ECOPHYSIOLOGY OF *LEUCOSPERMUM* R. BR. SEED GERMINATION IN FYNBOS

by

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Thesis submitted for the degree of Doctor of Philosophy in the Department of Botany at the University of Cape Town

June 1996

Statement

The conception, planning, execution and writing of this study was my own except in the instances mentioned below.

Some of the chapters are adapted from published papers which were coauthored with my supervisors, Neville Brown and John Manning, as well as Jonathan Cutting, Prof Hannes van Staden and my statistical consultant Frikkie Calitz. Their contributions were mainly complementary to the relevant main studies.

Four months after fire: seedlings of *Leucospermum cordifolium* emerge from under a stone, in Strandveld fynbos, presumably from an abandoned ant nest.

ABSTRACT

A primary goal in the study of natural seed regenerating systems is to understand how seed dormancy and germination is controlled by the environment. The aim of this thesis was to develop a general model for major seed anatomical, physiological and ecological factors interacting in *Leucospermum* R.Br in fynbos. The work is a collation of studies on several seed biological aspects and *Leucospermum* species with the main focus on the horticulturally used *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade. The study thus eclectically investigates diverse study fields and plant material. The result, as far as the practical limits of such a project allowed, is a first integrated ecophysiological model for seed regeneration in a group of fynbos species.

Seed ontogeny. Anatomy of the developing *L. cordifolium* seed was studied as a background and to search for possible links between seed structure and function. The outer integument was shown to form the seed coat: the outer epidermis becomes tanniniferous and the inner epidermis develops into a crystalliferous palisade. Examination of the literature reveals that the crystal palisade layer of the outer integument has been erroneously assumed to constitute an endocarp. This finding indicates that a re-interpretation of all published information on the seed coat in indehiscent Proteaceae is necessary.

Germination requirements. The first objective, of characterization and quantification of germination requirements in *Leucospermum*, was to achieve full control of germination of the notoriously unresponsive seeds. In two studies to maximize germination in *L. cordifolium* a tetrazolium viability test indicated that all viable seeds germinated. A combination of treatments was subsequently tested to achieve complete germination in six species from contrasting habitats (*L. cordifolium, L cuneiforme, L. erubescens, L. glabrum, L. reflexum* and *L. vestitum).* The most effective treatment combination stimulated all viable and a significant proportion of subviable achenes to germinate. Treatment effects on germination percentage were cumulative in most species and also resulted in similar germination rate patterns, suggesting common physiological mechanisms for germination in this species group.

The second objective was to relate experimental *Leucospermum* germination requirements to nature. Scarification and stratification requirements of *L. cordifolium* seeds were investigated. It was found that prolonged stratification at sub-optimal germination temperature does not play a role in fynbos, but that scarification of the seed coat layers does.

Temperature requirements in *Leucospermum* seed germination were studied using the same species as before. *Leucospermum* seeds generally required alternating temperatures for successful germination but the optimum low and high temperature requirements of individual species differed markedly. These requirements correlated positively with mean late autumn air temperatures representative of the natural habitats of species. It was concluded that diurnal alternating temperature requirement is a character syndrome in *Leucospermum* in which the constituent base and ceiling temperature requirements are adaptive, independent and genetically stable characters. The concept is proposed of diurnal bi-temperature requirement in *Leucospermum*, as distinct from optimal temperature amplitude requirement.

Hormonal regulation of seed dormancy and germination in relation to the environment. Seed hormonal changes were explored in relation to known dormancy and germination characters in a difficult-to-germinate species, *Leucospermum glabrum.* The endogenous levels of gibberellins (GAs) and cytokinins (CKs) changed phasically during normal germination under a single alternating temperature regime. The early increases found in GAs are thought to control the induction of the germination process. The CK pattern, correlated with known ultrastructural and morphological changes in the embryo, suggests that CKs control at least three major processes of germination *sensu stricto* following induction: 1) early mobilization of protein and lipid reserves in the axis and later in cotyledons, 2) cotyledon expansion which causes the endotesta to split permitting radicle protrusion and 3) later, radicle growth.

A correlated experiment was performed with exogenously applied GAs and CKs under different dormancy-enforcing conditions. It was concluded that synthesis of or tissue sensitizing to, both hormone classes GAs and CKs depends on moderately low temperature as the primary environmental requirement, in the presence of oxygen. For GA synthesis a secondary, daily pulse of high temperature is also required. Inhibiting hormones, specifically ABA, appear not to play a role.

Ecological aspects. This chapter foremostly addressed the question - why is there a germination flush in fynbos after fire? Experiments were conducted to test the reaction of germinating *Leucospermum* seeds to smoke treatment. Negative results suggested that smoke does not play a role in *Leucospermum* seed germination in nature.

A new seed biological phenomenon in *Leucospermum* was investigated. This is the extensive breaking, following desiccation treatment, of the seed testa layers which in intact, soil-stored seeds impose dormancy on the embryo by means of oxygen exclusion. Heat-desiccation treatments resulted in extensive breaking of the exo- as well as the endotesta of seeds. The results suggest that in nature desiccation by fire could break the exotesta and the endotesta as well, if fire is followed soon by rain, wetting being required in addition to fracture the desiccated endotesta. The viability of embryos was not affected by heat. A positive relationship is therefore predicted between heat intensity of fire and emergent seedling numbers. The promotive influence of heat desiccation was

found to be similar for three species tested (*L. cordifolium, L. glabrum, L. reflexum*) and this could not be ascribed to physiological changes caused by high temperature *per se.*

In a seed storage study low temperature-stored seeds maintained a high viability and vigour for c. two years but ambient temperature storage led to a marked decline after 1 year ending in almost complete mortality after 4 years of shelf storage. By contrast a seed batch stored for 4 years in the soil maintained a reasonably high viability and vigour of 60% of the fresh-seed response. This first experimental demonstration of longterm seed survival during soil-storage suggests a form of cellular repair during periodic wet periods as a survival mechanism in *Leucospermum.*

In a study of germination extending over seasons, seed bed emergence was recorded in the first and four subsequent winters. Sporadic germination occurred in all treatments, only during autumn and early winter each year, and more pronounced in intact (non-oxygenated) seeds. The model of long-living, soil-stored seeds, in which the testa is gradually scarified and which leads to the extension of germination in an erratic emergence pattern, is supported.

Seed structure and ecological function. In order to synthesize an ecophysiological model from the above results, the development and fate of structures with ecological function was studied, using light microscopy and SEM. The model developed related to the individual seed as well as the recruiting population in fynbos. The elaiosome was shown to be instrumental in myrmecochory only and the seed is therefore the true germination unit in *Leucospermum.* The woody endotesta protects the embryo indefinitely in the soil. The ecological roles of individual testa sublayers were elucidated. The effects of repeated desiccation/hydration cycles during soil storage cause the testa layers of the long-lived seeds of previous flowering seasons to become fully scarified. In contrast seeds of the latest flowering season will be in various stages of incomplete scarification, especially the exotesta, leading to differences in degree of embryo oxygenation. This appears to regulate extended (polymorphic) germination of some seeds in the young-seed component of the seed bank. During germination the endotesta breaks in a specific pattern along weak structural lines which facilitates emergence of the radicle. The desiccation-scarification mechanisms in *Leucospermum* was shown to be unique in seed science.

The interaction of characters within the seed, and externally with a series of ecological cues, constitute a complex regulatory pattern for dormancy control in *Leucospermum.* Several adaptive equilibria in *Leucospermum* reproductive strategies in fynbos are identified. These are shown to be the result of conflicting selective pressures. Consideration of the nature of character syndromes and apparent levels of genetic variability in seed populations suggest that *Leucospermum* may have evolved recently in the Cape.

GENERAL PREFACE

The aim of this thesis was to develop a holistic model for seed regeneration of *Leucospermum* in fynbos. Although the seed biology of the genus has been considerably researched, the information is fragmented and incomplete. The present work complements and extends the existing data pool. An integrated working explanation of the strategies and fates of *Leucospermum* seeds in nature is attempted from this.

Many similarities in seed biology exist between the fynbos group of *Leucospermum* species and even with species in the section *Alatosperma* of *Leucadendron*. These include a wide range of shared characteristics (e.g. ecological, anatomical, physiological traits - see literature review). Phylogenetically these *Leucospermum* species are considered closely related and recently radiated (Rourke, 1972). For the purpose of model construction, at least the group of *Leucospermum* species tested here is assumed to share the same seed regeneration syndromes, albeit with different degrees of expression.

L. cordifolium was focused on because of the wide research attention it has received in amenity horticulture. Five other species, from diverse fynbos habitats, were included in some studies to investigate variation in the genus. However a common basis was assumed. For example, in a study of hormonal-environmental relationships *L. glabrum* was focused on, *L. cordifolium* being included for partial comparison. This was thought desireable because the latitude of the environmental factors which control dormancy in *L. glabrum* is comparatively narrow (e.g. its narrow temperature requirements - see Chapter 4.4).

The thesis is necessarily also an integration of several study fields. The search for a holistic model necessitated eclectic treatment of a variety of topics: from physical seed coat properties through anatomical and structural seed characters to hormonal-physiological control - all in relation to seed ecology.

This aspect explains the apparent stand-alone nature of chapters, some of which have been published as independent studies. The linking thread however is the essential role each study plays in understanding the dormancy and germination ecology of this group of *Leucospermum* species.

The above approach explains a necessary degree of overlap, especially in the Introductions to various chapters. Chapters 3 and 7, for instance, describe different aspects of seed coat anatomy, the former with emphasis on phylogeny of Proteaceae and the latter on structure-function relationships.

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CHAPTER 1

ECOPHYSIOLOGY OF *LEUCOSPERMUM* R.BR. SEED GERMINATION IN FYNBOS

I. INTRODUCTION: DEFINING THE PROBLEM

In the past 25 years numerous publications (and theses) have appeared on seed germination physiology of the South African Proteaceae (reviewed by Van Staden and Brown, 1977), especially on *Leucospermum*. This information often tended to be generated as narrow areas of study. Physiological investigation appears to have often focussed on hit-and-miss attempts to break the notorious dormancy of Proteaceae seeds. Thus little reference is found to ecological function of seeds in physiological work. No integrated ecophysiological seed study for any species in fynbos, nor apparently in other mediteranean-type ecosystems have been conducted (cf. Le Maitre and Midgley, 1992; Keeley, 1991; Gutterman, 1993; Bell *et al.*, 1993).

In Proteaceae seed biology, the bridging of seed ecology and physiology has been recognized as an important objective (Van Staden and Brown, 1977). It is widely thought, in seed physiology, that certain environmental cues trigger specific physiological processes (metabolic sequences) in the embryo, which in turn regulate germination: the breaking of dormancy and the resumption of growth (Bewley and Black, 1982). In Cape Proteaceae this has been specifically postulated for *Leucospermum*, *Leucadendron* and *Protea* (Van Staden and Brown 1977; Bond 1984).

It is hoped that a working seed regeneration model for *Leucospermum* could focus attention on the following fields relating to fynbos seed biology and generate new interest:

1. Fynbos seed biology as a challenging and stimulating research discipline. The harsh fynbos environment has led to complex, highly adapted responses in seeds (Kelly *et al.*, 1992) and successful study of one subject is likely to attract attention to other seed regeneration systems.
2. Fynbos conservation. Identifying the links between the organism and its environment can effectively demonstrate the sensitivity of fynbos to human interference. This applies especially to the vulnerable seed regeneration phase.
3. Fynbos horticulture. Research in this field provides utilitarian value for fynbos and is presently recognised as a conservation factor of significance. Moreover, horticultural

research can contribute to the basic botanical knowledge of seeds (cf. Brown and Van Staden, 1973b; Brown *et al.*, 1986).

The information currently most lacking in the field of *Leucospermum* seed germination ecophysiology appears to be:

- a sound knowledge of seed structure (anatomy) which underlies ecological function;
- a (re-)appraisal of seed germination characteristics in *Leucospermum*;
- hormonal regulation of germination;
- seed bank dynamics;
- a holistic model for *Leucospermum* seed regeneration in fynbos.

These topics are scantily covered in the literature of nut-fruited Proteaceae seeds (see review below). They are consequently the areas of focus in the present study.

II. LITERATURE REVIEW. SEED REGENERATION IN CAPE PROTEACEAE WITH NUT-LIKE ACHENES

CONTENTS

Introduction

Although important physiological processes which regulate dormancy and germination in achenes ('seeds') of Cape Proteaceae species have been elucidated in recent years, the control of these processes by environmental factors remains unknown. These factors can be assumed to act as cues in initiating specific metabolic events in germinating seeds (Van Staden and Brown, 1977). A sound knowledge of environmental, i.e. ecological, influences is indeed useful in formulating meaningful approaches to physiological and other research problems (Bond, 1984; Rebelo and Rourke, 1986). A synthesis of existing ecological and physiological information is, therefore, appropriate. Of the celebrated diversity of plant life of the Cape Floristic Region the family Proteaceae, with its 300-odd members, has probably been studied best (Brits, 1984). Even within this group no working ecophysiological hypothesis for seed regeneration has yet been proposed. Comprehensive (holistic, life history approach) case studies of the seed biology of other mediterranan-type species are also generally lacking, although much needed - e.g. in California chaparral (Keeley, 1991) and in the (mediterranean) Australian heathland (Lamont *et al.*, 1985).

A wide range of stress and disturbance factors impact on fynbos (Moll *et al.*, 1984) and extensive adaptive responses to these have been recognised (Taylor, 1977; Kruger, 1979a). The variety and general severity of these factors indicate that the ecology and physiology of Proteaceae seed germination may be complex. These factors are common to the Cape fynbos, for example seasonal drought, low nutrient status of soils, cyclical fire and spatial competition in dense plant communities (Kruger, 1979b; Yeaton and Bond, 1991; Le Maitre and Midgley, 1992; Bond *et al.*, 1992). Such factors are strong macro-ecological denominators which suggest that seed regeneration characteristics in certain groups of Proteaceae may be broadly similar. Such adaptive plant responses to the environment shared by a group of species are referred to as character syndromes (Angevine and Chabot, 1979 *sensu* Stebbins, 1974).

The seed storage strategy employed by Cape Proteaceae indicates a major division in its seed biology (Brits, 1983; Rebelo and Rourke, 1986). This dichotomy is reflected in two distinct achene types which occur in fynbos Proteaceae. One type of achene is rounded (often ellipsoid), relatively hard and nut-like and stored in the soil, whilst the other type is winged, plumed or hairy (often flattened) and relatively soft (Brits, 1983). The latter type is produced mostly by serotinous species, i.e. species in which seeds are stored in the living plant canopy (Bond, 1980; Brits, 1983; Bond, 1985). In the former type seeds are stored in the soil and germination is characterized by the splitting of the seed coat, due to cotyledon expansion, which is followed by protrusion of the radicle (Brown and Van Staden, 1973c; Brown and Dix, 1985). In serotinous species germination is first indicated by penetration of the seed coat by the radicle (Brown and Van Staden, 1973c). This distinction is drawn at both the generic and subgeneric (in *Leucadendron*) levels. Serotinous genera comprise *Protea* L., *Aulax* Berg. and the greater part of the Section Alatosperma of *Leucadendron* R. Br. - approximately 37% of the Cape Proteaceae - the rest (excluding *Brabeium* L.) being nut-like (Brits, 1983; 1986b; Lamont *et al.*, 1985).

Significant exceptions within and intermediates between the two major groups occur (Brits, 1983; Bond, 1985). For example, *Protea aristata* Phill. is apparently only weakly serotinous; on the other hand *Leucadendron argenteum* (L.) R. Br. (Section Leucadendron), the silver tree, which produces large nut-like achenes, is also weakly serotinous (Williams, 1972). The seed biology of these exceptions is poorly understood (Rebelo and Rourke, 1986) and will not be dealt with here.

Within the nut-like group a distinction is drawn between myrmecochorous species eg. *Leucospermum*, and non-myrmecochorous species, the latter comprising most members of the Section Leucadendron of *Leucadendron*. Myrmecochorous achenes produce elaiosomes (fleshy parts of the pericarp instrumental in seed dispersal by ants) which are absent in the latter achene types (Bond and Slingsby, 1983; Slingsby and Bond, 1985). Dispersal into, and storage within the soil in non-myrmecochorous nut-like achenes have not yet been characterized (Le Maitre, 1988).

Contrasting germination syndromes are proposed for the main groups of serotinous and nut-like achenes (Brits, 1986b).

Overview of germination research on Proteaceae with nut-like achenes

Nut-like achenes do not germinate, or germinate poorly, in mature fynbos vegetation but seedlings recruit *en masse* during the first winter after fire (Rourke, 1972; Williams, 1972; Boucher and McCann, 1975; Brits, 1987). This is a recruitment pattern typical of species with soil-stored seeds in fire-prone mediterranean ecosystems such as fynbos (Le Maitre and Midgley, 1992), Australian heathlands (Bell *et al.*, 1993) and California chaparral (Keeley, 1991). Intact seeds of these fynbos Proteaceae germinate very poorly in cultivation (Knight, 1809; Thorns, 1943; Vogts, 1959).

This group of seeds have a strongly similar biology. Similarities exist with respect to their morphology (Vogts, 1982), anatomy (Jordaan, 1945; Venkata Rao, 1971; Johnson and Briggs, 1975), and aspects of their germination physiology (Van Staden and Brown, 1977; Brits, 1986b). Underlying these resemblances are common seed maturation, release, dispersal time and method, and storage characteristics in the myrmecochorous genera (Bond and Slingsby, 1983; Vogts, 1982; Brits, 1986a; 1986b; 1986c; 1987), which are at least partially paralleled in the nut-like non-myrmecochorous leucadendrons.

These similarities suggest a common germination physiology in species with nut-like achenes. Seed structure in related plant families living in similar environments, is correlated with germination and dormancy characters (Atwater, 1980). For California chaparral, Keeley (1991) proposed similar heat stimulated germination in structurally similar seeds. The principle of evolutionary convergence shaped by common selection pressure(s), leading to correlated seed characters between plant groups, is assumed in the following discussion.

Harper (1977) proposed a classification of seed dormancy types which is still widely quoted (e.g. in Keeley, 1991) and which applies to *Leucospermum*. The main categories are 1) innate (or primary) dormancy 2) induced and 3) enforced dormancy. *Innate dormancy* refers to a fully dormant state in which the seed is released. *Induced dormancy* manifests after seed release as a result of environmental conditions impacting on the seed - this is an acquired dormancy referred to as "secondary dormancy". *Enforced dormancy* - temporarily enforced on non-dormant seeds by environmental conditions other than the absence of "suitable" germination conditons, e.g. by allelopathic substances released from the plant canopy. A physiological interpretation of the three kinds of dormancy, applicable to the embryo only, is given below. The following types of innate dormancy can be distinguished:

1) embryos are rudimentary and require a period of after-ripening/growth before germination can commence;

2) an embryo imposed physiological condition regulates dormancy (e.g. lack of a growth promoter) which is changed by an environmental stimulus (cue) e.g. stratification;

3) dormancy is imposed by leachable inhibitors in the diaspore e.g. in the fleshy pericarp in drupes;

4) a seed coat-imposed physical condition regulates dormancy (e.g. mechanical constriction) which is changed by environmental agents (scarification) e.g. through microbial digestion.

Most species appear to have a single dormancy mechanism which inhibits germination in nature. Dormancy will be referred to as "complex" in the present discussion when:

1) several types of dormancy operate in the same seed - e.g. heat pulse of the seed coat (innate, coat-imposed dormancy) followed by embryo stratification (innate, embryo-imposed dormancy) are required in montane chaparral species of *Ceanothus* (Quick, 1935) prior to incubation/germination;

2) several environmental conditions (cues) can act singly, or additively, or synergystically, to break one dormancy type - e.g. both charate and heat pulse in certain chaparral shrubs (Keeley, 1991) or charate and light in the chaparral species *Adenostoma fasciculatum* (Keeley, 1987) breaks innate dormancy partially;

3) several functional seed characters are receptive to the same dormancy breaking

environmental cue, e.g. where functionally different exo- and endotestas are both scarified by heat pulse.

Observation confirms that the *Leucospermum* seed is a "multifunctional organism" (Manning, 1987) employing complex dormancy mechanisms of all three these kinds. Dormancy appears to be of the innate type (Harper, 1957; Roberts, 1972) since seeds are released in the fully dormant state (Brits and Van Niekerk, 1986).

Temperature requirements in germinating seeds.

The breaking of dormancy in *Leucospermum cordifolium* is strongly dependent on moderately low seasonal air temperature (Brits and Van Niekerk, 1986). The low temperature requirements of *L. cordifolium* and *Serruria florida* Knight embryos are of a diurnal, not seasonal, nature (Brits, 1986a). This is not a stratification requirement as in the case of *Malus* (i.e. a cold avoidance syndrome in which stratification is characteristically a prolonged separate phase of after-ripening at sub-optimal temperature, during which germination does not occur (Lewak and Rudnicki, 1977)). Low temperature requirement in *L. cordifolium* is rather the opposite, i.e. a mechanism to promote germination during the favourable cool, moist western Cape winter season period. The avoidance of germination during the dry, warm, unfavourable summer season period is a common germination strategy in plants adapted to mediterranean-type climates (Angevine and Chabot, 1979; Bell and Bellairs, 1992; Gutterman, 1993).

Diurnal high temperature is also required for maximal germination. The parameters for daily fluctuating temperatures were estimated at 9 and 24 °C for intact achenes of *L. cordifolium* and 7 and 20 °C for those of *S. florida* respectively, reflecting general climatic differences between their respective habitats. This suggests close adaptations in species with nut-like achenes to their particular environmental temperature regimes (Brits, 1986a).

Substantial percentages of *L. cordifolium* seeds also germinated in the laboratory under comparatively unfavourable temperature regimes (Brits, 1986a), which is in apparent contrast to the situation in the natural habitat (Rourke, 1972). Temperature regime alone therefore seems to be insufficient to impose complete dormancy on seeds in unburnt fynbos. It is therefore likely that an additional factor contributes to dormancy regulation in nature.

A small proportion of intact *Leucadendron daphnoides* and *Leucospermum cordifolium* seeds appear to be non-dormant and will germinate both at 25 °C or in mid-summer in the seed bed if moisture is available (Brown and Van Staden, 1973c; Brits and Van Niekerk, 1986). This suggests the presence of significant genetic variability in dormancy characteristics in nut-like achenes.

Temperature regimes in fynbos soil

The amplitude of diurnal soil temperature fluctuations in the surface soil after a burn is of a higher order than in unburnt fynbos (Brits, 1986a), and corresponds closely with germination temperature requirements in *L. cordifolium*. This is due mainly to the attainment of high day temperatures in burnt-exposed soil. Night temperatures are also lower in burnt fynbos soil, due to increased radiation from the exposed soil surface (Brits, 1986a).

The effective germination depths of *L. cordifolium* and *L. cuneiforme* (Burm. f.) Rourke seeds, buried in their habitat by ants, vary on average from 30 to 45 mm. Temperature fluctuations at these depths in burnt fynbos, especially during early winter, correspond reasonably closely with the estimated temperature requirements of *L. cordifolium* seeds (Brits, 1987).

Extensive laboratory research on the influence on germination of stratification at 5 °C, followed by incubation under alternating temperatures of 10 x 20 °C, have been conducted (e.g. Van Staden and Brown, 1977; Brown and Dix, 1985). Nightly temperatures in the habitat of *L. cordifolium* and *L. cuneiforme* during the winter germination season (May to September), in burnt-exposed soil, generally range from 1 to 11 °C, (Brits, unpublished) with an average of approximately 6 °C (Brits, 1986a). Seeds are clearly not subjected to an extended period of sub-obtimal temperature. The nightly low temperature regime in nature therefore encompasses both the stratification and the lower incubation temperature levels often used in the above-mentioned laboratory studies. The optimum low temperatures of 9 °C estimated for *L. cordifolium* and 7 °C for *Serruria florida* (Brits, 1986a) indeed suggest that the above-mentioned two lower laboratory temperatures may have produced similar physiological effects during experiments. A range of fluctuating temperatures is probably almost equally effective in stimulating germination, e.g. approximately 4 - 10 °C and 20 - 28 °C respectively in the case of *L. cordifolium*.

Oxygen requirement in germinating seeds

Seed coat imposed dormancy by means of oxygen exclusion, was demonstrated in *Leucospermum cordifolium, Leucadendron daphnoides* (Thunb.) Meisn. (Van Staden and Brown, 1977) and *Leucadendron tinctum* Williams (Brown and Dix, 1985), and is a characteristic of most Proteaceae with nut-like fruits (10/14 species of three genera tested), but not of serotinous species (13/15 species of three genera tested) (Brits, 1986b). This type of dormancy therefore probably fulfills a common ecophysiological role in Proteaceae with nut-like fruits. Dormancy is apparently attained during the final stages of seed maturation (Brits, 1986c).

Germination percentage was strongly increased by a single 24 h treatment of *L. cordifolium* achenes with relatively low concentrations (0,01 to 0,1%) of hydrogen peroxide (Brits and Van Niekerk, 1976). The mediating role of oxygen found in the positive effect of laboratory stratification on germination of intact *Leucospermum cordifolium* and *Leucadendron daphnoides* achenes in air (Van Staden and Brown, 1977) implies that even slight elevations in oxygen partial pressure within the embryo are effective. It seems therefore, that a relatively slight increase in the level of oxygen available to embryos is sufficient to initiate germination under suitable environmental conditions. Whilst relatively slight increases in oxygen partial pressure (by means of scarification) promoted germination, incubation in pure oxygen indeed suppressed germination temporarily in *L. tinctum* (Brown and Dix, 1985). This species is closely related to *L. daphnoides* in which, by contrast, germination was also stimulated strongly by incubation in pure oxygen (Brown and Van Staden, 1973c).

Seed dispersal, germination and establishment strategies in Proteaceae with nut-like achenes

Successful seedling recruitment of a species in nature depends on effectively coordinated seed dispersal, germination and seedling establishment.

Seed release.

