induced compounds (such as stigmasterol and some fatty acid), is in accordance with the findings that for up to 14d of clean storage, seeds of *A. marina* were less susceptible to fungal attack than those in the newly harvested state. Although β-1,3-glucanase and chitinase activity was still high in 10d-8 seeds, the capacity for new possible antifungal compound production decreased (4 to 13 compounds in axes and 3 to10 in cotyledons), indicating that these seeds were more susceptible (less capable in defence) to the effects of the fungus than those of fresh seeds and seeds that have been stored clean for 4 and infected for 8 days (4d-8).

It should be noted that β-1,3-glucanase activity decreased markedly with prolonged storage (12d clean and 18d clean) (Fig. 3.27), in contrast to the chitinase activity that remained relatively unchanged (Fig. 3.28). Only in combination are these two enzymes highly effective in limiting fungal growth, especially that of *Fusarium* spp. (Mauch *et al.*, 1988). *Fusarium* hyphal cell walls comprise randomly orientated chitin microfibrils embedded in a carbohydrate matrix consisting mainly of glucans (Griffin, 1981). Apparently, β-1,3-glucanase and chitinase must act simultaneously for complete digestion of the fungal cell walls and thus destruction of the pathogen. Therefore, high activities of both enzymes might be necessary for successful defence response of *A. marina* seeds against *F. moniliforme*. This could explain the high susceptibility of 8d infected seeds in which β-1,3-glucanase activity was apparently induced in response to fungal infection, but chitinase activity remained unchanged compared with the fresh seeds (Figs. 3.27-3.28). At present, however, it is not known whether the same classes of β-1,3-glucanases and chitinases are present in fresh seeds and in those during storage and experimental inoculation. Fungal infection also stimulated production of possible antifungal compounds in seeds stored for 10d infected.

These seeds demonstrated the highest number of compounds, some of which are antifungal (between 5 to 30 compounds in embryonic axes and 3 to 20 in cotyledons). Most of these compounds are suggested to have been synthesised as a result of the possibly prolific fungal elicitors. In addition, it is known that chitinase and β-1,3-glucanase enzymes that are involved in degrading fungal cell walls, release elicitor-active carbohydrate molecules that trigger the synthesis of fungitoxic phytoalexins/antifungal compounds in the host plant (Chakravorty and Scott, 1991). Thus, it is possible that, in 10d infected, antifungal enzyme activity was related to the production of antifungal compounds.

The present study suggests that the decreased susceptibility of *A. marina* seeds during short-term clean storage relies on the ability to create an antifungal environment prior to infection (through synthesis and accumulation of preformed and induced possible antifungal compounds and antifungal enzymes), which would also be an effective strategy during germination in the natural environment. Although the defensive role of the observed antifungal compounds and β-1,3-glucanase and chitinase in *A. marina* seeds requires more elucidation, it is likely that they offer a measure of protection against *F. moniliforme*, especially in seeds that were stored clean for at least 4 days. It is apparent that these seeds are more resilient to the deleterious fungal effects, perhaps because concomitant with enhanced metabolism, they were able to use inherent defence mechanisms and rapidly acquire the ability to resist the virulent fungal infection. This ability, which was most effective after 4 days of clean storage, gradually declined under the stress of prolonged wet-storage conditions (such as 10d-8 and up to 18d). Although it is known that all recalcitrant seeds that are stored hydrated ultimately die (Pammenter *et al.*, 1994), the details why the seeds of 4d clean treatment were more resilient to fungal infection than others is unknown.

In attempting to find a mechanism, one must understand the possible interactions between *F. moniliforme* and hydrated-stored *A. marina* seeds. Fresh and 4d clean seeds showed no presence of fungal infection but they displayed inherent defence mechanisms that could prepare them for possible pathogen attack. When seeds were stored infected for 10 days (10d infected), they demonstrated an increase in production of possible antifungal compounds. Similarly, 4d-8 seeds were able to react to fungal infection by using preformed compounds and induced compounds with possible antifungal activity. However, this ability decreased in seeds of 10d-8 treatment. Although faced with seed defence mechanisms it appears that *F. moniliforme* was able to circumvent or overcome these defences.