In *Leucospermum* the hygroscopic reflection of bracteoles during wilting of the mature infructescence causes the release of achenes. The relatively heavy, smooth, rounded fruits fall directly to the ground under the mother plant canopy (Jordaan, 1972; Brits, 1986c). The entire seed crop is thus released during midsummer (Brits, 1986b). This is common in species with nut-like fruits. In myrmecochorous species the elaiosomes, a food source for ants, are in a turgid condition at the time of release (Brits, 1986c).

Seed dispersal.

The diaspore is an achene. In some myrmecochorous genera (e.g. *Leucospermum*) the entire pericarp functions as an elaiosome, whilst in others only a basal elaiosome is produced (e.g. in *Serruria*) (Slingsby and Bond, 1983; Rebelo and Rourke, 1986). The chemical attractants in elaiosomes are poorly known (Midgley and Bond, 1995).

Most seeds are not carried beyond 2m of the parent plant canopy and are buried at average depths of 20-40 mm (Slingsby and Bond, 1985; Brits, 1987). Seeds are directed to depositories in open spaces between plants (Yeaton and Bond, 1991). Short-distance, localized dispersal of many species with refractory (fire-recruiting) myrmecochorous seeds has also been reported in California chaparral (Keeley, 1991).

Circumstantial evidence suggests that seeds of *Leucospermum*, nut-fruited *Leucadendron, Mimetes* and *Orothamnus* are long-living, i.e. a seed reserve accumulates in the soil (Rourke, 1972; Williams, 1972; Rourke, 1976; Boucher, 1981).

Myrmecochorous *Orothamnus zeyheri* Pappe ex Hook, for example, apparently maintains viable seed banks in the soil for periods of up to 19 years (Boucher and McCann, 1975; Van der Merwe, 1977). However, no convincing quantitative results on the longevity of soil-stored seeds are available.

Fate of the seed coat.

The elaiosome/pericarp is either consumed by ants (Slingsby and Bond, 1981) or decomposed microbially in the ground shortly after dispersal. Apparently the thin pericarp in non-myrmecochorous *Leucadendron* species with nut-like fruits also decomposes, by natural weathering. In all cases, therefore, only the true seed remains in the ground. The thick, woody inner palisade layer of the outer integument (seed coat - Jordaan 1945) is durable, however, and appears to maintain its protective function in the soil. Thick, protective seed coats are a common characteristic of soil-stored seeds (Thompson, 1987). Circumstantial evidence suggests that older seeds, i.e. the major part of the viable underground seed bank, would in effect be in a scarified condition.

Progressive weathering of the seed coat is a plausible explanation for the often-observed phenomenon of extended germination in Proteaceae with nut-like achenes. This refers to the slow, erratic germination of intact achenes over the entire winter germination period observed in *L. cordifolium* (Brits and Van Niekerk, 1976;), *Leucadendron argenteum* (J.G. Smith, pers. comm.) and other Proteaceae with nut-like achenes sown in seed beds in winter. A new wave of germination often occurs in seed beds in the second winter germination season (Brits, unpublished) and in *Leucospermum glabrum* Phill. germination has been observed to continue up to the fifth winter season (D. Van Niekerk, pers comm.), thus extending germination over consecutive germination seasons. This phenomenon was also observed in *Conospermum*, a Western Australian non-proteaceous genus, and in *Persoonia* (Proteaceae) with hard, dormant seeds (Dixon, 1985). Differential disintegration of the seed coat in soil-stored seeds is discussed by Murdoch and Ellis (1992). Slow weathering may cause the outer layer (or layers) of the seed coat of myrmecochorous seeds (or pericarp plus seed coat in the case of non-myrmecochorous *Leucadendron* species with nut-like fruits) to become progressively more permeable to oxygen (Brits, 1986c), thus causing uneven germination within batches of intact seed. Presumably at least one year is required for the seed crop of any flowering season to become fully scarified in the ground.

Although the seed coat of intact (freshly dispersed) seeds is poorly permeable to oxygen, it is readily permeable to water, permitting the seeds of *Leucadendron tinctum* and *Leucospermum cordifolium* to become fully imbibed within 48 h after immersion in water (Brown and Dix, 1985; Brown *et al.*, 1986). In nature the seed of *L. cordifolium* can tolerate repeated imbibition and desiccation whilst remaining dormant in the soil (Brits and Van Niekerk, 1986).

Pre-fire soil conditions.

Progressive weathering (scarification) of the outer layer(s) of the seed coat in nature could result in an oxygenating effect similar to that of sulphuric acid scarification of intact seed (Brits and Van Niekerk, 1976). Naturally scarified, long living seeds in the soil would, however, require an (unknown) oxygen depleting factor or factors which would maintain dormancy indefinitely in unburnt fynbos, but which would be removed by the effects of fire (Brits, 1986b).

Oxygen utilization in the top soil may increase significantly in unburnt fynbos during winter. The resulting unfavourable oxygen partial pressure or O_2/CO_2 ratio in the soil may suppress germination (Brits, 1986b). This may be caused by high seasonal respiration rates of the newly formed proteoid root system and other superficial roots, microbial decomposition of both the substantial leaf litter layer on, and the mass of old proteiod roots within the surface soil, oxygen "scavenging" substances e.g. organic compounds in the superficial humus layer, and high respiration rates of ants in subterranean nests where seeds are hoarded.

Fire.

Fire in fynbos occurs episodically after 4-40 year intervals and is concentrated in the dry hot summer season (Le Maitre and Midgley, 1992). Fires in fynbos are generally less intense than in other mediterranean-type ecosystems (Keeley, 1992). Unlike the coat-imposed dormancy in many Fabaceae (Villiers, 1972), e.g. *Virgilia*, that in *Leucospermum cordifolium* seeds appears not to be broken by the direct heat from fire (Pool, 1985; Brits, unpublished). Fire could have a direct effect on the embryo of fynbos seeds (cf. Musil and de Wit, 1991). Although Williams (1972) reported a positive effect of direct heat on germination in *Leucadendron tinctum* and *L. sessile* achenes, this could not be confirmed in the case of *L. tinctum* (Brits, unpublished). However, Bond *et al.* (1990) found that the recruitment of *Leucospermum conocarpodendron* (L.) Buek seedlings was positively correlated with inferred fire intensity in fynbos. The mechanism for this has not yet been explained. It is not yet known if a smoke-derived stimulus (de Lange and Boucher, 1990; Brown, 1993) functions as a germination cue for *Leucospermum* in nature.

Direct heat pulse and charate are the only known fire-related stimuli in chaparral (Keeley, 1991) and this appears to apply to fynbos as well. Charate-stimulation of germination has also not been explained (Keeley, 1991). The fact that Proteaceae achenes are insensitive to light (Horn, 1962; Brown and Van Staden, 1973c) precludes increased light levels after fire as a germination factor. *Orothamnus zeyheri* recruited strongly with seedlings after hoeing of a locality containing only a few old moribund individuals (Boucher, 1981). This indicates that other indirect effects of fire, e.g. nutrients, soil pH, or ethylene do not influence seed germination significantly. Pool (1985) likewise found no indirect effects of fire such as ash, pH or root exudation, on seed germination in *Leucospermum cordifolium*. Post-fire soil temperature effects are, however, the exception (Pool, 1985; Brits, 1986a)

Summer or autumn fires may significantly reduce the oxygen depleting factors in fynbos soil, thereby increasing the oxygen partial pressure of the soil atmosphere (Brits, 1986b) - as often happens in nature (Mayer and Poljakoff-Mayber, 1982).

Post-fire soil conditions: winter.

Although rainfall in Cape fynbos varies considerably, more than 60% of precipitation in the western Cape occurs in winter; there is a transition eastwards to a non-seasonal pattern (Deacon *et al.*, 1992). The pattern of winter rainfall is determined by eastward moving cold fronts which are spatially separated.

After fire, the combined effects of especially high daily fynbos soil temperature (Brits, 1986a) and of increased availability of oxygen (Brits, 1986b) would effectively change the situation of old, viable *L. cordifolium* seeds in moist fynbos soil to one comparable with fresh acid-scarified seeds sown in an exposed seed bed. These conditions, particularly during the early part of winter, will stimulate the major part of the natural seed bank to germinate. Early germination of the greater proportion of *L. cordifolium* and *L. cuneiforme* seed banks was actually observed (Brits, 1987). The above interpretation is consistent with the observation that *Leucospermum* seeds germinate *en masse* only after the occurrence of fire (Rourke, 1972).

Germination of scarified seed - physiological interpretation.

How do scarified viable seeds in the soil react to environmental stimuli? It would appear as if endogenous growth regulators in proteaceous seed could serve as the link between environmental stimuli and the biochemical and ultrastructural changes which occur in the germinating embryo (Van Staden and Brown, 1977). The involvement of plant hormones in regulating seed germination has been conclusively proven in a number of plant species (cf. Hilhorst and Karssen, 1992; Villalobos and Martin, 1992).

Seed hormone theories involving the roles of gibberellins (GA) and abscisic acid (ABA) in dormancy and germination were recently reviewed by Hilhorst and Karssen (1992). They proposed a model of embryo dormancy and germination, based on direct experimental proof of hormonal action, and assumed in the present study:

1) primary embryo dormancy: this originates on the mother plant (Khan and Samimy, 1982) and is induced by embryo-synthesized, transiently present ABA mainly during the late maturation phase of the seed, but ABA is not necessarily present in the dormant seed itself (Le Page-Degivry, 1990); no germination occurs even when all conditions required for germination are provided; dormancy breaking requires after-ripening (seeds dry) or low temperature stratification (seeds imbibed);

2) secondary embryo dormancy: develops in response to some environmental stimulus after release from the parent plant (Khan and Samimy, 1982).

The breaking of primary and secondary dormancy is a separate physiological phase from that of germination *sensu stricto* (i.e. activation process of the embryo - Come and Thevenot, 1982). Dormancy breaking does not involve biosynthesis of endogenous GA, but arguably only the development of the capacity of tissue to respond to GA (Hilhorst and Karssen, 1992). (Thus exogenous application of GA can hasten the onset of germination by overriding the effect of diminishing ABA-induced dormancy during late after-ripening - Le Page-Degivry, 1990).
3) "Pseudo" or enforced dormancy: quiescence (Bewley and Black, 1982) is imposed on the embryo by the environment; external "cues", i.e. specific environmental conditions, are required for germination to commence, e.g. light, alternating temperature.

For the induction of seed germination *sensu stricto* endogenous GA is needed (Le Page-Degivry, 1990; Hilhorst and Karssen, 1992). Thus the provision of specific environmental conditions relieves enforced dormancy by inducing GA synthesis, or sensitizing tissue to GA (Karssen *et al.*, 1989), and germination commences without delay (Hilhorst and Karssen, 1992).

A wealth of results have accumulated on hormonal regulation of germination of nut-like Proteaceae achenes, in some cases with divergent interpretations (e.g. Van Staden and Brown, 1977; Brown and Dix, 1985; Brown *et al.*, 1986). It is realised that the roles of hormones require further elucidation, possibly by means of multifactorial experiments (Roberts, 1972). It nevertheless is possible to construct from the above information, and other research results, a working ecophysiological hypothesis as follows:
1) Three environmentally controlled requirements must be met for dormancy breaking and germination in *Leucospermum cordifolium* and *Leucadendron* species with nut-like fruits. (In nut-like *Leucadendron* the seed coat may in addition restrict the embryo mechanically (Brown and Dix, 1985)). These are diurnal 1) low and 2) high temperature (Brits, 1986a) and 3) an elevated oxygen partial pressure in embryos, relative to the condition in freshly dispersed, mature, saturated seeds (Brown and Van Staden, 1973c; Brits and Van Niekerk, 1976; Van Staden and Brown, 1977; Brown and Dix, 1985; Brits, 1986b; Brits and Van Niekerk, 1986).
2) The breaking of dormancy and the onset of germination are associated with simultaneously increased levels of two endogenous germination promoters, butanol-soluble cytokinin and acidic gibberellin-like substances (Brown and Van Staden, 1975; Brown and Dix, 1985). The influence of low and high temperature (i.e. 5/10 °C and 20/25 °C respectively) and oxygen in different combinations, on cytokinin and gibberellin levels under laboratory conditions, are summarised in Table 1.
3) Low temperature and elevated oxygen partial pressure in embryos when simultaneously present are apparently responsible for an increase in butanol-soluble ('free') cytokinin levels (Table 1), which are low in intact seeds (Van Staden and Brown, 1977). Oxygen-dependent interconversion of 'storage' to 'free' cytokinins is required for

germination; germination begins only after attainment of a threshold level of butanol-soluble cytokinin (Brown and Van Staden, 1973b). Cytokinin appears to be required for dormancy breaking and germination (Brown and Van Staden, 1975; Brown and Dix, 1985), i.e. gibberellin is effective in promoting germination only in the presence of an elevated level of cytokinin. Thus low temperature and elevation of oxygen partial pressure in the embryo are the primary environmental requirements for dormancy breaking and germination. This conclusion is consistent with the finding of Brits and Van Niekerk (1986) that *L. cordifolium* seeds oxygenated with H_2O_2 germinate well in seed beds in winter, but that germination of either intact untreated seeds during winter or of oxygenated seeds in summer remains poor.

Table 1. Effects of treatment with oxygen, low temperature (5 - 10 °C) and high temperature (20 - 25 °C), on endogenous levels of butanol soluble cytokinin and gibberellin, in intact (non-scarified) saturated embryos of *Leucospermum cordifolium* and *Leucadendron* species with nut-like achenes; based on data of Brown and Van Staden.

Factor(s)	Cytokinin level	Gibberellin level
Continuous low temperature only (stratification at 5 °C) (+ possible effect of slowly diffusing O_2)	HIGH Brown and Van St., 1973a Brown and Van St., 1975	LOW Brown and Van St., 1975
Continuous high temperature only (25 °C) (low/no oxygen - seeds intact)	LOWER than for stratification - does not reach threshold level required for germination Brown and Van St., 1973a Brown and Van St., 1975	LOW Brown and Van St., 1975
Oxygen + low temperature (10 °C) + high temperature (20 °C), diurnally alternated	HIGH Brown and Van St., 1973b Van St. and Brown, 1973b Brown and Van St., 1975 Brown and Dix, 1985*	HIGH Brown and Van St., 1975

*Apparently true only in the case of slightly to moderately increased oxygen partial pressure in the case of *Leucadendron tinctum*

4) Increasing cytokinins without gibberellins did not effectively overcome dormancy in *Leucadendron daphnoides* and *L. tinctum* (Brown and Van Staden, 1975; Brown and Dix, 1985) and *Leucospermum cordifolium* (Brown *et al*, 1986). An active role for gibberellins in dormancy-breaking is therefore indicated. It is deducable from Table 1 that increased gibberellin levels apparently require either elevated oxygen and diurnal high temperature, or low temperature in addition to these two factors. The necessity for elevated oxygen partial pressure in increasing endogenous gibberellin levels may further be inferred from results on *L. tinctum* (Brown and Dix, 1985). The possible involvement of low temperature bears some resemblance to the situation in the hazel nut (*Corylus avellana*), and possibly in other species as well, in which gibberellin levels in dormant seeds increase only after transfer of seeds from chilling to high temperature (Jones and Stoddart, 1977). Thus the main difference between the requirements for cytokinin and gibberellin production seems to be the additional requirement for daily high temperature in the case of gibberellin. This is consistent with the finding that high temperature in addition to low temperature are required for maximal germination in *L. cordifolium* and *Serruria florida* (Brits, 1986a).

5) Promoter hormone levels do not change phasically (i.e. step-wise, in an ordered sequence - Khan, 1977) but occur concomitantly (Brown and Van Staden, 1975).

6) High levels of growth promoters enable the embryo to utilise its food reserves and subsequently germinate (Van Staden and Brown, 1977).

Adaptive responses in seed regeneration characteristics

Dormancy and other specialized characteristics are evolutionary adaptations to stress factors in the natural habitat (Nikolaeva, 1977; Mayer and Poljakoff-Mayber, 1982). A major adaptation in nut-like achenes, discussed above, is the avoidance of germination during temporary favourable conditions in the dry summer season, by means of a low temperature requirement. However, recruiting seedling populations must also be buffered against the converse, i.e. temporary unfavourable conditions occurring within the winter germination period. Adverse conditions include decimation of seedling populations by herbivores and, especially, episodic periods of drought during the otherwise moist, cool winter (Brits, 1987). Thus the gene pool could be destroyed if the entire seed bank germinated at the first occurrence of favourable conditions. An extended seed germination pattern could therefore be an important adaptation in natural populations (Mayer and Poljakoff-Mayber, 1982).

Extended ("erratic") germination of fresh (i.e. intact) Proteaceae seeds is well known (e.g. Knight, 1809; Brits and van Niekerk, 1976). The phenomenon has been described in fynbos (Brits, 1987) and other unpredictable environments (e.g. Gutterman, 1993). The mechanism by which extended germination is regulated remains unknown. Uneven ripening periods within infructescences (e.g. in *Leucospermum* - Vogts, 1982) may determine variable seed coat maturities at the time of dispersal, and thus differences in permeability to oxygen amongst seeds of the same seed crop (Brits and Van Niekerk, 1986). This and direct genetic control of variability in seed coat thickness, as well as in levels of endogenous germination promoters, may regulate innate polymorphism in germination time. Polymorphic seed pools of refractory (fire-recruiting) taxa have also been reported for chaparral (Keeley, 1991). External factors, such as differences in rate of natural scarification in the soil and variable seed burial depth (Brits, 1987) are also likely to cause uneven germination in nature.

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CHAPTER 2: MATERIALS AND METHODS

This chapter gives Materials and Methods (M&M) not described in subsequent chapters. These are mostly M&M in published papers predating this thesis, compiled for easy reference. For ready access all new M&M are described once in the appropriate chapters below, in the earliest study where they are used.

1. Seed material

Seeds of *Leucospermum* were produced from cultivated plants at Tygerhoek Experimental Farm, Riviersonderend, South Africa, on Table Mountain Sandstone derived soil adjacent to natural fynbos (Brits, 1987). "Seeds" were harvested under controlled conditions after natural release in December as achenes, the true dispersal units, with a mature, turgid pericarp (elaiosome) present (Brits, 1986). Seed heads were dried in a single layer under summer conditions in shade or in an oven at 40 °C. Seeds were hand sorted to ensure the use of fully developed embryos (Vogts 1976; Van Staden and Brown, 1973) and thoroughly mixed.

Seed material occasionally had to be purchased from other sources.

2. Seed germination characteristics:

2.1 Tetrazolium viability testing method

The tetrazolium test of Brits and Van Niekerk (1976) was used in modified form. A sample of 150 dry seeds was scarified in (c) H_2SO_4 for 10 min and washed under running tap water for 5 min. The seed embryos were then isolated from the the softened seed coat, taking care not to bruise embryonic tissue. Embryos were randomly divided into replicates of 25 and transferred to 6 petri dishes with distilled water, for 24 h. The seed pellicle, which is discernable as a thin membrane around the embryo, was removed. One cotyledon per embryo was then removed with a scalpel and, together with its transected half of the embryonic axis, transferred to a petri dish in a 0,1% solution of 2,3,5,triphenyl tetrazolium chloride. After a further 24 h period of soaking at 22 °C in the dark, the tetrazolium solution was poured off and the embryo-remnant washed and examined. The degree of colouring was scored on a scale of 0 (no colour/ embryonic axis not coloured) to 10 (cotyledons and embryonic axis fully coloured). Where necessary, data were analyzed as a completely randomized design.

2.2 Acid scarification method

Dry seeds, with the pericarp intact, was mixed with (c) H_2SO_4 (minimum purity 98%) at 22 °C in glass beakers, at a concentration of 15 ml acid per 100 seeds (Brits and Van Niekerk, 1976). The mixture was stirred at 1 min intervals for 7,5 or 10 min. The acid was discarded by pouring the mixture through a sieve. The mixture was immediately washed with running tap water, taking care that seeds did not overheat in the process. Seeds were washed under running tap water for 15 min, applying light rubbing four or five times during the process, to remove the acidic remains of the pericarp. Seeds were then dried lightly with paper towel.

2.3 H_2O_2 seed oxygenation treatment

A fresh 1% hydrogen peroxide solution was prepared. Seeds (usually freshly acid scarified or with pericarp removed manually) were mixed with the solution at a concentration of 20 ml solution per 100 seeds (Brits and Van Niekerk, 1976). Seeds were allowed to imbibe the solution for 24 h in the dark (22 °C). Seeds were briefly washed under tap water before sowing.

2.4 Smoke treatment of seeds

The "smoke-tent treatment" of de Lange and Boucher (1990) was used. Smoke was generated in a 130 l drum using a mixture of dry and fresh plant material gathered in the surrounding vegetation in late summer. The smoke was blown, by using bellows, through a pipe into a c. 1 m^3 plastic tent erected over trays with planted seeds, for 30 min. The system allowed the smoke to cool before it entered the tent.

2.5 Seed disinfection

Seeds were routinely disinfected before transferring to the petri-dish incubation regime. The justification for this somewhat unnatural approach was the intensive human handling of seeds which is not experienced in nature, together with the confined, damp conditions in the petri dish where seeds are incubated for several months. Experience has shown that fungal infections often kill isolated whole replicates (petri dishes) of seeds during experiments, leading to inflated coefficients of variation (Van Staden and Brown, 1973; Brits, 1986). Seeds were treated with either of the following methods.

Hot water treatment. Intact seeds were placed in cheese-cloth bags and completely submerged in hot water at 50 °C, for 30 min (Benic, 1986). The water was forced-circulated and care was taken to use an easily penetrable mass of seed in each bag.

Fungicide treatment. Seeds were mixed with thiram (75% a.i.) wp fungicide at a concentration of 0,05 g powder per 100 seeds in a small polyethylene bag (Benic, 1986). Vigorous shaking of the bag ensured a thin, even covering of seeds with powder.

2.6 Floating and sinking test of seeds

The floating test described by Van Staden and Brown (1973) was applied in a modified form. Samples of 200 seeds per species were scarified in (c) H_2SO_4 for 10 min and washed under running tap water for 5 min. The seeds, the pericarp of which had been partially digested by the acid, were then transferred to water to determine the fraction of floating and sinking seeds.

The original test of Van Staden and Brown is flawed in that small air bubbles tend to adhere to the dry pericarp in some seeds when immersed, causing many heavy seeds to float together with light (poorly developed) seeds. For this reason an alternative method was used to scarification of seeds before testing. In this method seeds were immersed in lukewarm (40 °C) water for 2 h and the softened pericarp removed manually before conducting the test. Care was taken to equalize immersion times of different test samples.

3. Plant growth regulator assays of seed samples

Abbreviations:

ABA, abscisic acid; BA, 6-benzyladenine; CK, cytokinin; DHZ, dihydrozeatin; DHZR, dihydrozeatin riboside; GA, gibberellin; HPLC, high performance liquid chromatography; iP, isopentenyladenine; IPA, isopentenyladenosine; PGS, plant growth substance; RIA, radioimmunoassay; Z, zeatin; ZR, zeatin riboside.

The description below details the methods used for radioimmunoassay of plant growth substances, referred to in Chapter 5.

3.1 Extraction and purification of plant growth substances

Samples (0,5 g) of cotyledon and axis material from each harvest date (incubation period) were homogenised in 1 ml 80% methanol with a mortar and pestle, the mortar was washed, and the homogenate made up to 10 ml, with 80% methanol, and the PGSs extracted in the dark for 24 h at 4 °C with stirring. Samples were filtered through a Whatmans GF/A 1,6 µm glass microfibre filter and passed through a 500 mg C_{18} Octadecyl 4 µm (Bond Elut - Analytichem) cartridge. The cartridge was activated in advance with 5 ml 100% methanol and conditioned with 5 ml 80% methanol and, following passing of the sample, the columns were eluted further with 10 ml 80% methanol. Filtrates were reduced to dryness in a Savant vacuum concentrator at 35 °C and stored at -60 °C prior to HPLC separation.

3.2 High-Performance Liquid Chromatography (HPLC) isolation of PGSs

For HPLC the dried, partially purified sample extracts were dissolved in 800 µl acetic acid HPLC starting buffer. A mixture of 100 µl of 10 000 dpm [^{3}H]DHZ (38 Cu mmol^{-1}

from Amersham International plc) and 100 µl 15 000 dpm [^{14}C]ABA was added to samples to determine recoveries. Three scintillation vials were spiked at the same time, for use as controls in recovery determination, capped and kept at 4 °C until required. Samples were filtered through a 0.45 µm HV filter (Waters) and whole samples (0,5 g fresh mass equivalent) injected into the HPLC. Samples were chromatographed using a Waters (Milford, MA) gradient HPLC fitted with a Zorbax 250 x 10 mm, C_{18} 5 µm semiprep ODS column (Dupont, Wilmington, DE) and a U6K variable volume injector, at 20 °C room temperature. The column was eluted with a gradient starting at 10% MeOH, in degassed 0.1 N acetic acid buffered to pH 3.4 with triethyl amine, and changing to 50% MeOH over 70 min at a flow rate of 1 ml.min^{-1}. The gradient was then held constant at 50% methanol for 10 min and then taken to 100% methanol over 10 min and held at 100% methanol for a further 20 min. Authentic Z, ZR, DHZ, DHZR and IPA standards were run simultaneously and UV traced at 260 nm (Chapter 5, Figure 2) to confirm the stability of retention times of CKs. GA and ABA retention times were determined by injecting [^{3}H]GA$_1$ and [^{14}C]ABA respectively. Samples were chromatographed in a sequence of one run UV traced standards and one blank run to rinse the column followed by four samples. 2-min fractions were collected on a Gilson fraction collector from 10 to 100 min into 20 ml Packard scintillation vials and, together with the three spike vials, reduced to dryness in a Savant vacuum concentrator. Immunohistograms were obtained from fractions collected in 8-12 min windows in the HPLC run corresponding to the elution times for individual PGS authentic standards (e.g. Chapter 5, Figure 2a) and verified by comparing immunohistograms with authentic PGS retention times (Chapter 5, Figure 2B-E).

3.3 Quantitation of PGSs by RIA

Radioimmunoassay was used to determine the concentrations of Z-, DHZ- and isopentenyl-type CKs (Cutting *et al.*, 1983; Hofman *et al.*, 1986; Cutting, 1991), GAs (Wand *et al.*, 1991) and ABA (Cutting *et al.*, 1986; Hofman, 1990). Serial dilution tests were carried out on all fractions showing immunological activity. HPLC vial contents were redissolved in 2 ml 100% MeOH. These were partitioned, in 600 µl aliquots, to determine the CKs Z/ZR, DHZ/DHZR, IPA/2iP, and the GAs and ABA separately, as well as a 200 µl aliquot to calculate recoveries. All radioimmunoassay quantitations were done in triplicate. Radioactivity was determined on a Packard 1900CA Liquid Scintillation Analyzer. Raw data was analysed using an on-line computer and the Securia data reduction radioimmunoassay package (Packard Instrument Company, 1986 publication no. 169-3016). Recovery of [^{3}H]DHZ and [^{14}C]ABA were determined simultaneously with PGS quantitations. The PGS concentrations were adjusted accordingly. Successive approximation using HPLC and extract dilution curves showed the absence of possible contaminants.

3.4 Bioassay of CKs in non-imbibed Leucospermum glabrum *seeds*

RIA of CKs was supplemented by bioassay of non-imbibed embryo axes and cotyledons. Samples (0,5 g) of *L. glabrum* embryo axes and cotyledons were prepared, extracted and HPLC separated (1 min fractions) as described above for RIA. Cytokinin activity of fractions was estimated by the soybean callus bioassay (Miller, 1965), following established procedures.

4. Statistical procedures for field, laboratory experiments and RIA

4.1 Experimental lay-out

Experiments in incubators were routinely carried out in petri dishes, each dish being a treatment replicate. Dishes were moved twice-weekly to different positions to equalise incubator environment as far as possible. This arrangement gave a completely randomized design as the basic layout in incubator experiments.

In seed beds replicates were allocated to fixed positions. The basic design was therefore randomized blocks.

Specific experimental designs, mostly factorial, and relevant analytical procedures for data, are given below for individual studies.

4.2 Transformation of percentage data

Percentage data were routinely analysed for normality of distribution (Shapiro-Wilk's test for non-normality). Angular transformation of percentages was carried out in non-normal data sets.

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CHAPTER 3: SEED ONTOGENY

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Preface

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- Mature ovule
- Seed coat development

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CHAPTER 3

SEED ONTOGENY

PREFACE

A study of seed anatomy seemed appropriate as an introduction to the subject of *Leucospermum* seed ecophysiology. The seed covering structures of South African Proteaceae were being misinterpreted - and misnamed - throughout the considerable seed literature. Correction of this was required as a basis for further investigation.

There was also the need to search for possible links between seed structure and function. Where, for example, is the seat of oxygen exclusion from the embryo and are there clues to the dormancy mechanism(s)? Although a study by Jordaan (1945) correctly identified seed structures it was ignored in subsequent literature. Clearly a fresh investigation was necessary to interpret function as well. Characterization and quantification of seed characters, comprising the bulk of this thesis, provide the basis for understanding function. The study of seed structure in relation to function is therefore placed at the end of the thesis (Chapter 7).

3. SEED COAT DEVELOPMENT IN *LEUCOSPERMUM CORDIFOLIUM* (KNIGHT) FOURCADE (PROTEACEAE) AND A CLARIFICATION OF THE SEED COVERING STRUCTURES IN PROTEACEAE

Introduction[*]

Despite the horticultural popularity of the Proteaceae, detailed anatomical investigations of the seeds are relatively uncommon (Corner 1976; Venkata Rao 1971). Furthermore, it appears that even in those species examined some considerable confusion exists in the interpretation of various layers of the seed covering structures. Seed structure can prove very useful in reflecting phylogenetic relationships between taxa (Corner 1976), and it is essential that the homologies of the layers be correctly established.

Schwarzbart (1905) was the first to study, and misinterpret, the structure of the seed of Proteaceae. He examined mature fruits, as have many subsequent authors, and erroneously considered the thickened outer integument to comprise the endocarp. Although Jordaan (1945) commented on this error and correctly interpreted the covering structures of the seed in *Leucospermum conocarpodendron* (L.) Buek (as *L. conocarpum* R. Br.), *Leucadendron salignum* Berg. (as *L. adscendens*), *L. lanigerum* Meisn. and *L. rubrum* Burm. f. (as *L. plumosum* R. Br.), his work has apparently been almost entirely overlooked by virtue of the language and journal in which it was published. Much of the subsequent studies on seed morphology in Proteaceae were conducted by Venkato Rao (1971 and references therein), who, following Schwarzbart in his terminology, ensured that this misinterpretation continued thence uninterrupted into the literature. Midgely (1987) recognized and commented briefly on this problem in *Leucadendron* but did not concern himself further with it.

In view of the extent to which this misinterpretation has persisted in subsequent studies and the resultant ramifications in attempts at phylogenetic analyses, it is essential to clarify the nature of the seed covering structures in Proteaceae.

Adapted from Manning and Brits (1993).

Material and methods

Seed of *Leucospermum cordifolium* (Knight) Fourc. at various stages of development was collected directly into FPA [50% ethanol, 5% propionic acid, 10% formaldehyde (40%), 35% H_2O]. Seed was produced from plants growing at Tygerhoek Experimental Farm, Riviersonderend, South Africa. Fixed seed was dehydrated in ethanol and embedded in wax following established procedures. Sections were stained with Alcian Blue and Safranin. Material for SEM examination was freeze-fractured in liquid nitrogen, mounted onto aluminium stubs and sputter coated with gold-palladium for viewing in a Cambridge S200 at 10kV.

In the text the following standard abbreviations are used: *oi* = outer integument; *ii* = inner integument; *oe* = outer epidermis; *ie* = inner epidermis.

Results

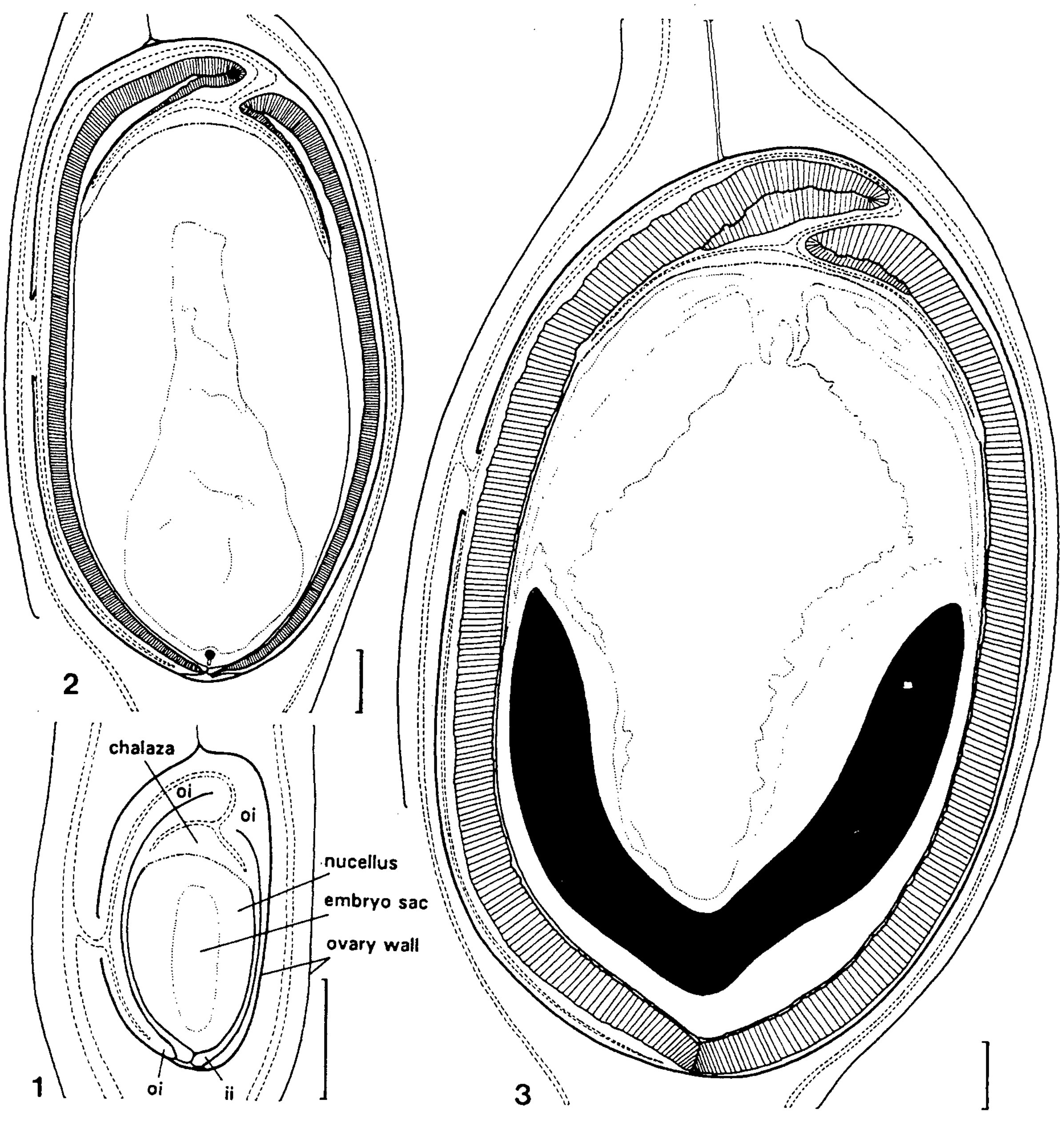
Mature ovule

Ovary unilocular, placentation parietal with a single sessile ovule, attached medially to an adaxial placenta, ovule adnate to the inner ovary wall except at the micropyle. Ovule (Figure 1) hemitropous, bitegmic, with the inner integument much shorter than the outer at the chalaza through hypertrophy of the chalaza, endostomal and crassinucellate; vascular bundle bifurcating as it enters the ovule, one branch travelling through the *oi* and terminating near the micropyle, the other, main branch, travelling through the raphe to the chalaza where it is abruptly recurved as it enters the heteropyle then splitting into 10-12 radiating branchlets extending almost to the base of the *ii*; hypostase shallowly cupuliform, densely staining. Integuments (Figure 4): *oi* 8-10 seriate on adaxial (raphal) side but 2-seriate on abaxial (antiraphal) side; *ii* mostly 3-seriate but 4-seriate at the base and at the micropyle through periclinal division of the middle layer; cells of *oi* c. isodiametric, densely staining on the antiraphal side and the inner 4 layers on the raphal side and extending along the vascular bundles to the hypostase; cells of *ii* brick-shaped except at the base where the cells of the *oe* are c. isodiametric and resemble those of the *ie* of the *oi*, densely-staining. The ovary wall consists of c. 10 rows of parenchyma.

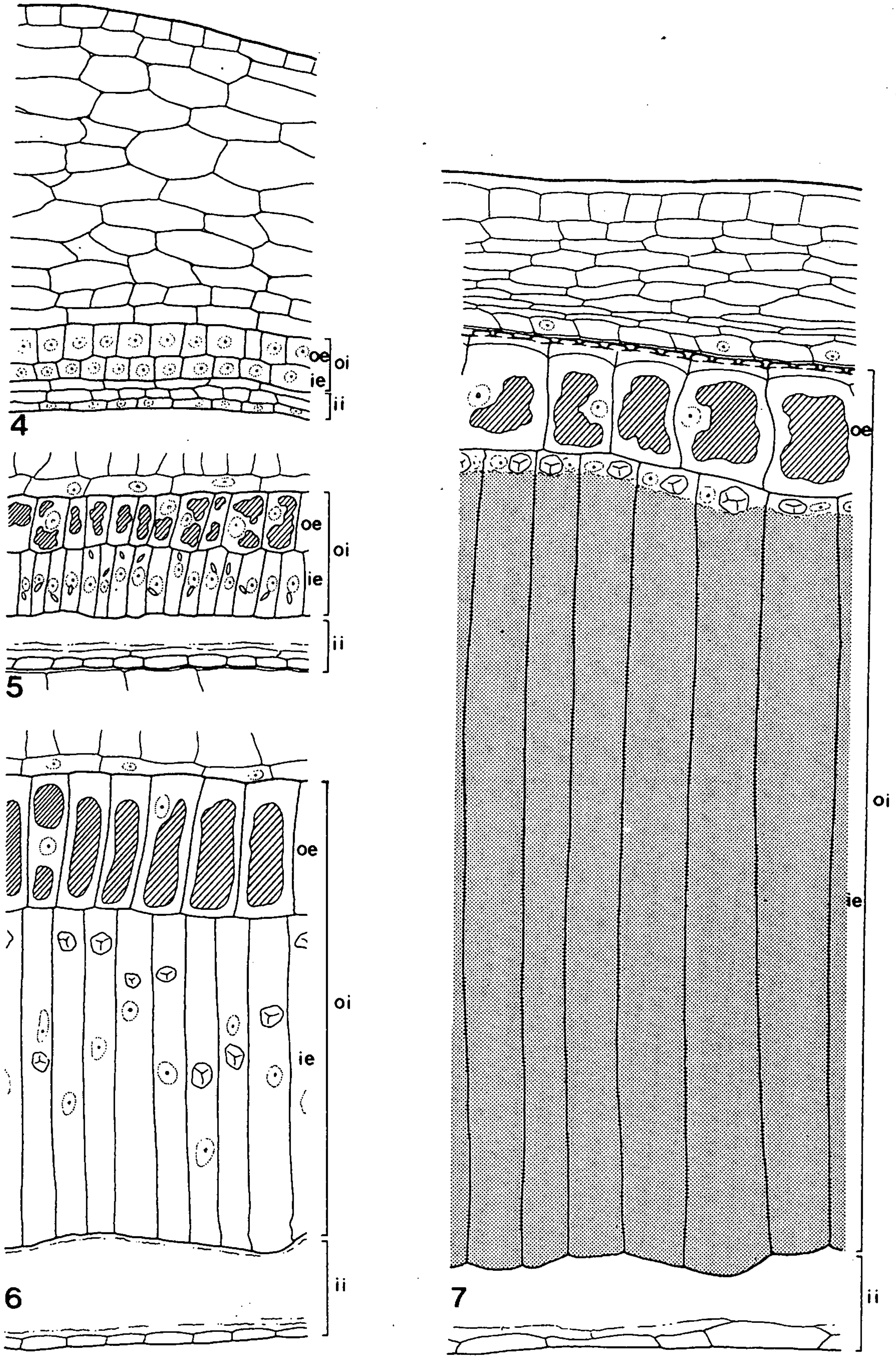
Seed coat development

During development (Figures 2 and 3) the ovule remains adnate to the ovary wall and is shed as an achene: the *oi* remains attached to the pericarp, while the embryo sac separates from the now complex seed covering (comprising pericarp plus *oi*) along the disintegrating *ii*, the *ie* of which remains attached to it. The pericarp is composed largely of thin-walled parenchymatous cells which are flattened at maturity although the epidermal cells are c. isodiametric and have a thick cuticle (Figure 7). The *ii* disintegrates during development (Figures 4-7), with only the *ie* somewhat persistent.

The *oi* is persistent, and at maturity comprises c. 11 layers at the raphe, three layers at the antiraphe from chalaza to halfway to the micropyle, and thence only two layers. During development the *oi* overgrows the *ii* at the micropyle and occludes the aperture



Figures 1-3. Seed development in *Leucospermum cordifolium.* Figure 1. Mature ovule. Figure 2. Seed 3 weeks after anthesis. Figure 3. Fully-formed seed 5 weeks after anthesis. Scale bar = 500 µm.



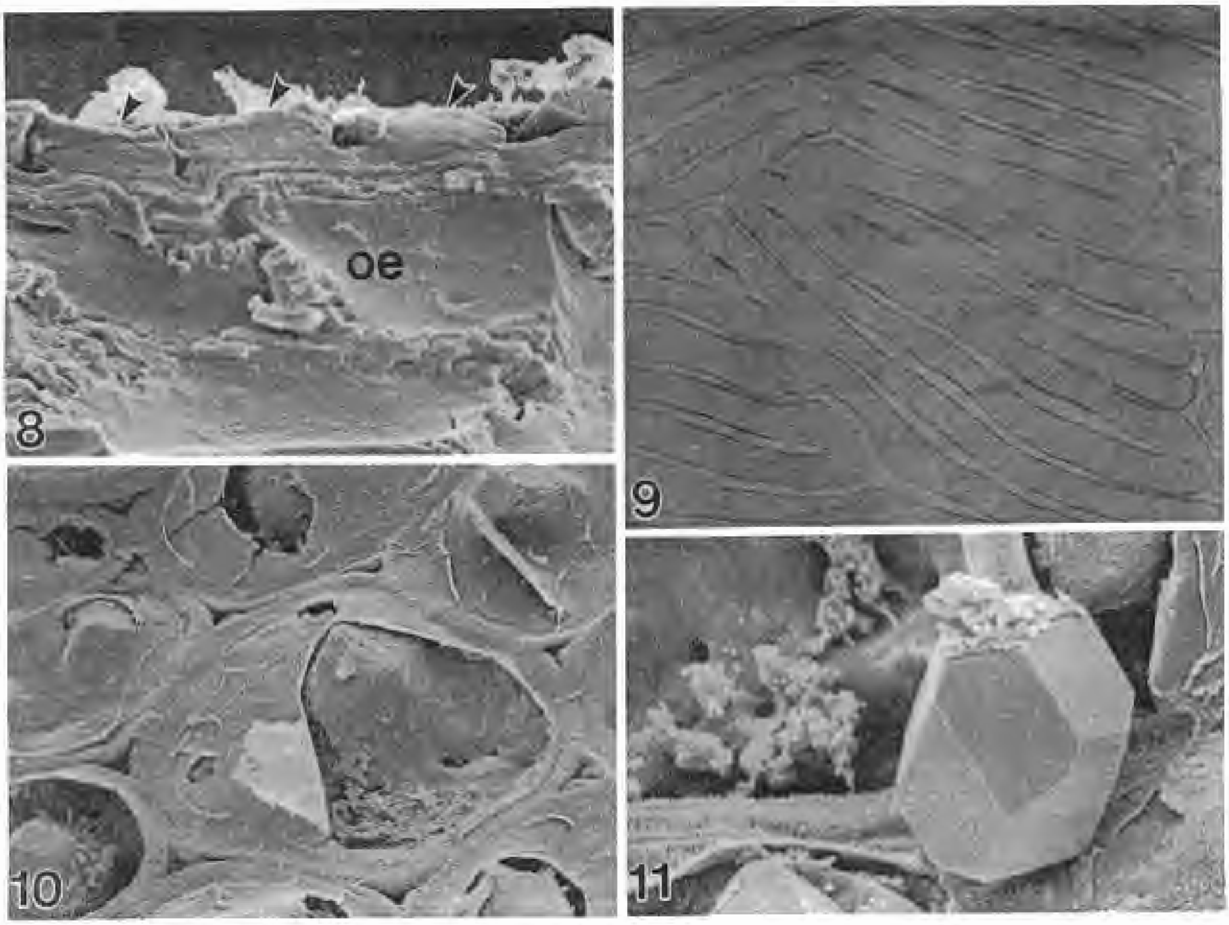
Figures 4-7. Seed coat development in *Leucospermum cordifolium.* Figure 4. At anthesis. Figure 5. At week 2. Figure 6. At week 3. Figure 7. At week 5. Scale bar = 10 µm. (oi = outer integument; ii = inner integument; oe = outer epidermis; ie = inner epidermis).

and the mature seed is thus exostomal (Figure 3). All the cells of the *oe* of the *oi*, chalaza and hypostase are tanniniferous at maturity. The cells of the *oe* (= exotesta) of the *oi* elongate radially and become radially brick-shaped at week 3 before becoming subsequently somewhat flattened and c. isodiametric at week 5 and further flattened at maturity (Figures 4-8). The cell lumen becomes occluded with tannin, present already at week 2. The cells of the *ie* (= endotesta) undergo extreme radial elongation and become columnar and form a palisade: at maturity these cells are radially oriented at the poles of the seed but incline slightly towards the micropyle around the rest (Figure 3). At week 2 a single rod-shaped crystal is present in each cell near the centrally situated nucleus (Figure 5). These crystals become prismatic and begin to migrate centrifugally to the tips of the cells at week 3, followed by the nuclei (Figure 6). At week 5 the cell lumina become completely occluded with crystal sand except at the extreme tips where the prismatic crystal (Figures 10 and 11) immediately internal to the *ie* are located (Figure 7). At the base of the *ii* a single row of cells of the hypostase undergo similar radial elongation, becoming columnar and occluded with crystal sand but lacking prismatic crystals. At maturity the cuticle on the testa develops longitudinal ridges resembling the whorls on a finger tip (Figures 8 and 9). During development the pericarp undergoes no significant structural changes apart from the development of a thick cuticle.

At maturity the fruit is thus shed with the thin, soft but turgid pericarp adnate to the thick and hard testa, enclosing the embryo and endosperm which bear the tenuous remains of the tegmen. The fruit is thus an achene.

Discussion

The ovule in *Leucospermum cordifolium* is unremarkable in Proteaceae in the following integumental characters: bitegmic, *oi* biseriate, *ii* 3 seriate, and endostomal. The radiation of the vascular bundle in the chalaza is also typical. The development of the seed coat is non-multiplicative and the coat strictly endotestal. The ovules in *Leucospermum* R. Br. (Jordaan 1945; this study) and *Leucadendron* R. Br. (Jordaan 1945), and possibly related genera, are unusual in the presence of extensive chalazal tissue in the zone between the bases of the two integuments. In the ovules of most other Proteaceae illustrated in Venkato Rao (1971) the two integuments arise at more or less the same level, and at this stage at least do not exhibit this chalazal hypertrophy. A significant exception is the ovule of *Persoonia* Sm., in which there is a tegmic pachychalaza, and which subsequently develops into a pachychalazal seed (Stroschen 1986c). Such hypertrophy of the chalazal region is discussed by Bouman (1978). The interpolation of this tissue between the integumentary bases distinguishes it from the true pachychalazal condition, in which the chalazal hypertrophy occurs between the nucellar and integumentary tissues, and in consequence of which much of the nucellus is surrounded by chalazal, rather than integumentary, tissue (Bouman 1978; Von Teichman and Van Wyk 1991). Corner (1976) refers to this interpolated tissue in Euphorbiaceae as a 'tegmic pachychalaza' to distinguish it from the fully pachychalazal condition because it affects only the interior of the tegmen and not the testa, while van Heel (1982) terms it an 'endochalaza' in Myristicaceae. Jordaan (1945) refers to this relatively massive tissue between the bases of the inner and outer integuments as the basal tissue, and concluded that it could equally well be referred to the chalazal or



Figures 8-11. Mature seed coat in *Leucospermum cordifolium.* Figure 8. SEM micrograph of fractured seed showing outer epidermis of outer integument (oe) covered with ridged cuticle (arrowed); x 1600. Figure 9. Light micrograph of cuticle showing longitudinal ridges; x 450. Figure 10. SEM micrograph of outer tangential face of crystal layer showing prismatic cystals protruding; x 1300. Figure 11. SEM micrograph of isolated prismatic crystal from crystal layer; x 5200.

nucellar tissues. In the ovule this tissue is separated from the nucellus by the densely staining hypostase and is clearly chalazal in nature. This extreme development of the chalazal tissue in the ovule in the zone between the bases of the outer and innner integuments is not restricted to Proteaceae, and is known in Euphorbiaceae: Crotonoideae (Singh 1854; Corner 1976), Myristicaceae (van Heel 1982) and Thymeleaceae (Bouman 1978). The post-fertilization separation of the outer and inner integuments by intercalary growth processes is of rather common occurrence in mature seeds (Bouman 1978), but chalazal hypertrophy in the ovule is clearly less common. The extent of the tegmic pachychalaza is readily visible in mature seeds of *Leucospermum cordifolium* from which the testa has been shucked, as a brown cap (due to the extensive tannin deposition) to the otherwise pale kernel. In the intact seeds the thickened testal layers surround the whole embryo and obscure this, unlike in truly pachychalazal seeds where the chalazal seed coat is externally very distinct from the lighter coloured integumentary part of the seed coat (Von Teichman 1991).

Table 1. Misinterpretation of the seed covering structures in *Leucospermum* by various authors (Schwarzbart, 1905; Venkata Rao, 1971; Johnson and Briggs, 1975).

Ovule	Seed		Misinterpretations	
ovary wall	pericarp		exocarp	
oe of oi	exotesta		mesocarp	pericarp
ie of oi	endotesta (crystal layer)	seed coat	endocarp	
ii	tegmen		seed coat	

If the structure of the ovules in Proteaceae has not been in dispute, the further development of the seed coat has been much confused (Table 1). The single most conspicuous layer surrounding the seed in many Proteaceae (although it may not persist intact to maturity) is the endotestal layer of crystalliferous palisade, derived from the *ie* of the *oi*. This layer was called the crystal layer by Netolitzky (1926) and the layer of crystal cells by Corner (1976) and this designation for this type of endotesta is apt. Much of the confusion has resulted from a lack of developmental studies, and interpretations have been based on the mature structures alone. In the Proteoideae with achenal fruits, it appears that the adnation of the *oi* to the pericarp and the partial dissolution of the *ii* have obscured their respective identities. In a section through the mature fruit it is difficult to distinguish the pericarp from the *oi* and this in turn from the *ii* unless it had been studied developmentally.

In *Leucospermum, Leucadendron* and other African genera, Filla (1926) attributes the crystal layer (actually the endotesta) to the pericarp as the innermost layer of the pericarp. Venkato Rao (1971) was similarly misled, and of the opinion that in Proteaceae "the seed coats are thin, flimsy and devoid of mechanical tissues" and that protection is provided by the pericarp. This understanding led him to attribute, in all instances, any hard tissues to the pericarp, as a woody or stony endocarp. In fact, in Proteoideae with indehiscent fruits at least, the seed coat is decidedly rigid and it is the pericarp which is less substantial. The indehiscent fruit types in Proteaceae were subsequently classified by Venkato Rao (1971) according to the nature of what he termed the exocarp (actually the pericarp proper); if fleshy the fruit was identified as a drupe, if woody as a nut, and if leathery or membranous as an achene. In consequence he considered the pericarp to be stratified in those instances in which the seed coat (his endocarp) differed in texture from the pericarp (his exocarp). This confused nomenclature led inevitably to some uncertainties. Fruit with a corky "exocarp" and woody "endocarp" (*Brabajum* L., *Panopsis* Salisb., *Gevuina* Molina. and *Euplassa* Salisb.: Grevilleoideae and *Dilobeia* Thouars: Proteoideae) are termed in one instance "drupaceous nuts", while in another the fruit of *Dilobeia* is described more expansively as a "nut with corky exocarp".