Fungi have different ways to counter the antifungal compounds produced by the host plant. Some fungi may simply avoid plant antifungal compounds as in the example of the *Colletotrichum glaeosporioides*-avocado fruit interaction. Unripe avocado fruits contain a preformed antifungal diene that is believed to play a role in resistance to fruit decay. Despite this, *C. glaeosporioides* spores germinate on the surface of the fruits but the initiation of invasive growth coincides with the decrease of diene concentrations to non-toxic levels, which is one of the changes associated with the ripening process (Pruski and Keen, 1993). To circumvent or delay antifungal compound production, some fungi may evade recognition by plant surveillance systems (Osbourn, 1999). But inevitably the pathogens will directly encounter antifungal compounds during plant infection. In the case of *A. marina* seeds that produce constitutive and induced possible antifungal compounds, the question is not only whether these compounds are in the right place but also whether they are there in sufficient quantity at the right time. Although it appears that axes and cotyledons of, for example 10d infected seeds, contain a high number of putatively antifungal compounds, it is clear that they were in insufficient quantity to be able to stop fungal infection. Phytopathogenetic fungi have demonstrated the ability to degrade plant antimicrobial compounds through different mechanisms and for

many of these the degradation products have been shown to be less toxic to fungal growth than the plant compounds (Vanetten *et al.*, 2001). For example, *Phorma lingam*, the most common fungal pathogen of rapeseed (*Brassica napus*), is able to overcome the phytoalexins produced by the plant through various detoxification steps (Pedras *et al.*, 2001). Some fungi have been reported to detoxify more than one plant compound, as in the example of *Botrytis cinerea* that could detoxify the grapevine phytoalexins resveratrol, bean phytoalexins and various saponins (Pezet *et al.*, 1991; Osbourn, 1999). A study has also shown that saponin detoxification may not simply be the inactivation of preformed antimicrobial compounds, but additionally, the degradation products generated by the detoxification process may themselves act as suppressors of the inducible plant defence responses (Talbot, 2003). This highlights the fact that seemingly straightforward enzymatic functions could result in very unpredictable cellular consequences during the complex plant-microbe interactions.

If seeds of *A. marina* were stored for a short period of time, and if fungal contamination was kept minimal, the highly metabolic seeds showed increased resilience to fungal infection, possibly as a result of their ability to use the already existing inherent antifungal mechanisms in conjunction with increased capacity to produce induced antifungal compounds. In long-term hydrated storage, seeds exhibited an increasingly heightened vulnerability to fungal attack perhaps as a result of the seeds becoming debilitated in storage, which permits an aggressive pathogenic strategy to counteract defence mechanisms.

This study provides evidence that *A. marina* seeds produce compounds that inhibit *F. moniliforme* growth and that these compounds (including antifungal enzymes) are likely to offer some seed protection to fungal attack. However, further support for the role of these antifungal compounds in the protection against *F. moniliforme* and for the fungal mechanisms of tolerance or

detoxification are needed to better understand this complex fungus-plant interaction.

Chapter 4

Some implications of associated mycoflora during hydrated storage of recalcitrant seeds of *Avicennia marina* (Forssk.) Vierh.: an overview and future prospects