Since the treatise by Venkato Rao (1971) is singularly influential to students of the Proteaceae, it is worthwhile exploring his interpretation more fully. It is not certain, however, that the nomenclatural confusion was restricted to those genera with indehiscent fruits. While illustrated (Venkato Rao, 1971) as part of the "pericarp" in *Orothamnus* Hook., *Adenanthos* Labill., *Petrophila* R. Br., *Aulax* Berg. and *Leucadendron* (Proteoideae), the distinctive palisade layer containing prismatic crystals is also (this time correctly) attributed to the testa in *Grevillea* J. Knight and *Hakea* Schrader (Grevillioideae) but to the tegmen in *Embothrium* Forster and Forster f. (Grevillioideae). In the grevillioid genera this layer is evidently crushed at maturity and so apparently does not develop the crystal sand found in *Leucospermum* and other proteoid genera. Despite this difference in ultimate fate, it is necessary to establish absolutely whether or not the palisade layer is homologous in all genera in which it occurs. Corroboration of the homology of the crystalliferous palisade throughout the Proteaceae is forthcoming from the studies on fruit development in *Persoonia*

(Persoonioideae), *Hickbeachia* F. Muell. and *Macadamia* (Grevillioideae) (Stroschen 1986a-c). In all these genera the crystalliferous layer is identified as endotestal, and together with the present study indicates its homology throughout the family. In view of its occurrence in members of all three subfamilies, it appears that a crystalliferous endotesta is characteristic of the Proteaceae and plesiomorphic, and its absence should be viewed as secondary and an apomorphy.

Macadamia F. Muell. (Grevillioideae) has been singled out as the only genus in the family in which the seeds exhibit a hard seed coat, but this was incorrectly interpreted (Venkato Rao, 1971) as of perichalazal origin and thus not homologous with the seed coat in other Proteaceae. In fact, although the seed in *Macadamia* is pachychalazal, a significant portion of the seed coat is integumental and this portion is endotestal with a crystalliferous inner epidermis (Joubert 1986; Strohschen 1986a). The chalazal part of the seed coat develops a similar crystalliferous layer and is externally indistinguishable, and the seed coat is uniformly woody. The structure of the integumental part of the seed coat in *Macadamia* is thus homologous with the seed coat of other Proteaceae, and far from being exceptional in the family by virtue of its hard seed coat, *Macadamia* is perfectly normal. The pachychalazal nature of the mature seed is also apparent, although not stated, in *Hicksbeachia* and *Persoonia* (Stroschen 1986b,c).

Thus the fruit wall in *Leucospermum* and *Leucadendron* at least, and probably throughout the family, has erroneously been construed to comprise the pericarp proper plus the adnate outer integument, which separates from the inner integument as the latter disintegrates, and the seed coat to comprise merely those scant remnant layers of the *ii* which adhere to the embryo sac. This interpretation results in the view that the pericarp proper in *Leucospermum* is an exocarp while the outer integument layers (the testa proper) constitute a hard endocarp. The fruit of *Leucospermum* has accordingly been misinterpreted as a nut, supposedly derived from a drupe (Venkato Rao 1971), whereas it is actually an achene, and the drupes actually achenes with fleshy pericarp. The mature seeds of *Leucospermum cordifolium* are dispersed by ants (Slingsby and Bond 1981) and the pericarp functions as an elaiosome. When the seed is shed the pericarp is turgid and gelatinous. As the fruit wall is thus not strictly dry, the fruit may be considered as an unusual, somewhat fleshy, achene.

The immediate result of the misinterpretation has been complete confusion as to the identity of the fruit and seed layers, particularly in taxa with indehiscent fruits. Accordingly, Johnson and Briggs (1975), in their treatise on phylogeny in Proteaceae, following this interpretation, defined the crystal layer as the endocarp, and reported its presence in other species of *Leucospermum* examined by themselves. They thus interpreted the fruits of *Leucospermum* and *Leucadendron* (amongst others) also as nuts (type 6 fruits: 132) and were forced to conclude that "types of fruit, and particularly of endocarp development ... only partly correspond to groupings which are supported by other ... evidence". Whether this will be the conclusion when seeds of the Proteaceae are correctly interpreted remains to be seen. Reassessment of the identity of the crystalliferous palisade leads to the suspicion that the distinction between types 3, 5 and 6 fruits recognized by Johnson and Briggs (1975) is scarcely tenable. The ramifications of this misapprehension in phylogenetic speculations are extreme, and all further discussion relating to crystalliferous endocarps in that publication must be viewed with caution until it is established whether any of the genera referred to actually have such an endocarp. Naturally, were the crystal layers found to be invariably testal, then their homology would be unimpeachable, and some of the phylogenetic speculation justified, albeit that the tissues are misnamed. A survey of the distribution of a persistent crystalliferous endotestal palisade in Proteaceae is more likely to yield a character of phylogenetic significance than is the distinction between fleshy, woody or leathery pericarps, uncertain as it sometimes is.

It is clear that a complete re-evaluation of published accounts of the nature of the fruit and seed covering structures in Proteaceae is required before any meaningful phylogenetic discussions can be entertained.

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CHAPTER 4: GERMINATION REQUIREMENTS IN LEUCOSPERMUM

Contents

CHAPTER 4

GERMINATION REQUIREMENTS IN *LEUCOSPERMUM* SEEDS

PREFACE

This chapter attempts to characterize and quantify seed germination requirements in *Leucospermum* R.Br. To this end the variety of ways is investigated in which dormancy can be broken and germination induced. Oxygenation requirements of the embryo, for example, can be satisfied by incubation in gaseous oxygen, soaking seeds in H_2O_2 solutions, scarifying seeds in (c) H_2SO_4, or, in nature, by scarification via digestion by soil microflora and even by specialized desiccation mechanisms that break the testa. With such a varied expression of one character there is a risk of confounding interpretations, especially where aspects of one factor are investigated in isolation.

A considerable number of seed characters have been implicated in dormancy breaking and germination induction. Do we have the full picture or are there unknowns left? One measure of knowing would be to ask to what degree can the germination of viable seeds be controlled artificially in various species.

Even if dormancy breaking and germination factors are fully identified, the question remains: how do they interact within the seed? This has been covered scantily in the literature.

The roles of scarification and stratification in *Leucospermum cordifolium* seed germination

Study 1 attempts to distinguish between scarification and stratification requirements of *Leucospermum* seeds. These are two important ecophysiological principles in relation to the fynbos germination environment. The problem is addressed of relating the artificial conditions in the laboratory to the web of (often unknown) interacting variables in nature. In addition an attitude is considered critically, the (unconscious) submission of students of fynbos to paradigms of classical, northern hemisphere-centered Botany - in this case the preoccupation with the role of stratification in *Leucospermum* seed germination. Why should laboratory-bench botanists continue to investigate stratification effects on seeds when fynbos hardly offers any ecological parallels (except at high altitudes)? The practice of limited single-factor studies which circumvents the effects of interaction is examined. Single-factor studies have been a hallmark of past research. In a factorial experiment scarification treatments and stratification pre-treatment were compared under germination regimes in the laboratory and in a seed bed adjacent to the habitat of *Leucospermum*. The results suggested that prolonged stratification at sub-optimal germination temperature does not play a role in natural germination of *L. cordifolium* but rather that scarification of the outer seed coat layers does. Parity is shown of laboratory and seed bed conditions, paving the way for further laboratory and habitat-simulating studies.

Additive and synergistic effects of seed germination factors

Study 2 poses the question: how can germination be maximized under controlled conditions? Full control has not been achieved and is of both practical and theoretical importance. This is needed to test the working hypotheses developed on germination factors in *Leucospermum*. The additive and synergistic effects of known seed germination factors are not known and require investigation in attempting to maximize germination. This could preferably be done intensively on one species and the results then tested on others.

The list of factors known to promote seed germination in *Leucospermum cordifolium* is considerable. However the effects of these factors have mostly been tested in isolation, whereas in nature the equivalents of such artificial treatments are likely to occur in combination. It was therefore necessary to study possible interaction. It was thought preferable to again compare seed bed and laboratory germination, this time with respect to a new set of variables. The results showed, when viewed against tetrazolium viability test results, that the most successful treatments gave complete germination of viable *L. cordifolium* seeds. The slight advantage of acid scarification found was thought to be due to removal of mechanical constraint of the germinating embryo by the seed coat, resulting in successful germination of some of the weaker embryos.

Techniques for maximal seed germination in six *Leucospermum* species

Having found an effective maximizing germination technique for one species, the question arose of general applicability in the genus. The species chosen for this study, originating from widely different fynbos habitats, were *L. cuneiforme, L. erubescens, L. glabrum, L. reflexum* and *L. vestitum*, representing 5 taxonomical sections in the genus. In this study the most likely stimulative factors were combined in a cumulative series of treatments. The effects of cumulative treatments on germination percentage were additive in most species and also resulted in similar germination rate patterns, suggesting common physiological mechanisms for germination. The promotive effect indicated for "Promalin" (GA_{4+7} + BA) growth regulator on *L. reflexum* and *L. erubescens* germination suggested that these species were incubated under a non-optimal alternating temperature regime. Acid scarification combined with the other treatments gave maximal germination percentages and rates and stimulated a significant proportion of subviable achenes to germinate, as was reflected by tetrazolium viability test results. It appeared therefore that dormancy and germination in *Leucospermum* can be fully controlled. However the true requirements for alternating temperatures remained poorly known for the genus.

Requirements for alternating temperatures

The optimal temperature regime for *Leucospermum cordifolium* seed germination was previously estimated at 8 °C (16 h) x 24 °C (8 h). This regime corresponds reasonably well with ambient soil temperatures in the habitat during the late autumn seed germination season, following fire in the preceding summer. The question was: is alternating temperature requirement a character syndrome (commonly represented) in *Leucospermum*? The same species as above (from contrasting habitats) were used in this study.

The results showed that the requirement for alternating temperatures is indeed common in *Leucospermum*. In some species the requirements were very specific (e.g. *L. glabrum*) whilst in others there was more latitude (e.g. *L. cordifolium*). Species also differed in the temperature levels required and these correlated positively with the climatic regimes characteristic of their habitat.

In conclusion the work reported in this chapter implicitly questions an established assumption in the literature, in relation to seed germination in the fynbos environment. The relevant seed characters in *Leucospermum* are studied, for the first time, in interactive context. The resultant maximal germination responses, checked against viability tests, indicated that complete control is possible. The more important germination characters were quantified. This set the stage for investigation of hormonal control of dormancy and germination induction in relation to environmental requirements.

4.1 THE ROLES OF SCARIFICATION AND STRATIFICATION IN *LEUCOSPERMUM CORDIFOLIUM* SEED GERMINATION

Introduction

The application of "classical" stratification in the germination of Cape Proteaceae species with nut-like achenes has been researched considerably (Brown and Van Staden, 1973a; 1973b; 1975; Mitchell, 1983). By this is meant the procedure of incubating diaspores at sub-optimal low temperature for a prolonged period after which they are transferred to higher "suitable" (Deall and Brown, 1981) temperatures for germination *sensu stricto* (Come and Thevenot, 1982) to commence (Mayer and Poljakoff-Mayber, 1982). The usefulness of the approach of stratification in understanding the role of seed dormancy in South African fynbos species has been questioned. The mediterranean-type climatic regime, to which Cape fynbos ecophysiology has adapted, differs largely from that to which a stratification requirement is an adaptive response (Brits and Van Niekerk, 1986).

Brits (1986b) found that daily alternating moderately high and low temperatures are required for the germination of *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade achenes. However, seasonally alternating temperatures, i.e. average ambient seasonal temperature paralleling sequential prolonged cool or warm periods, have little effect on germination. It was therefore proposed that the low temperature requirement is not a stratification requirement *sensu stricto* and should be distinguished from such (Brits, 1986b).

Brown and Van Staden (1973a; 1973b; 1975) and Mitchell (1983) however, found a slight stimulatory effect of continuous low temperature on the germination of *Leucadendron daphnoides* Meisn., another proteaceous species with nut-like achenes. The question arises whether this is a physiological effect of low temperature *per se* (i.e. dormancy breaking during wet low-temperature after-ripening) or whether it is a physical, possibly oxygen mediated, effect on the embryo.

It has been shown that the *L. cordifolium* achene is strongly deprived of oxygen due to the impermeability of the intact seed coat (Van Staden and Brown, 1973; Brits and Van Niekerk 1976; Chapter 4.3). Circumstantial evidence suggests that natural scarification of the seed coat in the soil could lead to oxygenation, and therefore germination, of the embryo. Sulphuric acid scarification of intact seeds is an effective approximation of the natural process and strongly promotes germination (Brits and Van Niekerk, 1976; Chapter 4.3). Since the role of stratification can possibly be interpreted in terms of oxygenation, both stratification and scarification were investigated.

These two factors have thus far only been studied separately in proteaceous seeds (e.g.. Brown and Van Staden, 1973a; 1975). The extrapolation of such single factor laboratory test results to complex field conditions can be misleading. The effects of field factors are regulated by complex ecophysiological interactions under field, as opposed to laboratory conditions (Vincent and Roberts, 1977; Rebelo and Rourke, 1986; Keeley *et al.*, 1989). For example, of the 7 primary component characteristics in alternating temperature regimes operating in nature (Murdoch *et al.*, 1989) only four are represented in the fixed laboratory temperature regime. However, controlled seed bed germination adjacent to fynbos and within the autumn germinative season was found to be a reasonable simulation of post-fire germination conditions in fynbos (Brits and Van Niekerk, 1986). Thus the seed bed regime includes the optimal alternating temperatures required for *L. cordifolium* germination (Brits, 1986b). Therefore parallel factorial experiments were conducted in the laboratory and the seed bed, to study classical laboratory stratification in relation to the natural fynbos germination regime.

Materials and methods

Scarification

Freshly harvested, hand sorted achenes of *L. cordifolium* were divided into four lots of 800 and scarified as follows:

1. Control: intact seeds.
2. (c) H_2SO_4 treatment for 7,5 min at 22 °C, achenes washed in running water (Brits and Van Niekerk, 1976), soaked in distilled water for 24 h.
3. (c) H_2SO_4 treatment for 15 min as in 1.
4. Achenes soaked in distilled water for 24 h at room temperature, the gelatinous pericarp rubbed off manually. The isolated seeds were disinfected in 1,5% sodium hypochlorite for 10 min, washed in running tap water for 5 min, dried with paper towel and filed through the testa at the micropyle end until the embryo was just exposed.

Stratification

The above seed lots were each divided in half, one half incubated at 6 °C for 21 days and then germinated; and the other half germinated immediately after scarification treatment.

Germination regimes

All treatments were further halved and germinated under either of the following regimes:

1. Control: intact seeds.
2. In petri dishes on filter paper (Brits, 1986b) in an incubator under alternating temperatures of 6 °C for 8 h x 20 °C for 16 h - laboratory regime.

3. In standard exposed seed beds (Vogts, 1976) adjacent to mesic mountain fynbos - seed bed regime. Mechanically scarified seeds when sown were oriented randomly with respect to the scar, since orientating the scar downwards could depress germination (Van Staden and Brown, 1973).

Seeds were germinated in mid-March (autumn); seeds were stratified beforehand and all seeds were germinated simultaneously. The seed bed was not disinfected. Germinated seeds were recorded weekly for 10 weeks.

Statistical procedures

The trial was laid out as a completely randomized design. Four replications of 50 seeds were used per treatment. Radicle emergence in the laboratory or seedling emergence in the seed bed were used as germination criteria. Factorial analysis of variance was performed on angular transformed percentage data for each regime. Shapiro-Wilk's test for non-normality was performed on the data. The F-ratio test for homogeneity of residual variances were performed to test for comparable accuracy between regimes. A combined analysis of variance for the regimes is given in Table 1. Student's t-Least Significant Differences (LSD) at the 5% significant level were calculated to compare means for significant effects.

Table 1. Analysis of variance for laboratory and seed bed regimes combined

Source	d.f.	SS	MS	F Value	Pr > F
Regime	1	38,52	38,52	2,09	0,1568
Error(a)	6	71,13	11,86	0,64	0,6942
Stratification	1	49,92	49,92	2,71	0,1085
Scarification	3	1103,04	367,68	19,98	0,0001
Strat*scar	3	244,02	81,34	4,42	0,0098
Regime*strat	1	14,47	14,47	0,79	0,3812
Regime*scar	3	76,16	25,39	1,38	0,2651
Regime*strat*scar	3	144,76	48,25	2,62	0,0659
Error(b)	35	644,03	18,40		
Corrected Total	56	2386,06			

Abbreviations:
SS = Sum of squares
MS = Mean square
d.f. = Degrees of freedom
Pr = Probability

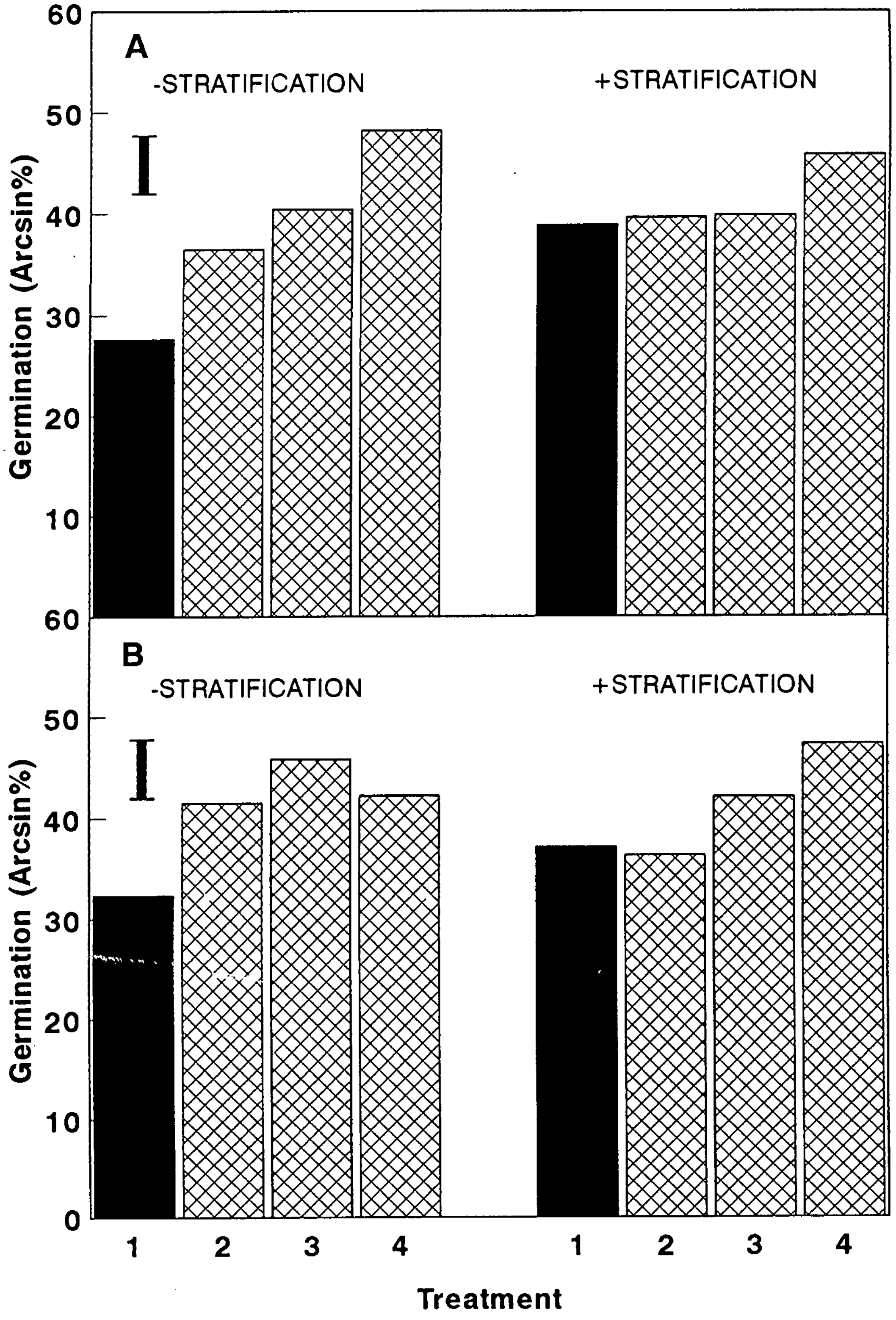

Figure 1. The influence of stratification and scarification treatments on the germination response of *Leucospermum cordifolium* achenes, incubated under laboratory (A) and seed bed (B) regimes. Scarification treatments were 1: control (solid bar); 2: 7,5 min sulphuric acid treatment; 3: 15 min sulphuric acid treatment; and 4: mechanical scarification. Small bars represent LSD (P=0,05)

Results

The data showed no evidence against normality. The test for homogeneity of error variances indicated no significant heterogeneity between regimes (P = 0,22) and the combined analysis (Table 1) is therefore valid. Regime x treatments interactions were not significant (Table 1). Germination regime means (laboratory 39,3%, seed bed 41,0%) did not differ significantly (Table 1). Scarification treatments differed highly significantly and probably contributed to the significant interaction with stratification (Table 1; Figure 1).

A positive relationship is evident between degree of scarification and germination response (Figure 1). In non-stratified seeds, controls (intact seeds) germinated markedly poorer than scarified seeds, the differences being statistically significant. However, when seeds were stratified, controls performed comparatively well, differing significantly from only the strongest (mechanical) scarification treatment (no. 4). Thus the scarification patterns (treatments 2-4) of stratified and non-stratified seeds were reasonably similar. The results differed mainly in the stratified control, which germinated at a comparable level to the weakest scarification treatment (7,5 min - Figure 1).

Discussion

The relatively small differences found between germination regimes, without significant interaction, indicate that laboratory germination resembled seed bed germination reasonably closely. Therefore, the most important requirements for natural seed germination can be assumed to be satisfied in the laboratory regime.

The response levels and upward trends for increasing scarification treatments 2-4 in stratified and non-stratified seeds (Figure 1) were roughly similar. This indicates that stratification *per se* did not influence germination response when seeds were partly oxygenated through scarification. Moreover, 6 °C stratification and 6 °C lower alternating incubation temperature exposed seeds to the same low temperature level. The fact that 6 °C had no autonomous effect supports the conclusion that low temperature here regulated germination *sensu stricto* only as part of an alternating temperature regime (Brits, 1986b).

If stratification *per se* in the controls did not act as a fundamental physiologically promotive principle, it could have had a secondary effect in intact seeds, such as oxygenation. Van Staden and Brown (1977) proposed that stratification led to a more effective absorption of gaseous oxygen by embryos, due to the higher solubility of oxygen at low temperature (Come and Tissaoui, 1973). This could have promoted germination up to the level of the weakest scarification treatment effect (7,5 min). The statistically significant, albeit modest, promotive effect was probably masked as seeds became

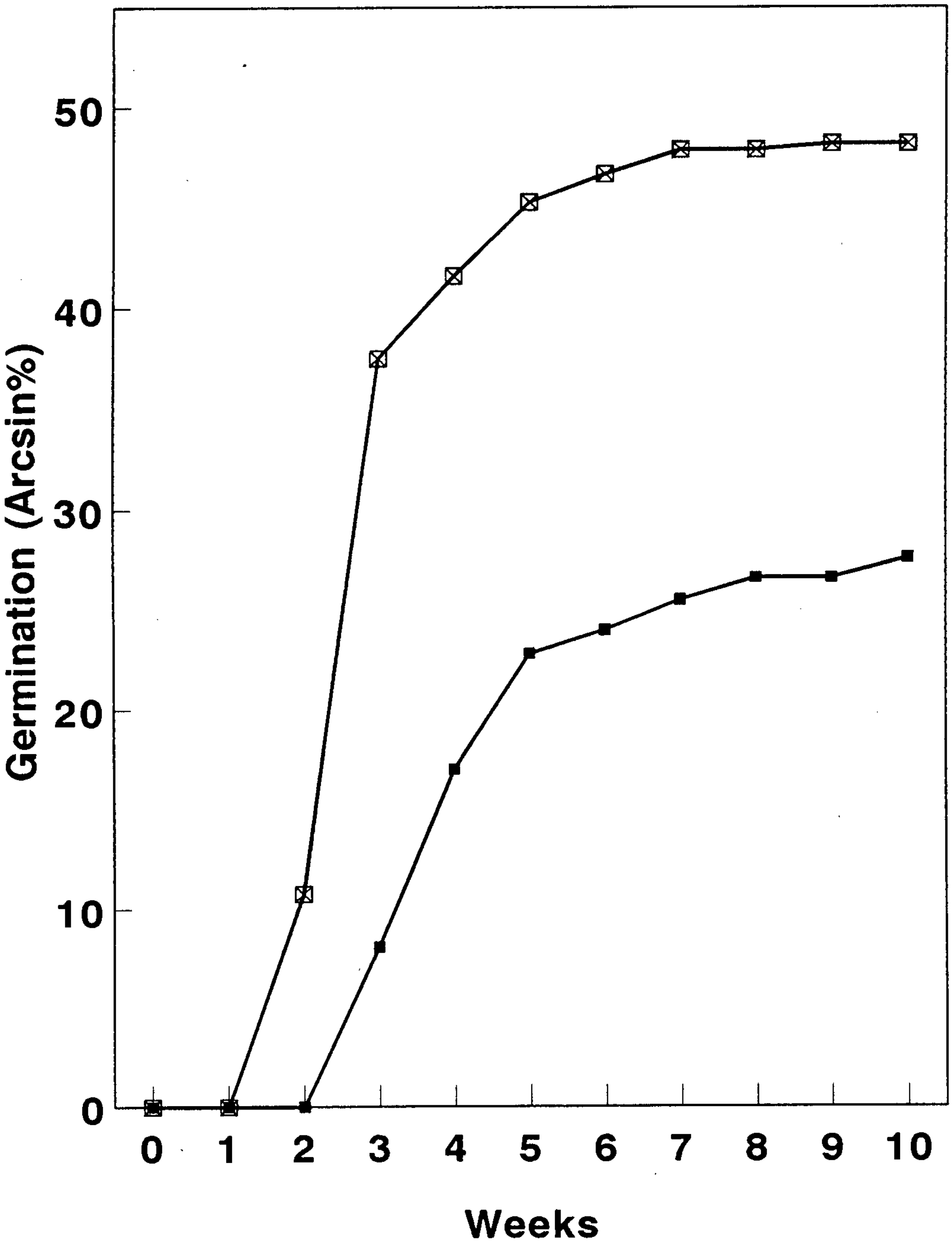

Figure 2. Germination patterns of non-stratified seeds incubated in the laboratory: —■— intact seeds, —⊠— mechanically scarified seeds.

increasingly oxygenated as a result of more effective scarification treatments (Figure 1).