4.1 Introduction

The most cost-effective and efficient method of plant germplasm conservation is seed storage (Withers and Engelmann, 1997; Engels and Engelmann, 1998). Orthodox seeds can be stored for long periods (if guidelines of low relative humidities [RH] and temperatures are observed), provided the seeds are of good quality and are sufficiently desiccated at the outset (Ellis and Roberts, 1980; Hong and Ellis, 1996). Storage of recalcitrant seeds is more complex, however, as such seeds must remain hydrated during storage to retain viability and could also be chilling-sensitive, being damaged even at temperatures that are well above 0 °C (Chin, 1980). Seed recalcitrance thus creates significant problems in terms of short- to medium-term storage for planting and the seed trade. The only way in which intact recalcitrant seeds can be stored appears to be wet-storage, which is strictly a short-term option (Chin, 1980; King and Roberts, 1980; Berjak *et al.,* 1989; Berjak and Pammenter, 2001). Such conditions of hydrated storage (high RH and relatively high temperatures) are also conducive to the proliferation of associated mycoflora (Berjak, 1996; Sutherland *et al.,* 2003).

Although fungal proliferation of wet-stored recalcitrant seeds has been generally accepted to be almost inevitable, there are no in-depth studies of mycoflora on recalcitrant seeds. This investigation was undertaken to ascertain whether the seed-associated mycoflora has any role in curtailing the wet-storage lifespan of recalcitrant seeds of *Avicennia marina*. A further question pertains to post-harvest seed resilience to fungal attack – i.e. do stored *A. marina* seeds become more susceptible to the deleterious fungal effects with increased storage time? A third question is whether it is possible to differentiate fungal-induced deteriorative changes from those inherent degenerative changes that could result from prolonged hydrated storage? Answers to these questions should help towards understanding whether the fungi trigger seed deterioration during hydrated storage or if inherent deterioration of the seeds actually facilitates fungal proliferation and invasion.

4.2 Do seed-associated fungi play a role in the curtailed wet-storage lifespan of *A. marina* seeds?

Generally, stored recalcitrant seeds of tropical seeds lose viability rapidly while those of temperate species may be viable for up to a year or more if they can be stored at cold temperatures (Berjak and Pammenter, 2001). However, irrespective of provenance and with only few exceptions, both temperate and tropical recalcitrant seeds harbour a range of fungal species as well as bacteria (Murray, 1974; Delatour, 1978; Mycock and Berjak, 1990; Singh and Singh, 1990; Kehr and Schroeder, 1996).

Avicennia marina seeds have been reported previously to harbour fungal species such as *Aspergillus, Penicillium* and *Fusarium*, as well as bacteria (Mycock and Berjak, 1990). In support of those earlier studies, the present investigation showed that *Fusarium* spp. eventually becomes the dominant fungal species, predominating in, and on, seeds tissues, including the pericarp, during wet-storage. Since the pericarp is a source of fungal inoculum, Goddard (1994) proposed that its removal might extend the lifespan of stored recalcitrant seeds. The current investigation did not, however, support this proposal as no differences in seed survival were observed for *A. marina* seeds stored under the same conditions, with or without pericarps.

Although surface sterilisation with NaOCl and periodic fungicidal treatment significantly decreased fungal infection at least for 4 days, *Fusarium moniliforme* had infected cotyledonary surfaces by the seventh day of hydrated storage. This was not surprising, as this particular fungus appeared to be the dominant pathogen of a number of species of recalcitrant seeds of southern African provenance (Mycock and Berjak, 1990). Seed viability declined to 70% later during storage, after 21 days, when *F. moniliforme* was known to be present within internal cotyledonary tissues.

The fungal effect on the experimentally infected seeds was more rapid and more dramatic, none of these seeds surviving for 7 days in hydrated storage (comparing to untreated seeds that lost viability by 16 days). It appears that the fungal infection was very aggressive, contributing towards the rapid decline in experimentally infected seed viability. The difference between the naturally- and experimentally-inoculated seeds was that *Fusarium moniliforme* gradually became dominant in the former, while newly-harvested seeds were inoculated immediately with this pathogen. It is therefore suggested that competition by less virulent fungi initially tempered the effects of *F. moniliforme* in the naturally-infected seeds. In contrast, upon experimental infection of seeds had been treated with fungicide, *F. moniliforme* would immediately have been pathogenic to the seeds in a situation virtually free of any competition by other fungi.

In the present study, hydrated storage lifespan of Previcur-treated seeds was extended significantly (to 21 days) when compared with the experimentally infected seeds. *Avicnnia marina* seed viability has previously been maintained for up to 80 days by encapsulating surface-sterilised seeds in an alginate gel that not only alleviated water stress, but also inhibited fungal growth (Motete *et al.*, 1997; Pammenter *et al.*, 1997).

The present study demonstrated that if the fungal proliferation can be minimised, then the hydrated storage lifespan of *A. marina* seeds could be extended. As a corollary, in the light of the results of Motete *et al.* (1997) and Pammenter *et al.* (1997), if all fungi could be eliminated, then a further, but not indefinite extension of seed lifespan should be possible.

4.3 Do recalcitrant *A. marina* seeds become more susceptible to fungal infection with increased storage time?

In the present investigation, newly harvested seeds that harboured fungi showed the presence of pre-formed antifungal compounds and β-1,3-glucanase

and chitinase. Pre-formed antifungal compounds are generally plant metabolites that exist in their biological active forms or as inactive precursors and are activated only by pathogen attack (Walton, 1997). β-1,3-glucanases are abundant, highly regulated enzymes that are distributed widely in plants (Leubner-Metzer and Meins (1999). In pathogen-free plants, these enzymes are implicated in various physiological and developmental processes such as pollen germination and tube growth (Roggen and Stanley, 1969), fertilisation (Ori *et al.,* 1990), cell division (Fulcher *et al.,* 1976), fruit ripening (Hinton and Pressey, 1980), seed germination (Vögeli-Lange *et al.,* 1994; Leubner-Metzger *et al.*, 1995) and bud dormancy (Krabel *et al.,* 1993). Constitutive chitinases are involved in non-defense processes such as somatic embryogenesis (Domon *et al.,* 2000; Helleboid *et al.*, 2000) and tissue protection from frost damage (Hiilovaara-Teijo *et al.,* 1999). In addition, some chitinases and other PR proteins have been suggested to offer cold tolerance to recalcitrant chestnut (*Castana sativa*) seeds (Collada *et al.,* 1992; Garcia-Casado *et al.*, 2000). Some chitinase and β-1,3-glucanases may also play a role in seed germination (Petruzzelli *et al.,* 2003), as they are expressed in tomato endosperm prior to radicle emergence (Wu *et al.,* 2001). It is likely, however, that the primary role of β-1,3-glucanases and chitinases is to protect seeds and vegetative organs from microbial infection (Gomez *et al.*, 2003).

In this study, while clean seeds remained fully viable during 14 days of hydrated storage, with viability declining to 70% only by 21 days, the experimentally infected seeds lost their ability to germinate by day 7. When the seeds were stored clean for 4 days, a period during which there was no apparent associated fungal presence, they appeared to become somewhat resilient to the adverse effects of *F. moniliforme*. *Avicennia marina* seeds are metabolically active upon shedding and they initiate germination during 4 days of hydrated storage (the present study; Farrant *et al.,* 1986). Concomitant with these events, it is likely that these seeds, which could be considered to be seedlings after 4 days storage, acquire an enhanced ability to counteract possible fungal

attack. In the present study, this ability was maintained for up to 10 days of clean hydrated storage.

Also apparent from the current investigation was that 4-day clean seeds (and even 10d clean seeds) have the ability to develop inherent defence mechanisms, typified by the production of antifungal compounds and PR-proteins. Although 4-day clean seeds produced the same number of antifungal compounds (4-6 - in embryonic axis) as fresh seeds, it is possible that three of these compounds were new. The isolated compounds are part of general phenylpropanoid metabolism that is responsible for the biosynthesis of a class of secondary metabolites involved in defence against pathogenic fungi (Chakravorty and Scott, 1991). In addition, a significant increase in β-1,3 glucanase and chitinase was observed in 4d clean seeds. As no fungal presence was apparent on 4d clean seeds, which had effectively already commenced germination, this increased level of enzymatic activity could form part of a strategy to create an antimicrobial environment prior to pathogen infection, rather than a requirement for germination.