Acid scarification for 7,5 min does not effect complete oxygenation of embryos, indicated by further stimulation of germination when additionally soaked in H_2O_2 (Brits and Van Niekerk, 1976). On the other hand mechanically scarified achenes of *L. cordifolium* (embryo exposed to air - Van Staden and Brown, 1973) and *L. daphnoides* (Brown and Van Staden 1973c) germinated vigorously and to a similar extent to excised embryos or achenes incubated in pure oxygen. This indicated that mechanical scarification led to maximal oxygenation in proteaceous nut-fruited seeds. The graded effect here of increasing scarification treatment, also found by Van Staden and Brown (1973) can therefore be attributed to increasing degrees of oxygenation of the embryo. This emphasizes the strong impermeability of the intact testa to gaseous oxygen. It suggests that germinating embryos could be sensitive to small variations in oxygen partial pressure occurring in the soil during natural germination. The intact testa is however fully permeable to water (Brown *et al.*, 1986).

In nature the oxygen-impermeable *Leucospermum* testa is gradually scarified in the ground by microflora (Chapter 6.4). Natural scarification is also regulated by specialised testa desiccation mechanisms (Chapter 6.2). These processes occur at different rates in seeds (seed-to-seed variation - Murdoch and Ellis, 1992; Chapter 7). Thus the long-living, dormant embryos when imbibing water during winter rainy spells become increasingly oxygenated during longterm soil-storage as well as in the seed bed. Assuming an oxygenation threshold level requirement for induction of germination *sensu stricto*, the graded scarification of seeds in fynbos predicts an erratic germination pattern (polymorphic germination - Cavers and Harper, 1966; Keeley, 1991). Intact seeds are indeed known to germinate irregularly (Figure 2) and in the seed bed this process can extend over several germination seasons (Chapter 6.4). The prevention of simultaneous germination of the entire seed bank can have survival value in unpredictable environments (Cavers, 1983; Gutterman, 1993). Dry spells occur episodically during the winter germinative season in fynbos (Brits, 1987).

The results therefore indicate a positive role for scarification in natural germination of *L. cordifolium* but not for stratification; differential scarification of seeds can be linked to the ecological function. Thus the stimulating effect of stratification was presumably not due to key endogenous processes during the period of cold storage but to a secondary, physical effect on oxygen availability. It highlights a fundamental ecophysiological difference between the requirements for pre-germination stratification and mild low temperature during germination. Stratification is a form of after-ripening which adapts seeds to hostile winters, i.e. to conditions unfavourable for germination (Mayer and Poljakoff-Mayber, 1982). This has been studied extensively in classical physiology of northern-hemisphere taxa such as Rosaceae species. However, fynbos ecophysiology does not implicate a function for stratification as experienced in the severely cold northern hemisphere regions. Fynbos experiences a temperate climate (excepting high altitude fynbos). Night temperatures often drop to 6 °C during the winter germination season and this is relatively close to the 9 °C level optimal for *L. cordifolium* germination (Brits, 1986b). These mild low temperatures are linked to high moisture availability during the winter season (Brits and Van Niekerk, 1986). Thus a stratification requirement *sensu stricto* and the mild low temperature requirement in *L. cordifolium* are adaptive responses to opposing germination principles, unfavourable and favourable, of contrasting habitats.

4.2 EFFECTS OF COMBINED OXYGENATION TREATMENTS, ACID SCARIFICATION AND GROWTH REGULATOR TREATMENT ON THE GERMINATION OF *LEUCOSPERMUM CORDIFOLIUM* (Salisb. ex Knight) Fourcade SEED UNDER SEED BED AND LABORATORY CONDITIONS

Introduction

This study is the first of two attempts to gain full control over germination in *Leucospermum*. First a variety of treatment combinations are tested on the "standard" species, *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade. This species also broadly represents fynbos Proteaceae with "difficult" seed germination. In seed biological terms the South African members of the Proteaceae are divided into two classes, species producing achenes ("seeds") which are either serotinous or nut-like (Brits, 1986a). Nut-like achenes, such as in *Leucospermum* R.Br., have been known to germinate with difficulty under artificial conditions since the first attempts were made to cultivate these plants from seed (Knight, 1809).

Many solutions to the problem have been proposed, from seed sowing during the natural winter germination season (Vogts, 1959) to improved sorting of seeds (Horn, 1962). The problem of germination percentages as low as 10% is acute in a modern breeding programme with a species such as *L. cordifolium* (Brits and Van den Berg, 1990). In this species poor seed germination is compounded by the effect of a low natural seed set, which is often below 10% of pollinated florets on each flowerhead. This means that, even in compatible crosses, seedlings of less than 1% of artificially pollinated florets are regularly obtained.

The complex dormancy mechanisms of *Leucospermum* seeds are adaptive, functional responses to some of the severe stress and disturbance factors operating in nature (Kelly *et al.*, 1992; Chapter 6.2). Considerable research has been devoted to the germination physiology of nut-like seeds (reviewed by Van Staden and Brown, 1977). The main dormancy mechanisms identified in *L. cordifolium*, and methods known to break them, are as follows:

1. The intact seed coat is readily permeable to water (Brown *et al.*, 1986) but poorly permeable to oxygen (Van Staden and Brown, 1973). Seeds must therefore be soaked in 1% H_2O_2 before commercial sowing in seed beds (Brits and Van Niekerk, 1976; Brits, 1986a) or, in the laboratory, they may be incubated in oxygen (Van Staden and Brown, 1973).
2. Seed germination is strongly dependent on seasonal low temperature. Seeds must consequently be sown commercially in seed beds during autumn or early winter (Vogts,

1976; Brits and Van Niekerk, 1986). Alternatively, in the laboratory they may be incubated at an optimum low temperature of 8 or 9 °C (Brits, 1986b).

3. High temperature is also required for germination and must be alternated with low temperature on a daily basis (Brits, 1986b). Commercial seed beds must therefore be constructed in open sunshine (Vogts, 1976). In the laboratory the optimum high temperature of 24 °C must be maintained for 8 h per day followed by a period of low night temperature (16 h) as mentioned above (Brits, 1986b).

Poor germination in *L. cordifolium* is only partly due to dormancy. A considerable proportion of seeds has a low germinability (Van Staden and Brown, 1973), which is usually reflected in a large proportion of floating seeds found when a seed batch is placed in water. The majority of the so-called "floater" seeds, although they often appear normal, contain embryos which are either under-developed or which have been completely aborted (Van Staden and Brown, 1973).

Experiment 1: Maximising of germination percentage

In the quest to maximise germination percentage the following problems predominate:

1. Is germination in the laboratory just as effective as germination under natural, winter, seed bed conditions?
2. Can the massive fungal growth normally accompanying laboratory germination in petri dishes, and which leads to variable results (Brits, 1986b) be controlled, since seed borne pathogens have been found on proteaceous seeds (Benic, 1986).
3. Is oxygenation with O_2 gas just as effective as H_2O_2 oxygenation?
4. Cytokinin is regarded as a primary hormone involved in germination (Van Staden and Brown, 1977). Can treatment with synthetic cytokinin (6-benzyladenine) augment germination in addition to the effects of supplying the optimal environmental conditions required?

Germination factors have been investigated as single factors in many experiments (e.g. Van Staden and Brown, 1977; Brown *et al*, 1986) with a resulting paucity of factorial studies which could yield more information, e.g. on the synergistic action of factors (Vincent and Roberts, 1977). The optimal treatment levels and conditions had already been established in some of these experiments. A factorial experiment involving the above factors was therefore conducted (Table 1).

Methods

Fresh, hand sorted, intact seeds of *L. cordifolium* were disinfected in hot water at 50 °C for 30 min. (Benic, 1986). They were soaked for 24 h in 1% H_2O_2 (Brits and Van Niekerk, 1976) or in a 200 $mg.l^{-1}$ 6-benzyladenine (BA) solution (Mitchell, Van Staden and Brown, 1986), or in a combination of H_2O_2 and BA. Following soaking the soft, gelatinous pericarps were rubbed off, seeds were washed briefly in running water and allowed to dry off until the seed coat contained no free water. Seeds were then treated with thiram wettable powder (75% active ingredient) at a concentration of 0,05 g thiram powder per 100 seeds (Benic, 1986). Seeds not soaked in H_2O_2 were incubated in medical grade oxygen (Van Staden and Brown, 1973) in 1 l flasks with screw-on lids sealed with petroleum jelly, under the optimum temperature regime (Brits, 1986b;) flasks were flushed with oxygen twice per week. H_2O_2 treated and control seeds were either incubated in petri dishes under the optimum temperature regime in the laboratory (Brits, 1986b) or were sown in a standard disinfected seed bed (Vogts, 1976) in mid-March (early autumn). Six replications per treatment with 33 seeds per replicate were allocated in both the laboratory and seed bed studies, laid out as completely randomized block designs. Radicle emergence in the laboratory or seedling emergence in the seed bed were used as germination criteria. Germination was recorded weekly for 20 weeks and analysis of variance was performed on percentage data. Orthogonal contrasts were calculated to compare groups of means.

Table 1. Experiment 1: treatment numbers in a factorial germination trial with *L. cordifolium* seeds

Incubation regime	Exogenous cytokinin	Seeds oxygenated		Seeds intact: non-oxygenated	
		O_2	H_2O_2	Non-disinfected	Disinfected
Laboratory	BA	1	3	-	6
	Non-BA	2	4	5	7
Seed bed	BA	-	8	-	11
	Non-BA	-	9	10	12

The tetrazolium viability test of Brits and Van Niekerk (1976) was modified by scoring the degree of colouring of cotyledons on a scale of 0 (no colour/embryonic axis not coloured) to 10 (cotyledons and embryonic axis fully coloured).

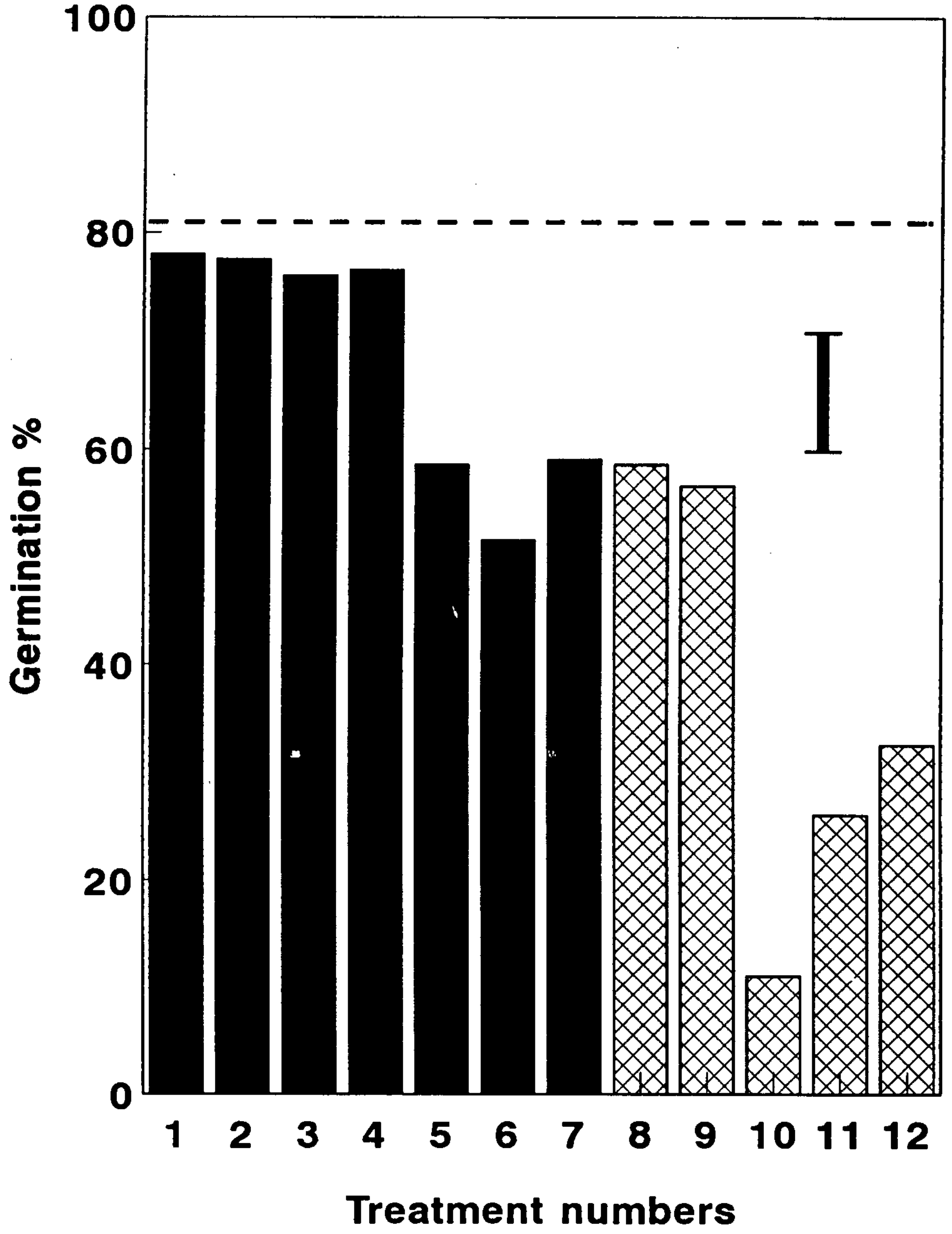

Figure 1. Mean germination percentage for *L. cordifolium* seeds following 12 treatments (see Table 1); small bar represents LSD (P=0,05). --- percentage embryos with good colouring in tetrazolium viability test. Solid bars, laboratory regime; cross-hatched bars, seed bed regime.

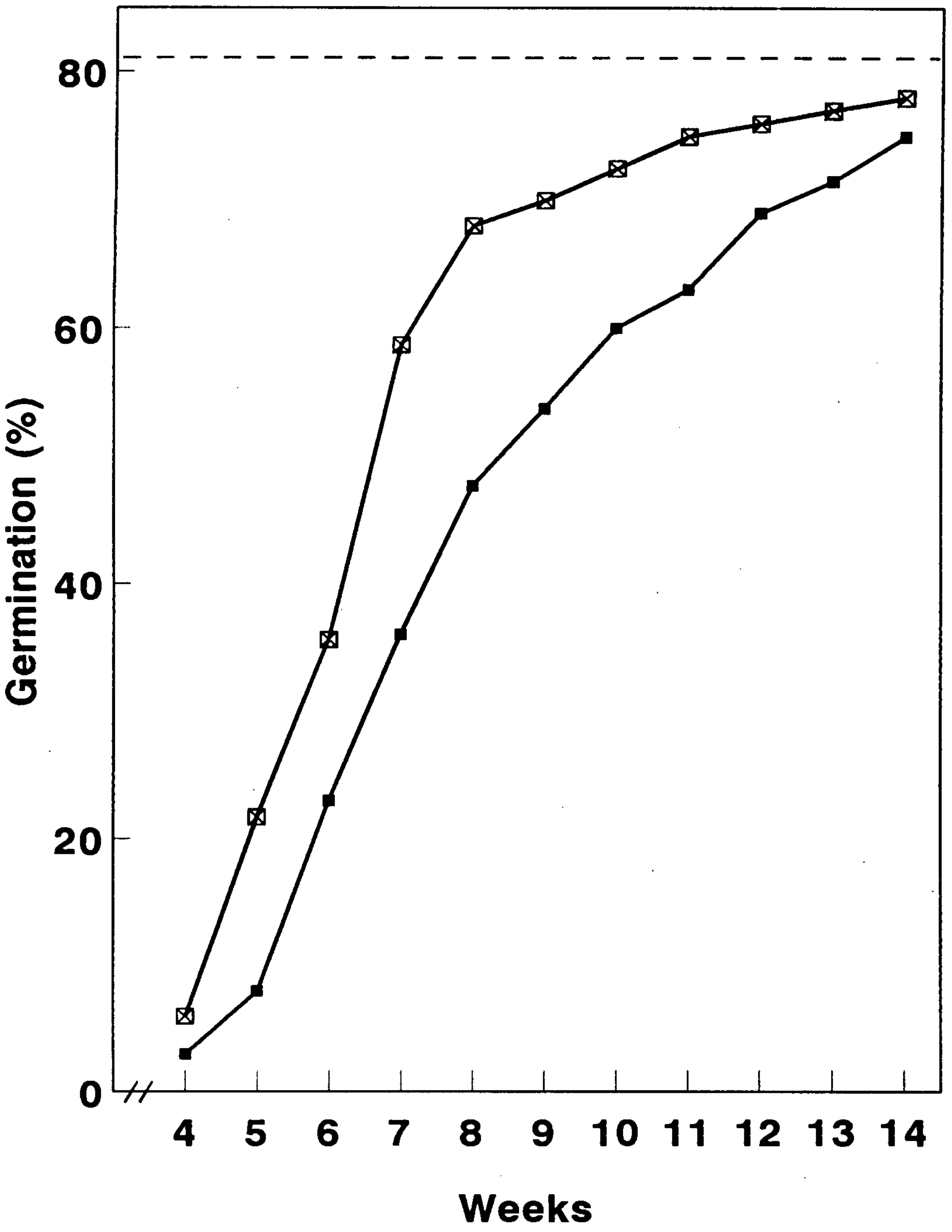

Figure 2. Average germination rate for *L. cordifolium* seeds incubated in oxygen (⊞——⊞) (treatments 1 and 2); or pre-treated by soaking in 1% H_2O_2 (■——■) (treatments 3 and 4). ---- % embryos with good tetrazolium colouring.

Results and discussion

Germination results are presented in Figure 1. Seed viability gave a high score of 81% ± 4% embryos with good colouring (more than three quarters of the cotyledon including the embryo axis coloured); 9% ± 3% with intermediate colouring and 10% ± 4% with poor (less than half of the cotyledon coloured) or no colouring. It was assumed that only embryos with good colouring were highly germinable.

Laboratory vs. seed bed (Treatments 3 vs. 8; 4 vs. 9; 5 vs. 10; 6 vs. 11; 7 vs. 12): In the main treatments seeds germinated much better under laboratory conditions than in the seed bed ($P<0{,}001$). Unfavourable temperature variations may have induced continued or secondary seed dormancy in the seed bed, as was suggested by a second wave of germination observed in this seed bed in the following winter. In contrast, the high germination percentages obtained in the laboratory approached complete germination of viable seeds, as is clear from a comparison with the viability estimate. Although the criteria for germination differed, favouring laboratory germination performance, preemergence losses can clearly be controlled more effectively in the laboratory, especially in the case of weaker embryos.

Disinfection vs. controls (Treatments 5 vs. 7; 10 vs. 12). In petri dishes in the laboratory (5 vs. 7) disinfection had no effect on germination. Untreated seeds were nevertheless covered with thick fungal growth, in contrast with a clean appearance of disinfected seeds. However in the seed bed (10 vs. 12) disinfection significantly improved germination ($P<0{,}01$). Disinfection could therefore be regarded as the preferable treatment in both cases.

Oxygenation (O_2 and H_2O_2) vs. controls [Treatments (1, 2, 3, 4) vs. (5, 6, 7); (8, 9) vs (10, 11, 12)]. The effect of oxygenation was significant in all comparisons ($P<0{,}001$) and confirms previous results (e.g. Van Staden and Brown, 1977; Brits and Van Niekerk, 1986).

O_2 gas vs H_2O_2 [Treatments (1, 2) vs. (3, 4)]. No significant differences were obtained, in the laboratory, with incubation in oxygen compared with H_2O_2 treatment ($P=0{,}70$). However, the rate of germination of the former was higher (Figure 2) indicating that incubation in oxygen may favour embryos in which germinability is reduced (i.e. with low germination energy). Incubation in oxygen is therefore preferable.

BA vs control (Treatment 1 vs. 2; 3 vs. 4; 6 vs. 7; 8 vs. 9; 11 vs. 12). Soaking in BA clearly had no effect on germination percentage ($P<0{,}10$). This suggests that endogenous cytokinin levels did not limit germination.

This experiment showed that germination in the laboratory under controlled temperature conditions was more favourable than under ambient climatic conditions in the seed bed. Oxygen treatment of the seed resulted in a higher percentage of germination and oxygen gas treatment versus hydrogen peroxide did not differ statistically. The results also indicate that BA had no favourable effect on germination.

Experiment 2: Refinement of germination technique

Since seeds did not react to exogenous cytokinin application, gibberellins could have been the other group of plant growth regulators limiting germination. Gibberellins are also involved in *Leucospermum cordifolium* seed germination (Brown *et al*, 1986). The growth regulator "Promalin" contains the gibberellins GA4, GA7 as well as benzyladenine, and has been used to stimulate the germination of proteaceous seeds (e.g. Brown *et al*, 1986).

The above results also suggested that all highly germinable seeds had germinated, but that marginally weaker embryos, indicated by the seed proportion with intermediate tetrazolium colouring (9%) did not germinate. Such sub-viable embryos may be constrained by the hard, thick seed coat which does not play a role in seeds with a high germinability (Brits and Van Niekerk, 1986). It is known that H_2SO_4 softens the seed coat and at the same time renders it more permeable to oxygen (Brits and Van Niekerk, 1976).

Table 2. Experiment 2: treatment numbers in a factorial germination trial with *L. cordifolium* seeds

Exogenous gibberellin + cytokinin	Scarification	Oxygenation	
		Air	O_2
Non-Promalin	Non-H_2SO_4	1	4
	H_2SO_4	2	6
Promalin	Non-H_2SO_4	3	7
	H_2SO_4	5	8

Methods

Three factors were combined in a factorial design with eight treatment combinations, each factor being tested only at the optimum and the control levels (Table 2). The optimum level for each factor had been established previously.

Seeds of a different *L. cordifolium* seed source were scarified in (c) H_2SO_4 for 8 minutes and washed (Brits and Van Niekerk, 1976). These as well as untreated control seeds were disinfected in hot water at 50 °C for 30 minutes (Benic, 1986). Seeds were then soaked for 24 h in either a 200 $mg.l^{-1}$ "Promalin" solution (Brown *et al*, 1986) or in oxygenated distilled water. Following soaking all seeds were cleaned by rubbing off the remains of the soft, gelatinous pericarp and by washing briefly in running water (Brits and Van Niekerk, 1976). Seeds were allowed to dry off and were then treated with thiram w.p. as in Experiment 1. Seeds were incubated in oxygen in 1 l flasks or in air in petri dishes, under the optimal temperature regime, as in Experiment 1. Eight replications per treatment combination with 25 seeds per replicate were used in a completely randomized design. Germination was recorded weekly as the number of seeds with newly emerged radicles, for 20 weeks. Germination percentages and rates were calculated, the latter by means of the formula of Heydecker (1973):

$$\text{Rate of germination} = \frac{\sum_{i=1}^{k} ni}{\sum_{i=1}^{k} Di.ni} .100$$

where k = final week of germination
Di = week of recording
ni = number of seeds germinated in week Di
i = week 1 to week k

Germination percentage and rate data were subjected to analysis of variance and orthogonal contrasts were calculated to compare groups of means.

Results and discussion

Oxygenation (Treatments 4, 6, 7, 8 vs. 1, 2, 3, 5). The results are presented in Figure 3. Incubation in oxygen significantly improved both germination percentage ($P < 0,001$) and germination rate ($P < 0,001$).