The 4d-8 seeds showed an apparent considerable increase of antifungal enzymes (hence induction of new isoenzymes should not be excluded) and antifungal compounds, of which some were induced compounds (by fungal presence). This response of heightened defence reaction is in accordance with the observation that for up to 14 days of clean storage, seeds showed less susceptibility to fungal infection than fresh seeds.

The proposed ability to resist fungal attack gradually declined with prolonged storage, with seeds clean-stored for longer periods becoming increasingly vulnerable to *F. moniliforme*. Although β-1,3-glucanase and chitinase activities were maintained at a high level in 10d-8 seeds, the production of antifungal compounds appeared to decrease, suggesting that these seeds would inevitably become more susceptible to mycoflora than fresh seeds and seeds stored for shorter periods.

From the present study it is proposed that in short-term hydrated storage, *A. marina* seeds show resilience to fungal infection through the apparent ability to create an antifungal environment prior to infection, which could also be an effective strategy during germination. The production of antifungal compounds and antifungal enzymes is suggested to offer some protection against aggressive fungal attack, especially in 4 day clean seeds. It is possible that the 4d clean seeds are best equipped to counteract fungus as when they prepare for germination, they also increase their β-1,3-glucanase and chitinase activity. When infected with *F. moniliforme*, these seeds already have an arsenal of defence compounds (antifungal compounds and/or antifungal enzymes), thereby enabling them to rapidly respond to fungal presence. Although heightened antifungal activity was observed in seeds that were inoculated immediately after harvest and stored infected for 10 days, it is possible that a 'trade-off' situation could have existed between seeds resisting the aggressive fungal attack or continuing with the germination process. In long-term storage, clean seeds gradually declined under the stress that has been suggested to accompany the prolonged hydrated storage condition (Pammenter *et al.,* 1994), and as a result may become more susceptible to *F. moniliforme*.

4.4 Is it possible to differentiate fungally-induced deteriorative changes from inherent degenerative events that may be the outcome of prolonged hydrated storage?

Intracellular and metabolic activity increase in the early stages of hydrated storage of recalcitrant seeds and cell division and vacuolation are an indication of ongoing metabolism (Berjak *et al.,* 1984; 1989; Farrant *et al.,* 1985; 1989; Pammenter *et al.,* 1994). Subsequent to this, intracellular deterioration occurs and, despite there being no significant water loss, seed viability is lost in prolonged hydrated storage (King and Roberts, 1980; Suszka and Tylkowski

1980; Farrant *et al.,* 1980; Fu *et al.*, 1990; Pammenter *et al.,* 1994; Pritchard *et al.,* 1995).

Several of the deteriorative ultrastructural changes observed in the current study have been previously reported for wet-stored *A. marina* seeds (Berjak *et al.*, 1989; Motete *et al.,* 1997). These include vacuolar confluency, mitochondrial and plastidial abnormalities, swelling of endomembrane cisternae, evidence of "unstacked" Golgi vesicles and plasmalemma withdrawal. These have been proposed to be a result of water stress that could occur during hydrated storage. In short-term storage, water could be directed from cotyledons to axis, but in long-term storage, however, water stress is proposed to occur if additional water is not provided to the seeds (Pammenter *et al.*, 1994). According to those authors, this water stress would probably result in deregulation of biochemical reactions and accumulation of free radicals, resulting in lipid and protein peroxidation with subsequent membrane damage, as described by Hendry *et al.* (1992). Uncontrolled free radical generation and the inability to repair consequent damage have been proposed to represent a significant contributing factor in the loss of recalcitrant seed viability during hydrated storage (Pammenter *et al.*, 1994).

Ultrastructural evidence of endomembrane system activity has been reported to be associated with ongoing metabolism in wet-stored *A. marina* seeds (Motete *et al.*1997). Its apparently heightened activity beyond a few days was, however, not reported. In this study, particularly in meristematic root primordium cells of embryonic axes of clean seeds, well-defined endoplasmic reticulum and the frequent occurrence of Golgi bodies with many associated vesicles could be indicative of increased protein synthesis. It is probable that some of the proteins synthesized could be PR-proteins that were measured in increased quantities during the hydrated storage period. It is common knowledge that ER-derived vesicles carrying proteins are processed through the Golgi complex and then directed to various destinations (Wolfe, 1995). Secretory vesicles fusing with the plasmalemma will release their contents extracellularly through exocytosis,

while other Golgi-processed vesicles have intracellular destinations, including fusion with lytic compartments (Wolfe, 1995; Becker *et al.,* 2003). It is suggested that either of these routes may be followed during constitutive PR-protein elaboration and during defence reactions in response to pathogen attack.

In addition to the heightened endomembrane activity that could reflect cells responding to exogenous (defence-related) signals, there were other ultrastructural observations may also (in part) be fungally-induced. *Fusarium moniliforme* synthesizes fumonisin B_1 (FB_1), a mycotoxin that has been found to be a virulence determinant in plant disease and which also induces a variety of cellular responses, including cell death (Gilchrist, 1998). An increase in vacuolation, membrane damage and mitochondrial deterioration observed in root tips of maize (*Zea mays*) embryos were interpreted as responses to treatment with various mycotoxins (McLean *et al.,* 1992; 1994a; 1994b).

The consistent presence of lipid bodies (a feature that was also not reported previously for wet-stored *A. marina* root primordium cells) throughout hydrated storage, is also currently proposed to be a fungal-induced reaction, as it has been observed in fungal-infected and aflatoxin-treated material (McLean *et al.,* 1994b; 1994c). In addition to this phenomenon, it is possible that the presence of densely-staining small vacuolar globules in some cotyledonary cells could also be part of some plant defence reaction to *F. moniliforme* infection, as the occurrence of similar globules has been linked to defence reactions during plant-microbe interactions (Kosslak *et al.,* 1997; Hu *et al.,* 2003).

Ultrastructural changes, regarded as signs of incipient subcellular deterioration in root primordia cells of *A. marina* seeds, have been reported to occur at different times: in gel-encapsulated seeds they appeared only after 50 days of hydrated storage (Motete *et al.* 1997; Pammenter *et al.,* 1997); in Benlate-treated seeds after approximately 12 days (Farrant *et al.,* 1986; 1989); and

currently in Previcur-treated clean seeds only after 21 days in storage (the time when fungal infection was also manifested).

Noting that in the previous research cited on *A. marina* immediately above no attempts were made to ascertain whether or not fungi were present, it is suggested that these differences in the timing of the onset of intracellular degradative symptoms could be related to fungal presence. The results of the present study suggest that if fungi are apparently absent, then cells maintain a normal ultrastructure for longer periods during hydrated storage, in contrast to cells of infected tissues that undergo early subcellular deterioration and lose viability rapidly.

An overview of the findings of the present study is presented in Table 4.1.