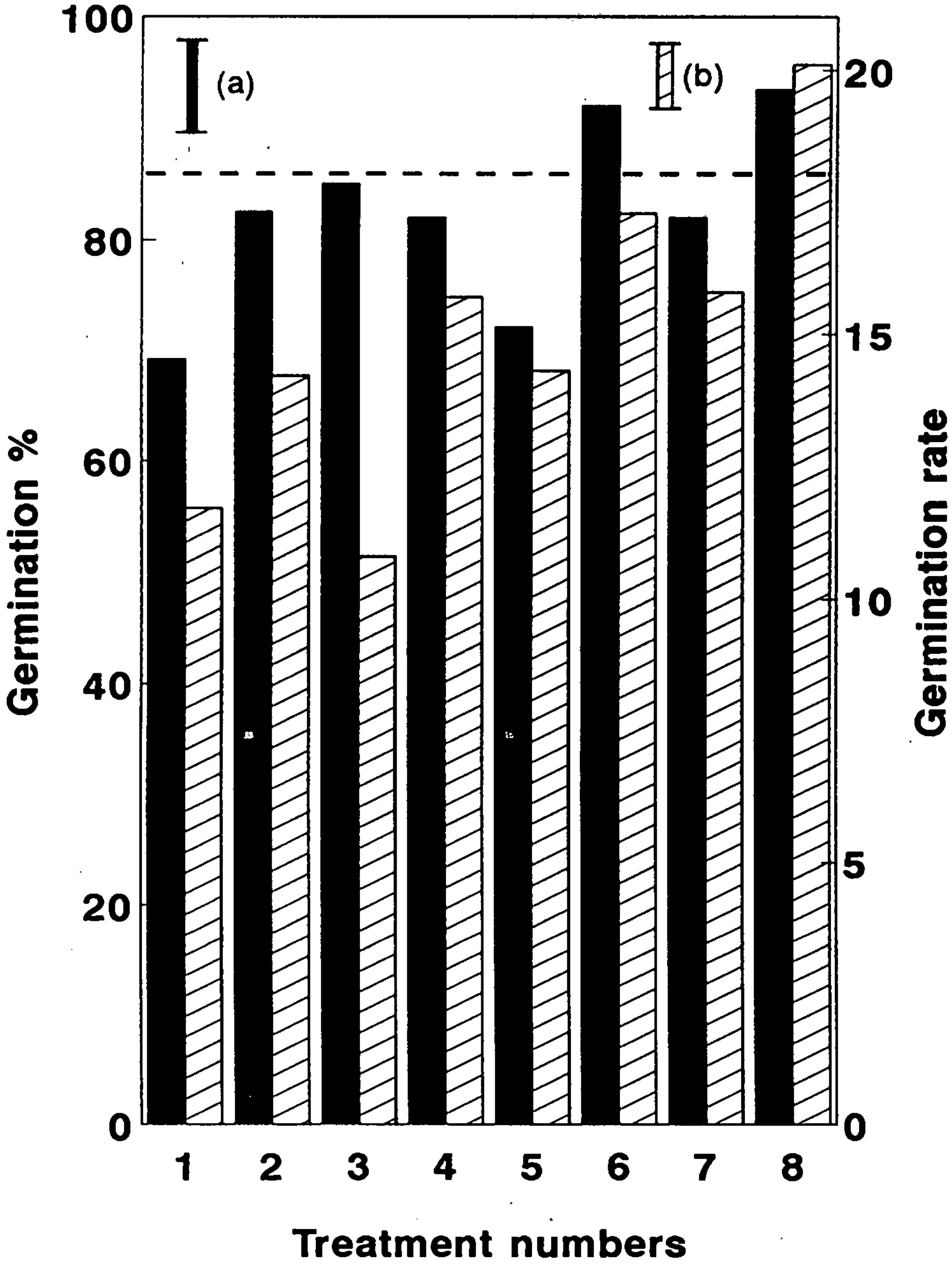

Figure 3. Mean seed germination percentage (■) and germination rate (▨) for *L. cordifolium* seeds following 8 treatments (see Table 2); small bars represent LSD (P = 0,05) for germination percentage (a) and rate (b). --- estimated percentage of seeds with high germinability.

H_2SO_4 scarification (Treatments 2, 5, 6, 8 vs. 1, 3, 4, 7). Scarification improved germination percentage in three of four ($P<0,01$); treatments; the interaction provided by treatments 3 and 5 can not be explained. The germination rate was improved significantly in all scarification treatments ($P<0,0001$). The fact that scarification improved germination percentage and rate despite the presence of oxygen at a presumably supra-optimal partial pressure (4 vs. 6 and 7 vs. 8; $P<0,001$) indicates that the seed coat restricted some embryos mechanically.

Promalin treatment (Treatments 3, 5, 7, 8 vs. 1, 2, 4, 6). Promalin improved germination percentage only where the oxygen partial pressure was sub-optimal (1 vs. 3), on a par with oxygen incubation alone (treatment 4). This suggests that "Promalin" provided an essential hormone(s) which cannot be synthesized with the impermeable seed coat present. Since benzyladenine (cytokinin) did not influence germination in the first experiment, it appears that gibberellin may be this hormone (Brown *et al*, 1986). The fact that incubation in oxygen with or without Promalin gave the same germination percentage and rate (4 vs. 7) indicates that high oxygen partial pressure cancelled the effect of the applied gibberellins in Promalin. It is therefore likely that gibberellin synthesis is dependent on elevation of the oxygen partial pressure in intact seeds. The fact that "Promalin" in combination with acid scarification and oxygen incubation (treatment 8) gave the highest germination rate as well as the highest combined score, indicates that these three factors acted additively in stimulating some marginally weak embryos to germinate. A tetrazolium test conducted in this experiment was unsuccessful; however, a comparison of similar teetrazolium treatments in this and the first experiment suggests that the seeds with a high germination energy could have totalled approximately 86%. The increased germination percentages of over 90% as well as the increased germination rates of treatment 6 and especially treatment 8 therefore suggests that sub-viable seeds (with a marginally low germination energy) were also germinated here.

It is clear that "Promalin", and especially the gibberellins in this mixture, in combination with acid scarification and oxygen incubation gave the highest germination percentage and rate. The results also indicate that Promalin i.e. the gibberellins in it, could substitute for the oxygen treatment. It seems that acid scarification can lead to increased germination in cases where seed coats restrict development of the germinating embryo.

Conclusions

Acid scarification normally increases the permeability of the seed coat to oxygen and thus improves germination. The above experiments indicate that scarification can also cause hard seed coats to split more easily and thus further increase germination percentage. This would especially be the case with poorly developed embryos.

The results also confirm that a low night (16 h) temperature and a relatively high day temperature are required for maximal germination. Since such conditions can more readily be regulated in the laboratory, improved germination is obtained compared with sowing in open seed beds.

Disinfection of seeds significantly improved germination and emergence of seedlings in open seed beds. Although not significant under controlled laboratory conditions, it resulted in far less contamination of seeds.

Results also showed that BA had no beneficial effect on germination; but Promalin, especially the GA in it, improved germination. This was especially the case in the absence of oxygen treatment.

Comparison with viability estimates indicates that all viable and even sub-viable *L. cordifolium* seeds can be germinated by disinfection and by acid scarification of the seed followed by soaking in Promalin and incubation under relatively low night and high day temperatures in oxygen. It can therefore be concluded that all requirements for dormancy breaking and germination were met in these seeds.

4.3 TECHNIQUES FOR MAXIMAL SEED GERMINATION IN *LEUCOSPERMUM* R.BR.

Introduction

Having maximized control over germination in *Leucospermum cordifolium* (Chapter 4.2) this study aims to broaden control to other species in the genus. In the study of *Leucospermum* R.Br. ecophysiology it is essential to develop a technique to fully control seed germination. This is required for testing the working hypotheses developed on the factors controlling the germination process.

Germination of the strongly dormant achenes of *L. cordifolium* was shown to be influenced by a variety of factors. These factors have often been investigated separately and few if any single treatments have succeeded in breaking dormancy completely (e.g. in Brown *et al.*, 1986). In the laboratory a combination of treatments were found to maximize seed germination percentage and rate in *L. cordifolium* (Chapter 4.2). Acid scarification, Promalin treatment and oxygen incubation, used in a factorial design, acted cumulatively in maximizing germination response.

The six *Leucospermum* species used in the present study originate from widely different habitats. Limited resources precluded the use of all combinations in a factorial trial with six species. Consequently the hypothesis was tested that oxygen incubation, Promalin soaking and acid scarification will act cumulatively where seeds are incubated under an assumed non-optimal temperature regime.

Materials and methods

The species used are given in Figure 1. *L. cordifolium*, of which the temperature requirements are known, was included as a control species. The achenes of *L. cuneiforme* and *L. vestitum* were harvested from unknown sources under non- controlled conditions and donated for research purposes. Achenes/seeds of the other species were grown at Tygerhoek Experimental Farm and harvested under controlled conditions after natural release in December.

Fresh, hand sorted, intact achenes of six *Leucospermum* species were scarified in (c) H_2SO_4 at 22 °C for 8 minutes and washed (Brits and Van Niekerk, 1976). These as well as untreated achenes were disinfected in hot water at 50 °C for 30 minutes (Benic, 1986).

Achenes were then soaked for 24 h in either a 200 $mg.l^{-1}$ "Promalin" solution (Brown *et al.*, 1986) or in distilled water. Following soaking, the seeds proper were

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Adapted from Brits (1990).

isolated and cleaned by rubbing away the remains of the soft, gelatinous pericarp and by washing briefly in running water (Brits and Van Niekerk, 1976). Seeds were allowed to dry off until the seed coat contained no free water and then shaken up with thiram (75% a.i.) w.p. fungicide at a concentration of 0,05 g thiram powder per 100 seeds (Benic, 1986). Seeds were placed on a single layer of damp filter paper on a 10 mm sterilized sand bed within 1 l flasks. Seeds were incubated either in air or in medical grade oxygen (Van Staden and Brown, 1973) under the optimum temperature regime for *L. cordifolium* (Brits, 1986b). Control flasks (air) were covered with loose glass lids whilst oxygen receiving flasks were closed airtight with screw-on lids sealed with petroleum jelly; flasks were flushed with oxygen twice per week. The cumulative series of seed treatments were thus:

1) Control: incubation in air
2) Incubation in oxygen
3) Oxygen plus Promalin presoaking
4) Oxygen plus Promalin plus acid scarification

Six replications of 25 seeds per species per treatment were used in a completely randomized experimental design. Germination was recorded weekly as the number of seeds with newly emerged radicles, for 20 weeks. Germination percentages and rates were calculated, the latter by means of the formula of Heydecker (1973).

The results were subjected to analysis of variance. Viability tests were conducted on 6 replicates of 25 embryos per species. The tetrazolium test of Brits and Van Niekerk (1976) was modified by scoring the degree of colouring of cotyledons on a scale of 0 (no colour/ embryonic axis not coloured) to 10 (cotyledons and embryonic axis fully coloured). In addition the percentage seeds sinking in water was determined in samples of 200 seeds per species, following sulphuric acid scarification and washing.

Results

In most of the 18 cases covering six species, cumulative treatments gave step-wise increases in germination percentage (Figure 1). In the three exceptions the difference between treatments were small and not statistically significant. Germination rate gave a characteristic response pattern to treatments (Figure 1). In treatments 2 and 3 the rates were slightly lower than the control treatment for most of the species. In the case of treatment 4, however, a significant increase in germination rate was found in all six species.

Analysis of variance of germination percentage gave a highly significant interaction ($P<0,001$) for treatments x species. This can be seen, for example, in the different response patterns of *L. cuneiforme* and *L. reflexum* (Figure 1). Inspection of data revealed three patterns of germination response to treatments, relative to the overall germination of species:

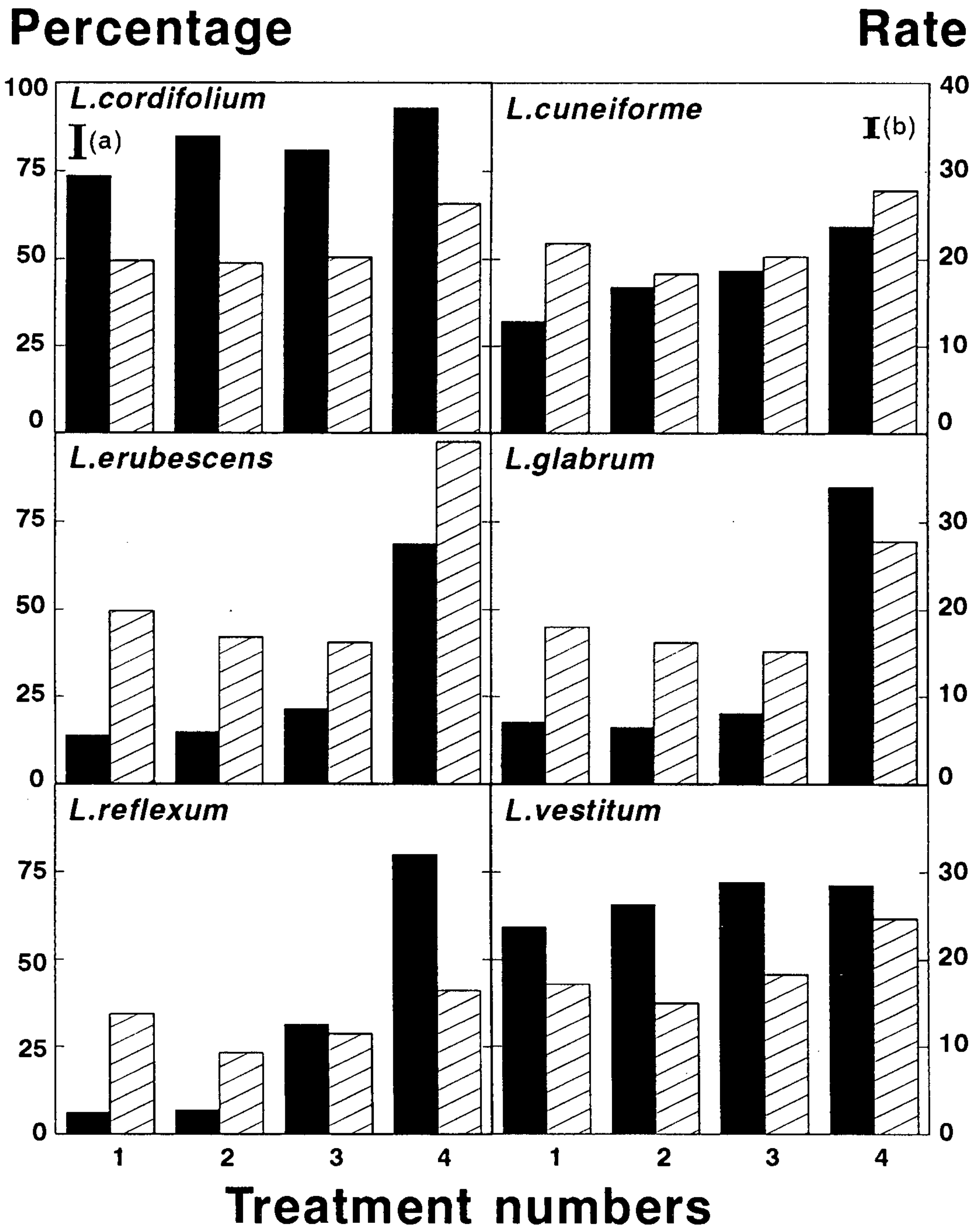

Figure 1. Mean seed germination percentage (■) and germination rate (▨) for six *Leucospermum* species following four treatments: 1, incubation in air; 2, incubation in oxygen; 3, oxygen incubation plus presoaking in Promalin; oxygen plus Promalin plus acid scarification; small bars represent LSD (P = 0,05) for germination percentage (a) and rate (b).

Table 1. Average percentage seed germination following treatment with 4 methods, for 3 *Leucospermum* groups; probability values (P) are given for comparisons of adjacent treatment means, using one-tailed t-tests. Seed viability was estimated by means of a tetrazolium test; sinker seeds denote percentage sinking in water (see Materials and Methods).

		Treatments								
Group	Species	Control %	P	Oxygen %	P	O_2 + Promalin %	P	O_2 + Prom. + H_2SO_4 %	Viability	Sinkers
A	*L. cordifolium*	73,6	0,08	84,7	$>0{,}5$	80,8	$<0{,}001$	92,7	94	90
B	*L. cuneiforme*, *L. vestitum*	45,7	0,02	53,8	0,08	59,3	0,07	65,2	56	55
C	*L. erubescens*, *L. glabrum*, *L. reflexum*	12,4	1,0	12,4	$<0{,}001$	24,2	$<0{,}001$	77,8	82	84

A: *L. cordifolium.* Relatively very high control value and very high maximal germination value with relatively little difference between treatments.

B: *L. cuneiforme*, *L. vestitum.* Moderately high control values and relatively low maximal values with little difference between treatments.

C: *L. erubescens*, *L. glabrum*, *L. reflexum.* Relatively very low control and oxygen treatment effects coupled with substantial reaction to Promalin and with relatively very high maximal values i.e. with very strong reaction to acid scarification.

Discussion

The fact that oxygen alone and in combination with Promalin gave, in most cases, a lower germination rate than controls, cannot be explained. The reduced germination rate in these treatments suggests physiological stress. This could have been due to oxygen toxicity, as was suggested for *Leucadendron tinctum* achenes germinated in pure oxygen (Brown and Dix, 1985). However in treatment 4, in which the seed coat permeability to oxygen was strongly increased by scarification, embryos would have been subjected to a higher oxygen partial pressure than in the case of treatments 2 and 3, contradicting this hypothesis.

The similar germination percentage patterns, in which germination percentage increased with cumulative treatments, as well as the similar germination rate patterns, suggest that the physiological processes involved in seed germination are common in *Leucospermum* species. Treatment 4 thus apparently provides a maximal seed germination technique for *Leucospermum*. That maximal germination was indeed realized is supported by the close correlation between viability estimates, percentage sinker seeds and treatment 4 values (Table 1).

In freshly harvested seed batches the viability and sinker percentage of seeds are apparently closely correlated (Table 1).

The high treatments x species interaction (different response trends within species groups) can possibly be explained by variable seed coat permeabilities to oxygen of groups (Table 1):

A: In *L. cordifolium* the seed coat would have been fully developed as was indicated by both the high viability and percentage sinker estimates as well as by the maximal germination obtained of almost 93% (Table 1). The present *L. cordifolium* population is semi-domesticated i.e. it has been cultivated as cut flowers from seed for at least 10 generations. Seed dormancy is genetically variable (Roberts, 1972) and artificial selection for a more permeable seed coat could therefore have occurred. This could explain the high germination percentage in control seeds. A high control germination percentage for this seed source, harvested in a different year, was also found in an earlier experiment (Chapter 4.2). In addition incubation of *L. cordifolium* seeds under its optimum germination temperature regime would have contributed to a relatively high control value.

B: *L. cuneiforme*, *L. vestitum*. A similar response pattern as was found in *L. cordifolium*, but at a lower level (Table 1) likewise suggests relatively permeable seed coats for these commercially harvested species. The low average viability and especially low percentage sinker estimates suggest that the seeds of these two species were prematurely harvested. Prematurely harvested seeds can result in incompletely developed, more oxygen permeable seed coats which produces both a relatively high control value and a poor response to oxygenation (Brits and Van Niekerk, 1986). The latter reactions are also given by floater seeds (with poorly developed embryos) as opposed to sinker seeds (Van Staden and Brown, 1973). Premature commercial harvesting of *Leucospermum* seed is a common practice. The conclusion above is supported by the relatively low maximal germination values obtained for *L. cuneiforme* and *L. vestitum*.

C: *L. erubescens*, *L. glabrum*, *L. reflexum*: seeds of these species, harvested under controlled conditions, gave a high average maximal germination percentage, viability estimate and percentage sinkers, as in *L. cordifolium* (Table 1). The very low control germination percentage, however, suggests that either a non-optimal temperature regime could have contributed to low control values, or that seed coats were much less permeable to oxygen than in *L. cordifolium*. Surprisingly incubation in oxygen alone (treatment 2) did not increase germination percentage. Oxygen could not have been inhibitory to these seeds as was suggested for *Leucadendron tinctum* Williams (Brown and Dix, 1985) since treatment 4, in which presumably the highest oxygen partial pressure was realised, gave maximal germination values approaching viability estimates. The results therefore suggest that non-domesticated *Leucospermum* species have highly impermeable seed coats in intact mature seeds.

Conclusions

The following roles are proposed for each in a series of cumulative treatments required to maximize germination percentage in *Leucospermum*:

1) Oxygen incubation increases the oxygen partial pressure required for germination of seeds with intact seed coats. Oxygen incubation enhances germination also in acid scarified seeds, as was found in *L. cordifolium* (Chapter 4.2) and scarification appears not to substitute fully for oxygen incubation in realizing maximal germination response.

2) Promalin pre-soaking provides essential hormone(s) which act as promoters of germination. Thus the level(s) of endogenous hormones are increased/complemented by applied hormones, especially where sub-optimal environmental factors prevent synthesis/interconversion of endogenous hormones. This may be the case where seeds are incubated at non-optimal low and high temperatures.

In *L. cordifolium*, which was incubated under an optimal alternating temperature regime, Promalin treatment gave no significant response (Table 1). *L. reflexum* in nature, however, germinates at substantially higher altitudes than *L. cordifolium* (Rourke, 1972) i.e. at lower environmental temperature. Its seeds would therefore require a relatively cool germination temperature regime (Brits, 1986b). *L. erubescens* on the other hand, grows on hot, dry, north facing slopes (Rourke, 1972) i.e. it germinates at higher average temperature in nature than *L. cordifolium*. Both *L.*

reflexum and *L. erubescens* gave substantially increased germination percentage with added Promalin compared to oxygen incubation alone.

3) Scarification augments germination by a) increasing testa permeability to oxygen, especially in the case of wild species and b) reducing mechanical constraint of the hard seed coat, thereby facilitating germination of embryos with a low vigour (Chapter 6.4).

Dormancy characters (e.g. high and low temperature requirement) are apparently variable in their expression in seed populations, depending partly on genetic (Roberts, 1972) and environmental (Gutterman, 1993) factors. For example, low temperature requirement in any seed population does not manifest as a single threshold low temperature level or duration of temperature for the breaking of dormancy of all seeds (Brits and Van Niekerk, 1986). Rather some moistened seeds will germinate even under constant high temperature, with increasing numbers responding to progressively lower temperature (Brits and Van Niekerk, 1986; Brits, 1986b). This could explain why the progressive fulfilment of environmental requirements gave step-wise increases in the germination response.

4.4 DIURNAL BI-TEMPERATURE REQUIREMENTS IN *LEUCOSPERMUM* R.Br. (PROTEACEAE) SEED GERMINATION AND THEIR ECOLOGICAL CORRELATES

Introduction

Diurnally alternating temperature requirement is a major factor in the germination ecophysiology of wild (non-domesticated) seeds (Probert, 1992). The subject has consequently become well-researched (Morinaga, 1926; Thompson, 1974; Thompson and Grime, 1983; Murdoch *et al.*, 1989). The approach in this study field has often been limited to the quantification of temperature requirements with little attempt being made to correlate thermoperiodicity with underlying physiological processes (c.f. Murdoch *et al.*, 1989). However, correlation of alternating temperature requirements with ecological determinants in natural species recruitment has been attempted (Thompson, 1974; Thompson and Grime, 1983). Again a limitation is often the lack of experimental demonstration of seed-environment interaction. Examples in the literature of proposed ecological function involving sensitivity of seeds to increased diurnal temperature amplitude in maximizing natural seedling recruitment, are:

- Weed seeds released from dormancy by soil disturbance. A small proportion of seed in the persistent, soil- stored seed bank is displaced to a position near the bare soil surface where temperature fluctuation is greater (Probert, 1992). Sensitivity to alternating temperature senses depth, effecting minimal losses of the deeper lying dormant seed bank (Thompson and Grime, 1983).
- Dense-canopy vegetation where sporadic disturbance creates gaps that could be colonized by pioneers. Soil temperatures fluctuate stronger within gaps resulting from increased daily radiation. Sensitivity to alternating temperature causes seed germination in suitably exposed terrain, in herbaceous weed species (Thompson *et al.*, 1977) and forest pioneer species (Vazques-Yanes and Orozco-Segovia, 1982).
- Marsh and wetland vegetation where a significant proportion of the habitat is flooded seasonally and which becomes suitable for recolonization only during the drier seasons (Thompson and Grime, 1983). The increased diurnal temperature fluctuation occurring in exposed ground, compared with flooded ground, is sensed by seeds.
- Water-dispersed seeds which establish on exposed stream/river banks following a period of floating (Thompson, 1974).
- Species of fire-disturbed habitats in mediterranean-type climates. A dense, severely competitive vegetation canopy is removed periodically by fire. This has apparently pressurized taxa to adapt to a strategy of rapid and massive seed germination after fire

(Keeley, 1991; Le Maitre and Midgley, 1992; Bell *et al.*, 1993). The soil-stored seeds of nut-fruited Proteaceae in the Cape Floristic Region sense the favourable post-fire condition by means of increased soil temperature fluctuation (Brits, 1986b; 1987).

Leucospermum cordifolium (Salisb. ex Knight) Fourcade and *Serruria florida* Knight seeds germinate maximally when incubated under alternating moderately low and high temperatures within a 24 h period (i.e. paralleling natural nocturnal/diurnal temperature fluctuations within the range of 7-11 °C and 20-25 °C - Brits, 1986b). Individual requirements appear to be correlated with the climatic temperature regimes associated with the natural habitats of these species. A cooler optimal regime was indicated for *S. florida*, which grows at a higher altitude, than in the case of *L. cordifolium* (Brits, 1986b). The two species have relatively narrow ecological amplitudes (Rourke, 1972; Vogts, 1982) in the natural Cape fynbos habitat, which is characterized by contrasting micro-climates (Rourke, 1972). Since *Leucospermum* species are dependent on temperature cues for germination, the question arises whether, and how closely, *Leucospermum* R.Br. species are adapted to their environmental temperature regimes. A group of 6 *Leucospermum* species with contrasting phylogeny (representing 5 of the 9 taxonomic sections in the genus) and originating from varied climatical backgrounds were used in this study (see Chapter 4.3). Three hypotheses are tested, relating to germination temperature requirements:

1) diurnally alternating temperatures are universal requirements (character syndromes *sensu* Angevine and Chabot, 1979) within *Leucospermum*;

2) the temperature requirements of individual species differ;

3) specific differences are correlated with ecological determinants, i.e. they are a function of selective pressures.

The chosen *Leucospermum* species were found to germinate maximally when incubated under an alternating temperature regime, in combination with oxygenation and other treatments (Chapter 4.3). No other dormancy factors, such as light requirement (Van Staden, 1966) are known in *Leucospermum*. It was assumed that *Leucospermum* seeds in the soil-stored seed bank must be scarified in the soil before oxygen dependent germination can take place (Chapter 4.3; Chapter 6.2). In order to simulate natural conditions, seeds were therefore acid scarified prior to incubation (Brits and Van Niekerk, 1976). The temperature range was based on previous results with *L. cordifolium* (Brits, 1986b). The choice of a single 16 h low x 8 h high diurnal temperature cycle relates to ambient temperature regimes in fynbos during autumn. This is in accordance with the need for relatively long periods at low temperature for optimal germination response within diurnally fluctuating temperature cycles (Thompson, 1974; Totterdell and Roberts, 1980).

Table 1. Origin of *Leucospermum* experimental seed sources, tetrazolium viability estimates for sources and maximal realized germination percentage.

Species	Tygerhoek Experimental Farm, Riviersonderend 34°9'S, 19°54'E		Other sources		Performance%	
					Tetraz.	Max.
L. cordifolium (Salisb. ex Knight) Fourcade	Domesticated cultivars + clonal selections of different ecotypes	(100%)	-		85	77
L. cuneiforme (Burm. f.) Rourke	Natural stands, Riviersonderend Mountain,	(25%)	"Die Bof" Nursery, George area	(75%)	37	23
L. erubescens Rourke	Clone T821005	(75%)	Kirstenbosch Bot. Gardens,	(25%)	63	17
L. glabrum Phillips	Mixed clones cv. Helderfontein	(33%) (67%)	-		78	51
L. reflexum Buek ex Meisn.	Mixed clones	(10%)	Kirstenb. Bot. Gards. Caledon Wildf. Gard. Clanwill. Wildf. Gard.	(23%) (11%) (56%)	54	25
L. vestitum (Lam.) Rourke	Mixed clones, mostly from natural stands, Clanwilliam	(100%)	-		66	36

Materials and Methods

The methods in this section generally follow those of Brits (1986b). *Leucospermum* seed was produced under cultivation within a typically mesic mountain fynbos area (Chapter 2). Seeds were harvested, sorted, and a few which were in short supply were supplemented by cultivated 1- 2 yr-old seed obtained from commercial seed suppliers (Table 1). Experimental plants had been cultivated for only one or two generations from seed following collection from the wild, excepting *L. cordifolium*.

Seeds were thoroughly mixed and divided into lots of convenient size for acid scarification treatment. Seeds were treated for 7 min in (c) H_2SO_4 (Brits and Van Niekerk, 1976), washed and soaked in distilled water for 24 h. Seeds were then air-dried and dusted with thiram wp fungicide (Chapter 2). Petri dishes (9 cm) were divided into 6 segments and 25 seeds per species (except 22 for *L. cuneiforme* and *L. erubescens*) were transferred to each segment per petri dish onto one layer of Whatman no. 1 filter paper. Each petri dish constituted one replication and this was repeated 6 times within each of the temperature regimes.

Temperature regimes

Seeds were incubated at 15 different temperature regimes. Where temperatures were alternated the lower and higher temperature was maintained for 16 and 8 h respectively. Mean daily temperature was calculated as 2/3 (lower temperature) + 1/3 (higher temperature). The regimes (°C), their means and amplitudes were:

	Regime	Mean	Amplitude	Regime	Mean	Amplitude
1.	4	4	0	9.	4X28	24
2.	10	10	0	10.	10X16	6
3.	16	16	0	11.	10X22	12
4.	22	22	0	12.	10X28	18
5.	28	28	0	13.	16X22	6
6.	4X10	6	6	14.	16X28	12
7.	4X16	8	12	15.	22X28	6
8.	4X22	10	18			

Alternating temperatures were obtained by transferring petri dishes with seeds from one to another of five incubators which were kept at constant temperature, with a daily variation not exceeding ±1 °C. A seed was considered to have germinated when the radicle had emerged 1 mm and germinated seeds were counted and removed weekly for 14 w.

A viability test with tetrazolium (Brits and Van Niekerk, 1976) was carried out on samples of all 6 species. The relationship between mean daily temperature, temperature amplitude and germination percentage was determined by means of multiple regression analysis. From these the turning points (maxima) were estimated of X_1 (mean daily temperature) and X_2 (temperature amplitude) for untransformed percentage data. Using the estimated turning points X_1' and X_2', the optimum low (base) and optimum high (ceiling) germination temperatures for the six species were calculated by transposition (Brits, 1986b):

Base temperature = $X_1' - 1/3X_2'$ and

Ceiling temperature = $X_1' + 2/3X_2'$.

Germination rates for treatments and species were calculated (Chapter 4.2).

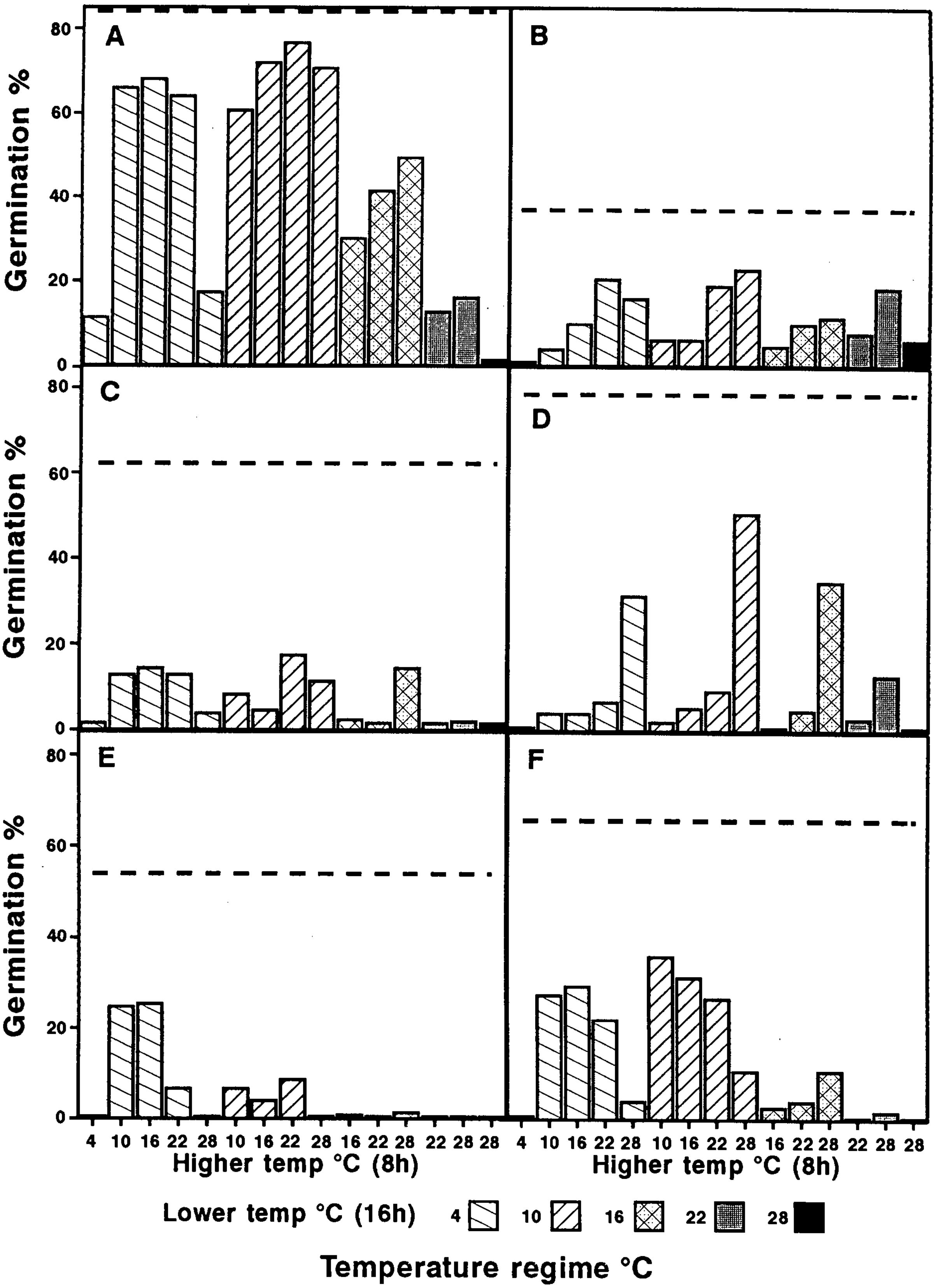

Figure 1. Mean seed gemination percentages for six species of *Leucospermum* incubated under 15 regimes of constant and alternating temperatures: A - *L. cordifolium* ; B - *L. cuneiforme*; C - *L. erubescens*; D - *L. glabrum*; E - *L. reflexum*; F - *L. vestitum*. LSDs = 9,6% (P = 0,05) and 12,7% (P = 0,01). - - - - Tetrazolium viability estimates.

Statistical methods

A 6x15 factorial experiment was carried out within a completely randomized design with 6 replicates. The factors were 6 species and 15 temperature regimes. Each experimental unit consisted of 25 or 22 seeds. The appropriate analyses of variance were performed on the percentage germination, logit transformed data and angular transformed percentages. Since variances did not differ significantly, only germination percentage were used for analysis.

Response surfaces were calculated on mean germination percentages after adding a few hypothetical points that were thought inappropriate to include within the present experiment. Pearson's correlation coefficients were calculated where needed. Genstat 5, version 2.2 were used for the above calculations. Student's t-Least Significant Difference were calculated to compare treatment means.

Results and Discussion

Germination percentages for species and treatments differed statistically highly significantly ($P < 0{,}001$) and the interaction found for species x treatments was likewise highly significant ($P < 0{,}001$). For this reason the mean percentage values for species and treatments are presented individually (Figure 1). Maximum germination response correlated positively with tetrazolium viability estimates ($r = 0{,}81$; $P = 0{,}05$), *L. erubescens* being the outlier (Table 1). However tetrazolium viability scores all over-estimated realized maximal germination percentages, some strongly (Figure 1). Apparently the discrepancy between tetrazolium estimates and realized germination increased with decreasing viability (Table 1). Thus tetrazolium became increasingly ineffectual with declining seed quality in predicting germination response. It is possible that the scarification time was insufficient to break seed coat dormancy fully, or that optimal temperature regimes were not realized (Chapter 4.3). However the inexplicable discrepancy between viability estimates and realized germination percentage leaves the possibility that an additional, unknown factor may co-control dormancy in *Leucospermum*.

Germination rates for treatments generally echoed percentage responses (Figure 2) illustrating that generally germination response was slower under non-optimal conditions. Multiple regressions of germination percentage as a function of mean incubation temperature and incubation temperature amplitude generally gave reasonable R^2 values when based on experimental values only, accounting for more than 60% of the variation in germination of individual species, with the exception of *L.erubescens* and *L. vestitum* (Table 3).

Within the 4 °C lower-temperature series, realized germination percentages tended to follow a parabolic trend, i.e. a clear maximum is discernable within series - e.g. in *L. cordifolium* (Figure 1). Parabolic trends (with clear turning points) are required within

Table 2. Series of 10 outlying incubation temperature regimes (°C) at which germination was postulated not to occur in *Leucospermum*, daily average temperatures being either too low or too high for germination to occur. Each regime was given an arbitrary value of 1% and used for calculating multiple regression for six species: daily low (16 h) and high (8 h) temperature, mean daily temperature X_1 and temperature amplitude X_2

Regime		X_1	X_2	Regime		X_1	X_2
Low	High			Low	High		
-2	4	0	6	-2	34	10	36
-2	10	2	12	4	40	16	36
-2	16	4	18	16	40	24	24
-2	22	6	24	22	34	26	12
-2	28	8	30	22	40	28	18

most temperature series of a species data set to be able to calculate optima (base and ceiling germination temperatures) using regression. However this was not the case in *L. cuneiforme*, *L. glabrum* and *L. reflexum*. Germination within these temperature series often failed to reach maxima (Figure 1). For example in *L. glabrum* realized germination percentages in all series were ascending throughout the temperature test ranges used and turning points clearly could not be estimated. A series of 10 outlying values at which germination is expected to be very low or not to occur at all, was therefore postulated (Table 2). For example, a base temperature of below 0 °C will conceivably lead to a zero response in most *Leucospermum* species, since prolonged sub-zero temperatures do not occur in the generally temperate fynbos montane climate during the germination season. In addition the sensitive seedlings could be frost damaged during emergence (personal observation). On the other extreme an average constant high temperature of 34 or 40 °C could be severely depressive since such high incubation temperatures tend to suppress germination strongly (Brown and Van Staden, 1971; Brits and Van Niekerk, 1986; Brits, 1986b). Average maximal temperatures of this order also do not occur during the autumn germinative season in nature (Table 4). The set of hypothetical temperatures and responses chosen were standardised over the experimental species. The resulting polynomial functions (Figure 3) allowed calculation of base and ceiling temperature estimates and these corresponded reasonably well to the

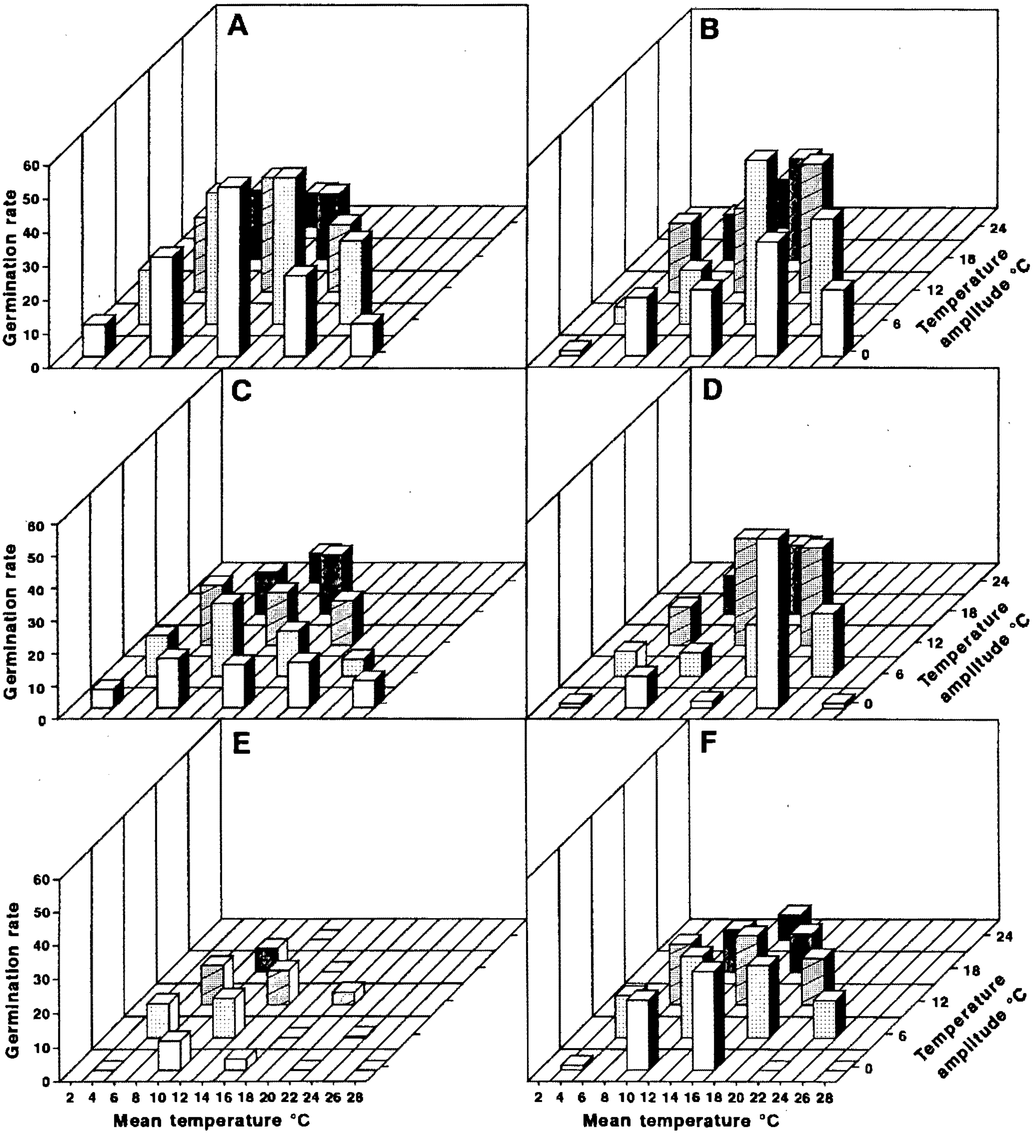

Figure 2. 3-D bar diagrams of mean seed germination rates for six species of *Leucospermum* incubated under 15 regimes of constant and alternating temperatures: A - *L. cordifolium* ; B - *L. cuneiforme*; C - *L. erubescens*; D - *L. glabrum*; E - *L. reflexum*; F - *L. vestitum*.

Table 3. Multiple regression equations, significance levels (SL), calculated maxima X_1' (mean temperature) and X_2' (amplitude), R^2 values based on experimental plus postulated germination percentage values; and R^2 values for regression based on experimental values only, for 6 *Leucospermum* species.

Species	Experimental + postulated					Exp. only
	Equation	SL	X_1'	X_2'	R^2 %	R^2 %
cordifolium	$Y = -14{,}1 + 10{,}2X_1 + 1{,}14X_2$ $- 0{,}377X_1^2 - 0{,}093X_2^2 + 0{,}045X_1X_2$	$<0{,}001$	14,26	9,53	68	68
cuneiforme	$Y = -12{,}2 + 2{,}64X_1 + 1{,}04X_2$ $- 0{,}077X_1^2 - 0{,}029X_2^2 - 0{,}018X_1X_2$	0,002	15,71	12,95	48	63
erubescens	$Y = -2{,}74 + 1{,}45X_1 + 0{,}47X_2$ $- 0{,}054X_1^2 - 0{,}021X_2^2 + 0{,}006X_1X_2$	0,015	14,24	13,24	36	39
glabrum	$Y = -19{,}1 + 3{,}31X_1 + 1{,}54X_2$ $- 0{,}102X_1^2 - 0{,}046X_2^2 - 0{,}002X_1X_2$	0,073	16,03	16,39	22	78
reflexum	$Y = +5{,}19 + 0{,}325X_1 + 0{,}101X_2$ $- 0{,}023X_1^2 - 0{,}011X_2^2 + 0{,}010X_1X_2$	0,518	8,81	8,49	-	62
vestitum	$Y = +2{,}95 + 2{,}68X_1 - 0{,}006X_2$ $- 0{,}113X_1^2 - 0{,}024X_2^2 + 0{,}027X_1X_2$	0,022	12,75	7,04	35	39

trends evident in the bar diagrams. *L. glabrum*, for example, appears to need a high ceiling temperatureboth from inspection of the bar diagrams and from the regression estimates (Figure 1, Table 4) and, by contrast, *L. reflexum* requires a low base temperature. The regressions yielding these estimates in three cases gave comparable R^2 values to those of regressions based only on experimental data (Table 3), the other values deviating where experimental data did not show sufficient parabolism. It is therefore proposed that the base and ceiling temperatures calculated from additional, postulated values be viewed as provisional, working estimates.

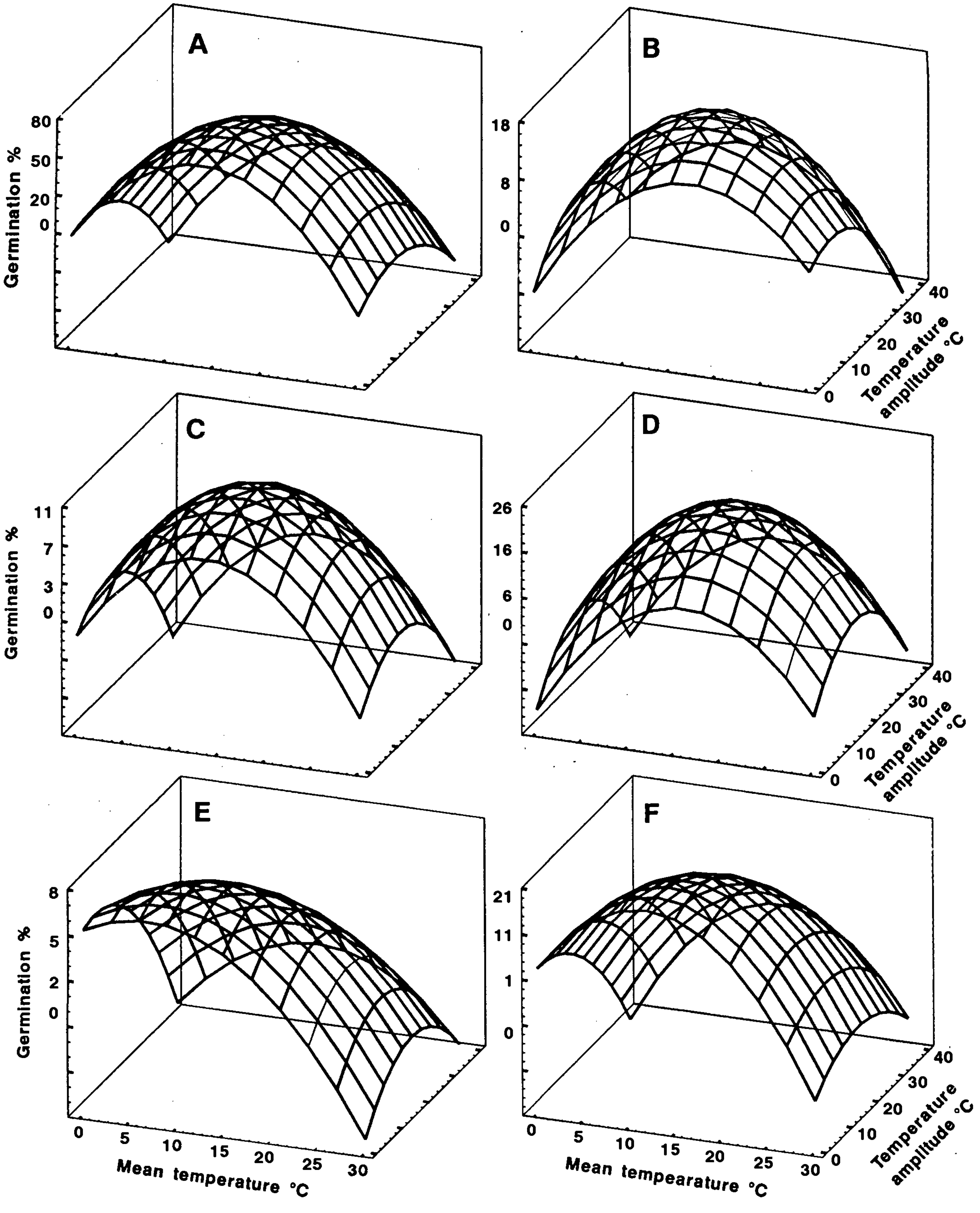

Figure 3. Bivariate polynomial regressions of the data presented in Figure 1, supplemented with postulated outlying values (see Table 2). A - *L. cordifolium* ; B - *L. cuneiforme*; C - *L. erubescens*; D - *L. glabrum*; E - *L. reflexum*; F - *L. vestitum*. For regression equations see Table 3.

Table 4. Habitat characteristics and elevation (m) (Rourke, 1972), average autumn air temperatures at nearby weather stations (May, 1983 - 1992) and estimated base and ceiling temperature requirements, calculated from regression, for 6 *Leucospermum* species, in decreasing order of base temperature (bold print).

Species	Habitat		Weather station data				Regression	
	description	m	Name	m	Min	Max	**Base**	Ceiling
cuneiforme	Southern foothils of Cape South Coast mountains	0-1000	Swellendam	125	9,7	22,1	**11,4**	24,3
cordifolium	Coastal areas - Strandveld	30-450	Driefontein	91	10,0	21,6	**11,1**	20,6
glabrum	Coastal, southern foothills of Outeniqua Mts.	150-450	Outeniqua	204	10,0	21,6	**10,6**	27,0
vestitum	Inland, north/west facing slopes	60-1200	La Plaisante	260	9,6	20,8	**10,4**	17,4
erubescens	Langeberg northern foothills	450-600	Die Vlakte	722	8,4	19,3	**9,8**	23,1
reflexum	Eastern Cedarberg (subalpine)	900-2000	De Keur	945	6,1	17,7	**6,0**	15,0
Average							**9,9**	21,2

L. cordifolium *estimates*

Poor repeatablity was shown in the case of *L. cordifolium* in which the present base and ceiling temperature estimates are substantially different from previous estimates (Table 5). Moreover a much wider latitude of response is evident in this study, i.e. *L. cordifolium* seeds germinated well under a wider variety of temperatures (Figure 1) compared with the responses found previously (Brits, 1986b). The response latitude of

L. cordifolium here is also exceptionally wide compared with the narrow response ranges found in the other species. Thus *L. cordifolium* exhibited much less dormancy than before, and also in relation to the other *Leucospermum* species (Figure 1). The between-experiment differences in *L. cordifolium* are possibly due to genetically different cultivated seed sources used for experiments. Seeds for the present work were obtained from stands of clonally propagated selections and cultivars that have been domesticated for a longer period of time than in the case of the previous seed source. Seed dormancy is genetically variable (Roberts, 1972) and the gradual genetic loss of seed dormancy in domesticated *L. cordifolium* through artificial selection could explain the difference (Chapter 4.3). This view is also consistent with the comparatively wide response latitude found in *L. cordifolium*, compared with other species in this study. The fact that, originally, selections from different ecotypes of *L. cordifolium* constituted the present seed source, could also have contributed to a wide response latitude. The present data suggests that different ecotypes have different responses which in a mixture could lead to a wider response latitude.

Other factors contributing to a variable germination response could have been environmental effects, especially harvest year (Table 5) and experimental incubation environment, as was shown for *Rumex* seeds (Totterdell and Roberts, 1980). However, oxygenation with H_2O_2 (previously) as opposed to oxygenation via H_2SO_4 scarification (this experiment - Table 5) did probably not interact with the temperature regime (Brits and Van Niekerk, 1986). Oxygenation treatments relieve seed coat imposed dormancy, in contrast to temperature which influences physiologically imposed dormancy, these two factors acting independently (Brits and Van Niekerk, 1986).

The extremely wide response latitude in *L. cordifolium* in this study suggests that the base and ceiling temperature differences with those values calculated before (Table 5 - Brits, 1986b) are not significant.

Table 5. Base and ceiling temperatures estimated for *Leucospermum cordifolium*, derived from different seed sources, harvest years and pretreatments

Seed source	Harvest year	Pretreatment	Reference	Base	Ceiling
Tygerhoek - source 1	c. 1983	None	Brits, 1986b	9,1	23,9
Tygerhoek - source 1	c. 1983	H_2O_2 oxygenated	Do.	8,5	22,7
Tygerhoek - source 2	1989	H_2SO_4 scarified	This study	11,1	20,6

Ecological roles of temperature requirements in Leucospermum *in fynbos*

Although species differed markedly, germination temperature requirements in *Leucospermum* are clearly all of a diurnally alternating character. These common requirements indicate two character syndromes in *Leucospermum* which are instrumental in recognizing the ephemeral early post-fire winter condition in fynbos (Brits, 1986b; 1987). This is the brief and isolated temporal gap in the natural fire cycle optimal for seedling recruitment. During this period moisture is available following the dry summer season when at the same time the habitat is temporarily cleared of its canopy of competing vegetation. A competitive and suppressive canopy establishes within one season following fire in fynbos (Brits, 1986b). Thus low temperature requirement avoids moisture stress by restricting germination to the moist winter season. Daily high temperature requirement, on the other hand, avoids competition by restricting germination to the brief phase coinciding with high winter topsoil temperatures post-fire when canopy competition is absent (Brits, 1986b).