Table 4.1. An overview of the findings of the present study

Treatment	Viability (% germination after 12 days)	Fungal contamination	Ultrastructural analysis	Defence mechanisms		Comment
				Antifungal compounds	Antifungal enzymes	
Fresh – untreated (without surface sterilisation or fungicidal/fungistatic treatment)	Low: - 24% seeds stored with pericarp - 20% seeds stored without pericarp	Several fungal species & bacteria in fresh state but *Fusarium* spp. become dominant in wet-storage	Contaminants were associated with all seed components, internally and externally	Not assessed		- Removal of pericarp did not affect seed storage lifespan - No seeds were viable by day 16
Fresh – treated (with surface sterilisation and Previcur treatment)	100%	Several fungal species on embryonic axis and cotyledonary surfaces	Low level intracellular activity apparent in freshly-harvested seeds	Some present	Activity detectable	Minimising fungal contamination considerably prolonged seed storage lifespan
4d clean	100%	No fungal contamination	Some enhancement of intracellular activity with increased degree of vacuolation	Similar to fresh material	Marked increase in production	Seeds were resilient to fungal infection
4d infected	55%	*F. moniliforme* on all seed parts	Marked general deterioration, but signs of preceding intracellular activity/response were evident : - high degree of vacuolation - long ER profiles - frequent Golgi bodies - lipid bodies	Not assessed		- The ultrastructure suggests increased protein synthesis (possibly PR) - The observed degradation is suggested to be fungally-induced
4d-8	55%	*F. moniliforme* on and in cotyledons	Evidence of ongoing metabolism with a degree of abnormality: - irregular nuclear profiles - occasional mitochondria with electron-transparent areas - degradation of some plastid content Fungal structures associated with cotyledonary cells	More compounds present than in fresh seeds	Higher activity than in fresh seeds	Seeds were more resilient to fungal infection than those inoculated immediately after harvest and then stored for an equivalent period
7d infected	0%	*F. moniliforme* associated with embryonic axis and cotyledonary tissues	Some embryonic axis cells shoed advanced damage and others maintained integrity but with abnormalities: - large vacuoles - nuclear lobing - long ER profiles - Golgi bodies with many vesicles - occurrence of lipid bodies	Not assessed	Higher β-1,3-glucanase activity than fresh but marked decrease in chitinase activity	- The ultrastructure suggests increased protein synthesis (possibly PR) - The observed degradation is suggested to be fungally-induced
10d clean	95%	*F. moniliforme* on cotyledonary surfaces	Increased intracellular activity with strong organelle development and with little sign of abnormality, except for: - occurrence of lipid bodies - some mitochondria had electron-transparent matrical regions	More compounds present than in 4d clean seeds	Increased activity but lower than 4d clean	- Some abnormalities (e.g. lipid bodies) could be induced by fungus, even if only cotyledons were externally infected
10d infected	0%	*F. moniliforme*	Marked general deterioration: - high degree of vacuolation - complete loss of mitochondrial organisation - dilated nuclear envelope and ER Fungal structures were present in	More compounds present than in fresh seeds	Low activity	- Although defence mechanisms in place, it appeared that the fungal infection was too aggressive - Ultrastructural deterioration appeared to fungally-induced
10d-8	10%	*F. moniliforme*	Marked general deterioration: - high degree of vacuolation - dilated ER profiles - nuclear lobing Cotyledonary cell necrosis associated with fungal presence	Compounds were produced but less than 4d-8	Activity remained high but lower than 4d-8	- The resilience was lost, seeds becoming susceptible to fungal presence - Some ultrastructural changes were a result of ongoing metabolism but others were fungally-induced
21d clean	70%	*F. moniliforme* on and in cotyledons	Most meristematic cells retained ultrastructural integrity characteristic of ongoing metabolism but with incipient signs of deterioration: - confluent vacuoles - nuclear lobing - abnormal membrane formation in mitochondria - Golgi bodies with many associated vesicles Some cotyledonary cells were intact but others were penetrated by fungal structures	Not assessed	Although reduced, activity was detected (at 18d clean)	Some ultrastructural changes suggested to be the result of ongoing metabolism, but others probably were fungally-induced

4.5 Future prospects

Over the last 30 years, considerable progress has been made in understanding the cascade of intracellular events involved in the phenomenon of seed recalcitrance. However, in spite of these advances, there remains a need to address our understanding of the interactions between recalcitrant seeds and their almost inevitably associated mycoflora.

In addition to the urgent requirement for the identification of fungicides that would be effective in curtailing specific fungal infection during short- and medium-term hydrated storage of recalcitrant seeds, the precise mechanisms of the plant-pathogen interactions need to be investigated.

We now know that *A. marina* seeds synthesize antifungal compounds and PR-proteins. Demonstration of effective antifungal activity *in vitro* of each of the observed compounds and proteins should give a more detailed indication of the significance of these seed-elaborated compounds as antifungal protectants. The localisation and assessment of concentration of these compounds within seed tissues relative to fungal location should also be an indication of their potentially protective role. In addition, we also know that some of these compounds are pre-formed and that others are induced, possibly by pathogen infection. Hence, the question as to whether these compounds were synthesised in the right place and also at the right time to be effective, is pertinent. It was shown in the present study that although presence of antifungal compounds was increased, seeds eventually became vulnerable to *F. moniliforme,* so the phenomenon of their failing efficacy in relation to fungal presence requires investigation. Although these are difficult problems, a start has been made in localising phytoalexins have been made in cocoa (Cooper *et al.,* 1996), sorghum (Snyder and Nicholson, 1990) and oat seeds (Osbourn *et al.,* 1994).

In order to understand how recalcitrant seeds respond to fungal attack, and when during hydrated storage the sequence of events in the resistance process is entrained, needs to be determined. The localisation of preformed antifungal compounds and antifungal proteins (as part of a primary response) could be followed by elucidation of the signalling process. What molecules elicit the defence response? Are these molecules of fungal or of plant origin? The signals and pathways involved in the initiation and maintenance of primary responses and systemic acquired resistance are still poorly understood, although there has been progress. Salicyclic acid has been identified as a key signalling component involved in the activation of certain plant defence responses (Druner *et al.*, 1997). There have been reports that water-stressed recalcitrant seeds lose the capacity to produce ethylene (Corbineau *et al., 1990;* Salmen Espindola *et al.;* 1994). However, ethylene production is suggested to be an important signal molecule in systemic acquired resistance (Abeles *et al.,* 1971). Those authors found that ethylene induces some PR-proteins such as β-1,3-glucanases and chitinases. Hence it is suggested that loss of the capacity to produce ethylene by water-stressed recalcitrant seeds in storage may be associated with the declining ability to maintain systemic resistance to fungal attack. This suggestion could be the basis of relevant future research. Calcium ions also appear to be required in the elicitation process, the concentration of free Ca^{2+} in mitochondria and chloroplast being subject to oscillations controlled by external and intracellular signals (Somssich and Halbrock, 1998). The rapid cellular influx of Ca^{2+}, with all its consequences, is among the earliest reactions observed in elicitor-treated cells (Greenberg, 1997). Since it is possible to localise Ca^{2+} intracellularly in seed tissue (Berjak and Mycock, 2004), the opportunity exists to explore this phenomenon.

Studies have shown that plants produce reactive oxygen species (ROS) in response to pathogen infection (Wojtaszek, 1997a; 1997b). It would be interesting to assess ROS production in support of the proposed hypothesis (e.g. Pammenter *et al.,* 1994) of seed viability loss during hydrated storage as a consequence of water stress and free radical damage.

The intra- and intercellular spatial organisation of the defence response is highly complex, as is assessment of the generation of reactive oxygen species that could be derived from more than one subcellular location (Allan and Fluhr, 1997). Some cytological investigations have demonstrated major structural rearrangements within infected cells that included rapid depolymerisation of the microtubular network around the penetrating fungus, and actin filament-mediated translocation of the plant cell nucleus and cytoplasm to this site (Somssich and Halbrock, 1998).

The question arises as to which of these numerous reactions that are associated with pathogen attack are actually required for pathogen defence and, which are a consequence of the deleterious effects of hydrated storage. A conclusive answer will require the concerted application of different approaches.

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