Relationship of estimates with natural temperature regimes

The relationship between estimated base and ceiling temperatures in *Leucospermum* species and climatic/ecological determinants (Table 4) was investigated. Estimated temperature requirements correlated positively with mean air temperature during May, at nearby weather stations. This was strong in the case of base temperature requirement and minimum air temperature ($r = 0{,}82$; $P = 0{,}04$ - Figure 4B). However, the correlation between ceiling temperature and maximum air temperature was weaker and not statistically significant ($r = 0{,}65$; $P = 0{,}16$ - Figure 4C). Low temperature is the most important environmental requirement and May (mid-autumn) was shown to be the month of maximum emergence in seed bed germinated *Leucospermum cordifolium* seed (Brits and Van Niekerk, 1986).

The resulting *Leucospermum* germination temperature requirements were thus related to the localized germination season climate of species. Average May temperatures for weather stations differed markedly (Table 4). Although mean minimum air temperatures reflect the altitude of weather stations (Table 4) they are also influenced by local topography and proximity to the coast. Coastal species (*L. cordifolium*, *L. glabrum*) showed more temperate requirements as opposed to inland species (*L. erubescens, L. vestitum*) which required lower base temperatures. The "subalpine" species (*sensu* George, 1981) *L. reflexum* had both the lowest base and ceiling temperature requirements. Environment related germination temperature requirements were also found for the subalpine *Banksia* L.f. (Proteaceae) species *B. canei* J.H. Willis (750 - 1500 m), *B. saxicola* A.S. George (>600 m) and some forms of *B. marginata* Cavanilles (up to 1400 m) which required stratification at 5 °C for germination. This was in contrast to species of lower altitudes which responded to

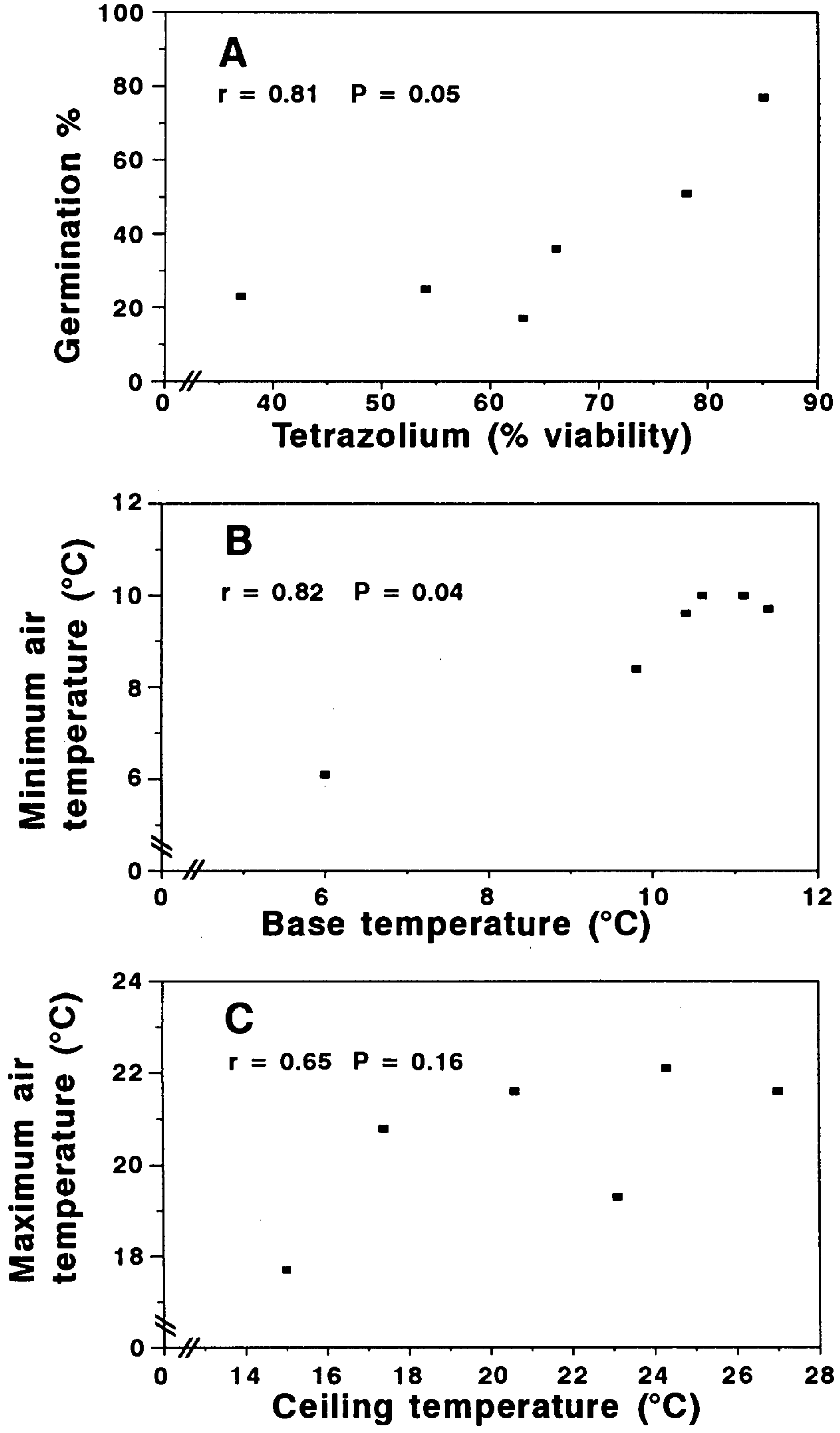

Figure 4. Scatter diagrams and correlations for *Leucospermum*:
A: Correlation between tetrazolium viability percentage estimates and realised maximal germination percentage.
B: Correlation between estimated base (optimum low) incubation temperature and average minimum air temperature during May (1983 - 1992) at weather stations close to the natural habitat of six *Leucospermum* species.
C: Correlation between estimated ceiling (optimum high) incubation temperature and average maximum air temperature, for six *Leucospermum* species, as in B.

higher germination temperatures (Salkin and Hallam, 1978). Trends in germination temperature requirements were also studied in 28 non-proteaceous perennial native Australian species (Bell and Bellairs, 1992). Generally the conditions yielding the highest germination percentages tended to coincide with those habitat temperature conditions most suitable for seedling establishment. The same applies for a wide range of plants in the Negev desert (Gutterman, 1993). In fynbos *Leucospermum* species these are the habitat conditions prevailing during May.

Embryo, seed coat and environmental factors interact in complex ways in the induction of *Leucospermum* germination (Kelly and Van Staden, 1992). It is therefore remarkable that the relatively close experimental correlations of base/ceiling temperature requirements with environmental temperature regimes were found despite the presence of several potential sources of error. Firstly, the fact that most seeds in this experiment originated from only one harvest year and from cultivated sources (Table 1) could have influenced the particular germination response found. In *Rumex*, for example, marked differences in germination percentage response occurred in seeds of the same species originating from either different ecotypes in the same harvest year or the same ecotype harvested in different years (Totterdell and Roberts, 1980). Moreover the weather station data used for *Leucospermum* are at best approximations of the local regimes prevailing in the habitats of species, the precise location of the original seed source populations being unknown. In particular the air temperature data of weather stations could be expected to differ from the appropriate fynbos soil temperature regimes. The weather station closest to the habitat of *L. erubescens*, for example, lies considerably higher than the altitude range in which the species occurs (Table 4). This may explain the overprediction of regression estimates when compared with the habitat temperature regime in this species (Table 4). The good statistical correlation found therefore confirms that fynbos *Leucospermum* species are narrowly adapted to their natural environments. Moreover, the experimental seed was produced under cultivation. Thus the expression of *Leucospermum* germination characteristics outside the habitat indicate that they are genetically stable (Salkin and Hallam, 1978).

Temperature requirements as adaptive responses

The advantage of narrow adaptedness to a particular environmental niche could be increased sensitivity of a seed population to a unique and short-lived co-occurrence of favourable conditions, thus improving responsiveness. Such rare and critical conditions are the brief overlapping period of fire-exposed soil and moist, moderate winter climate in fynbos. Moreover a narrow adaptedness in species could result in avoidance of seed loss to spurious germination cues during unfavourable conditions (Probert, 1992). This consequently maximizes the soil-stored seed bank (Thompson and Grime, 1983). Conversely, effective timing of germination during the critical phase of favourable conditions could maximize interspecific seedling competitive success, through the principle of pre-emption of space by early germination and establishment (Stebbins, 1950; Fenner, 1985).

The above proposals reflect on the question of whether, in *Leucospermum* seed germination, the requirement for temperature amplitude *per se* is functional in nature, i.e. as a secondary germination factor (*sensu* Murdoch *et al.*, 1989). Alternatively, do only the primary base and ceiling temperature requirements function: do they operate on specific and independent physiological levels? Here the separate roles identified for requirements of low temperature (drought avoidance) and high temperature (competition avoidance) suggest independent adaptive roles for these characters. Moreover the close relationship indicated between habitat climate and base/ceiling temperature requirements does not imply temperature amplitude *per se* as a physiological determinant. Low temperature was shown to be a primary and independent environmental requirement in *Leucospermum cordifolium* seed germination (Brits and Van Niekerk, 1986). This suggests a strong physiological role for low temperature. A separate physiological role for high temperature in *Leucospermum* has also been found (Chapter 6.2). Thus the ecophysiology of *Leucospermum* germination temperature requirements strengthens the impression that distinct physiological processes are regulated by different temperature cues. In apparent contrast most species with persistent seed banks, e.g. many weed species, require increased fluctuation *per se* of daily temperature. A proportion only of such seed banks are typically released from dormancy, often in conjunction with light requirement. The typical small-sized seeds of these species respond to a relatively wide range of fluctuating temperatures (Murdoch *et al.*, 1989; Probert, 1992). In contradistinction the large majority of the large-sized *Leucospermum* soil-stored seeds appear to germinate simultaneously, mostly from relatively even depths (Rourke, 1972; Brits, 1987). *Leucospermum* seed banks in fynbos are therefore essentially non-persistent (*sensu* Thompson and Grime, 1979), although the seeds are long-living. A physiological interpretation for alternating temperature requirement in species with persistent seed banks is unfortunately lacking (Probert, 1992). Nevertheless the case of *Leucospermum* highlights the differences between fynbos and northern hemisphere (boreal) seed germination ecophysiology.

It is therefore proposed that an "optimal temperature amplitude" parameter in the germination of this *Leucospermum* group of species should, in the seed ecological context, rather be interpreted as two independent temperature requirements. These requirements should therefore appropriately be characterized as a diurnal bi-temperature requirement. The same applies to the theoretical (non-)significance of "mean temperature" as a secondary factor (Murdoch *et al.*, 1989; Probert, 1992) in *Leucospermum* germination. Mean temperature apparently has no ecological (and physiological) significance in *Leucospermum*. In contrast, however, the parameter of median temperature is a highly functional concept. Dormancy in *Leucospermum* in fynbos is enforced physiologically at this temperature level (Chapter 5) as part of the seed's avoidance strategies (Brits, 1986b).

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CHAPTER 5

HORMONAL REGULATION

PREFACE

In the only review of seed germination in the South African Proteaceae, Van Staden and Brown (1977) advanced the ideal of understanding hormonal control in terms of appropriate environmental cues in fynbos. The preceeding chapters provide the foundation for such an investigation in *Leucospermum*. These include studies defining physiological germination requirements in *Leucospermum*. The characterization developed is validated by the high degree of control now possible over germination in several species.

The work below explores seed hormonal changes correlating with known dormancy and germination characters. This yields a predictive model of hormonal regulation on the level of the laboratory environment.

HORMONAL AND ENVIRONMENTAL CONTROL OF DORMANCY AND GERMINATION IN *LEUCOSPERMUM* SEEDS

Introduction

Early models on the roles of phytohormones in seed germination were based mostly on circumstantial evidence, i.e. on correlations between physiological / ultrastructural / morphological states of the embryo, and extractable endogenous hormone levels (Hilhorst and Karssen, 1992; Wareing, 1982). Only recently has direct evidence been presented for absolute dependency of germination on the availability of the growth promoting hormone classes, gibberellins (GAs) and cytokinins (CKs). This evidence was based on studies with hormone-deficient and hormone insensitive mutants (e.g. Karssen *et al.*, 1989) and studies with hormones of which the endogenous levels are restored artificially in the seed, starting from zero levels (Munoz *et al.*, 1990; Munoz *et al.*, 1992). Thus the proposed involvement of different hormones in the regulation of specific functions in seed germination and dormancy has gained conclusive experimental proof in a number of key studies (Hilhorst and Karssen, 1992; Karssen *et al.*, 1989; Villalobos and Martin, 1992).

Leucospermum plants are subject to severe stresses and disturbance factors in the natural fynbos habitat (Cowling, 1992). These all contribute to the complex regulation of seed germination (Kelly *et al.*, 1992). The seed biology of species within *Leucospermum* was found to be very similar, although species differ in their degree of reaction to environmental conditions (Chapters 1 and 4). The gross embryo morphology, storage reserves, universal dependence on low temperature for germination and ultrastructural changes during germination in species of several Proteaceae genera are also similar - including *Leucospermum* (Brown and Van Staden, 1973c; Mitchell, 1983). Good examples are two species with structurally related seeds, *Leucadendron daphnoides* (Thunb.) Meisn., and *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade, which have similar seed release periods and strategies and are anatomically both nut-fruited (Brits, 1986b).

Adapted from Brits *et al.*, 1995

These species require the embryo to split the testa before radicle protrusion can occur (Brown and Van Staden, 1973c; Van Staden and Brown, 1973b), they are subject to testa-imposed oxygen exclusion (anoxia) of the embryo in intact seeds (Brits, 1986b; Brits and Van Niekerk, 1976; Brown and Van Staden, 1973a; Van Staden and Brown, 1973a; 1973b) and germination responds strongly to alternating temperature regimes (Brits, 1986a; Brown and Van Staden, 1975a). In view of these similarities within *Leucospermum* the objective of this chapter is to characterise important hormonal-environmental features in two species and then use the results for a general model on *Leucospermum*.

Several studies, including studies on endogenous GA levels in *L. daphnoides*, produced convincing circumstantial evidence that in certain Cape Proteaceae:

1) normal germination (under optimal conditions) is associated with both quantitative and qualitative changes in endogenous GAs and CKs (Brown and Van Staden, 1975a; Mitchell *et al.*, 1986);

2) dormancy is due to a lack of the growth promoters GA and CK in the embryo and not the presence of inhibitors (Brown and Van Staden, 1973b; Brown and Van Staden, 1975a; Van Staden and Brown, 1977). Studies were also conducted of CK changes paralleling ultrastructural and morphological changes in germinating embryos of several species of Proteaceae (Van Staden and Brown, 1977).

Despite the above and other growth regulator studies, a physiologically well characterized model of germination and dormancy relief in species of Cape Proteaceae has not yet been developed, often because in experiments 1) the germination requirements of seeds were only partially met, seeds were thus incubated under dormancy enforcing conditions when attempting to study normal germination (e.g. intact seeds were not oxygenated when studying temperature effects in *L. daphnoides* in Mitchell (1983) and Brown and Van Staden (1975a); 2) individual studies concentrated mostly on a single hormone (Brown and Van Staden, 1973a; Mitchell, 1983) thus precluding a whole-seed physiological approach i.e. the principle that hormones should not be considered in isolation (Van Staden *et al.*, 1982); 3) thus far hormone levels in the whole proteaceous seed only were analysed, 4) using bioassays. Bioassays in general have limitations (Wareing, 1982) and the soybean assay in particular could be insensitive to certain cytokinin intermediate compounds in proteaceous seeds (Van Staden and Brown, 1973a). Recent work indicates, moreover, that within the dicotyledonous embryo the axis is the main regulator of germination, including the regulator of reserve mobilization in cotyledons, by producing a hormonal stimulus (Come and Thevenot, 1982; De Klerk, 1986, Ilan and Gepstein, 1980; Munoz *et al.*, 1990; Penner and Ashton, 1967). These limitations in previous studies precluded the elucidation of interactions between different hormones, and between embryo organs and hormones, during dormancy relief and germination (Come and Thevenot, 1982; Wareing, 1982).

In the search for a working eco-physiological model for *Leucospermum* seed germination the following questions currently dominate:

1) can dormancy breaking and germination induction in *Leucospermum* be distinguished physiologically;

2) how are the known external germination cues linked to the hormone system of seeds, and

3) how do hormones relieve dormancy and influence the different known stages (e.g. protein and lipid hydrolysis, testa splitting, meristem activation and radicle elongation) of germination *sensu stricto*?

This study is a first attempt to characterize and correlate 1) changes in the endogenous levels of some likely hormones functioning during normal germination, (all requirements met) e.g. GA_1 and GA_3 (Khan, 1971) in *Leucospermum glabrum* Phillips and 2) the germination responses to exogenously applied hormones, e.g. GA_{4+7}, under different known dormancy enforcing temperature regimes, in seeds of *L. glabrum* and *L. cordifolium*. GA_{4+7} are two active GAs (Karssen, 1976) which, together with applied GA_3, are known to strongly promote germination in *Leucospermum* (Brown *et al.*, 1986). Ethylene, which in *L. cordifolium* has given only variable and limited stimulation of germination (Brown *et al.*, 1986) was not included for study. A similar approach is used to that of Farrant *et al.* (1993) in a study of recalcitrant seeds. The roles of PGSs within the *Leucospermum* seed axis and its cotyledons are examined separately (the seed contains no endosperm), by means of radioimmunoassay (RIA). Analysing the considerable literature on Proteaceae germination (Chapter 1), a working model for dormancy and germination in *Leucospermum* is proposed.

Materials and Methods

The following standard abbreviations are used in the text:
ABA, abscisic acid; BA, 6-benzyladenine; CK, cytokinin; DHZ, dihydrozeatin; DHZR, dihydrozeatin riboside; GA, gibberellin; HPLC, high performance liquid chromatography; iP, isopentenyladenine; IPA, isopentenyladenosine; PGS, plant growth substance; RIA, radioimmunoassay; Z, zeatin; ZR, zeatin riboside.

1. Endogenous hormone experiment

Seed material

Six and 18-month old batches of hand-sorted *Leucospermum glabrum* seed were mixed and scarified in (c) H_2SO_4 (Brits and Van Niekerk, 1976). Seeds were washed (Brits and Van Niekerk, 1976), soaked in distilled water at 20 °C for 24 h, followed by superficial drying and fungicide treatment (Chapter 2).

Incubation regime and harvesting of seeds. This study was done prior to Chapter 4.4 and the optimal germination temperature for *L. glabrum* was provisionally estimated to be a 10 °C (16 h) x 26 °C (8 h) alternating regime. The effect of this regime on germination percentage and rate of scarified and non-scarified seed was compared with that of scarified seed incubated under constant intermediate (18 °C) and the constituting low and high temperatures respectively (Figure 1). Seeds were incubated on filter paper in petri dishes (Brits and Van Niekerk, 1976) and harvested at 0, 3, 7, 14, 28 and 42 d of incubation. Harvested seed groups were freeze-dried for 24 h, transferred to airtight containers and stored desiccated at -60 °C until all harvested groups could be extracted simultaneously for PGSs.

Dissection of embryos. Desiccated seed groups were removed from storage, allowed to attain equilibrium with room temperature and transferred to water for 1 min. to break the hard testa (Chapter 6.2). Following superficial drying with a paper towel seeds were again freeze-dried, for 12 h. Embryos were isolated and the 1.5 mm embryo tip containing the axis was removed.

Cytokinin, gibberellin and abscisic acid determination

The isolation and quantitation of the cytokinin, gibberellin and abscisic acid complement in the cotyledon and axis material was achieved as reported previously (Cutting and Lyne, 1993; Cutting *et al.*, 1991) using high performance liquid chromatography (HPLC) immunohistograms. The conditions used for extraction and purification of PGSs, HPLC separations and PGS quantitations by RIA, are detailed in Chapter 2.

2. Bioassay of CKs in non-imbibed Leucospermum glabrum *seeds*

RIA of CKs was complemented by bioassay of non-imbibed embryo axes and cotyledons. Samples (0.5 g) of *L. glabrum* embryo axes and cotyledons were prepared, extracted and HPLC separated (1 min fractions) as described above for RIA. Cytokinin activity of fractions was estimated by the soybean callus bioassay (Miller, 1965).

3. Exogenous hormone experiment

A 24 month-old batch of the same *L. glabrum* seed source used in the endogenous hormone experiment and an 18-month old batch of mature, hand-sorted *L. cordifolium* seed was used. The pericarp around seeds was removed either by soaking in water at 40

°C for 2 h, mild rubbing and washing, resulting in an intact testa (Chapter 6.2) or by (c) H_2SO_4 scarification (Chapter 2). Pericarpless intact and acid scarified seed were dried at 20 °C for 24 h and soaked in either of three PGS solutions or distilled water, for 24 h. Seeds were removed from PGS solutions, washed, and incubated in petri dishes on a damp, single sheet of Whatman no. 1 filter paper under either of three different temperature regimes in germination trays. From preliminary work a combined suitable alternating temperature regime for *L. glabrum* and *L. cordifolium* germination was estimated to be 8x26 °C. Six replicates (petri dishes) of 25 seeds per treatment were used. Seeds in which the radicle had protruded at least 1 mm were scored as germinated and germination was recorded weekly for 12 weeks.

The three factors and treatment levels of the experiment are summarized as follows:

Factor 1:	Temperature	Regime:
	1. Constant low:	8 °C
	2. Constant high:	26 °C
	3. Low x high alternating:	8 x 26 °C

Factor 2: PGS and oxygenation (= treatments 1-4 scarified)

1. Benzyladenine (BA)	150 $mg.l^{-1}$
2. GA_{4+7}	150 $mg.l^{-1}$
3. BA + GA_{4+7}	150 $mg.l^{-1}$ of each
4. Control 1	Exotesta scarified
5. Control 2	Exotesta intact

Factor 3: Species:

1. *Leucospermum glabrum*
2. *Leucospermum cordifolium*

Statistical treatment:

The same experiment was carried out three times, i.e. once for each temperature regime. Each experiment was a randomized blocks design which consisted of 10 treatment combinations with 6 replications (blocks, germination trays). The treatment combinations were 3 hormone combinations and 2 controls for each of two species, resulting in an overall treatment structure of the form 5x2x3. The results were analyzed using hierarchical analysis of variance with treatments randomized within blocks and blocks randomized within temperature regime. Analyses of variance were performed on the arcsine transformed (for normality) percentage germination data of each variable using Genstat 5 statistical software. Student's t-Least Significant Difference were calculated to compare treatment means.

Results

Endogenous hormone experiment

L. glabrum seeds incubated parallel with the harvested batches started germinating after d 14 and attained a relatively low maximum of only 28% at d 56 (Figure 1). HPLC separation of the partially purified extracts from *L. glabrum* axes and cotyledons yielded several peaks with immunological activity cochromatographing with GA 1/3, IPA/iP, Z, ZR, DHZ and DHZR (Figure 2).

Exogenous hormone experiment

1. *L. glabrum*. Germination response of this species was so poor (6.6% on average for the experiment) that treatment effects were rarely statistically significant. Poor germination was possibly due to deteriorating seed quality with age, in addition the low temperature of 8 °C in the alternating regime was sub-optimal for *L. glabrum* (Chapter 4.4). No treatment x temperature regime interaction was found (P = 0.73). The scarified control (7.6% average) germinated significantly better than the intact control (3.3% average, $P<0.01$). BA application did not suppress germination and GA_{4+7} application did not affect germination response significantly. On account of the very poor germination response the interpretation of applied PGS effects will be limited to the results with *L. cordifolium*.

2. *L. cordifolium*. Statistical interaction between temperature regime and treatments was highly significant $P<0.001$) and the results are therefore discussed separately for temperature regimes. Intact control seeds (treatment 5) germinated the poorest (average 13%, compare Figure 4), giving less than half the average germination response of acid scarified controls (treatment 4). The scarified control (treatment 4) under alternating 8x26 °C temperature gave the highest germination percentage, 57%, for the experiment as a whole, i.e. over all temperature regimes (Figure 4). The non-scarified control under the alternating regime (treatment 5) also gave a relatively strong germinating response, thus echoing treatment 4, compared with treatment 5 control values under the other two regimes.

Gibberellins

1. Endogenous levels. GA_1/GA_3 rose immediately and transiently in *L. glabrum* cotyledons only, peaking by d 3 of alternating temperature incubation, but remaining low in the axis (Figure 3).

2. Exogenous application. GA *vs.* no-GA treatments in *L. cordifolium* gave a strong interaction with temperature regime ($P<0.001$). Under alternating 8x26 °C temperature GA_{4+7} (treatments 2, 3) suppressed the germination of *L. cordifolium* markedly, when compared with the scarified control, treatment 4 (Figure 4). However under constant low 8 °C temperature GA treatments gave substantially higher responses than other treatments ($P<0.05$). Here the effect of GA application alone (treatment 2, 54%) approaches the maximal response level found under alternating temperature in the non-treated (control) treatment 4 (Figure 6). At constant high 26 °C temperature GA also promoted germination (Figure 4).

Cytokinins

1. Endogenous levels.

Free bases Z and ZR. Z initially measured at relatively high and intermediate levels in non-incubated (d 0, freshly imbibed) cotyledons and axes respectively of *L. glabrum* (Figure 3). In cotyledons Z levels dropped sharply to near-zero during the first days of incubation and then rose rapidly to a transient, intermediate level at day 7. Z levels finally fell in cotyledons, reaching a very low level immediately prior to the beginning of the visible germination phase (radicle appearance) at d 14 (Figure 1) and remained low for the rest of the germination period.

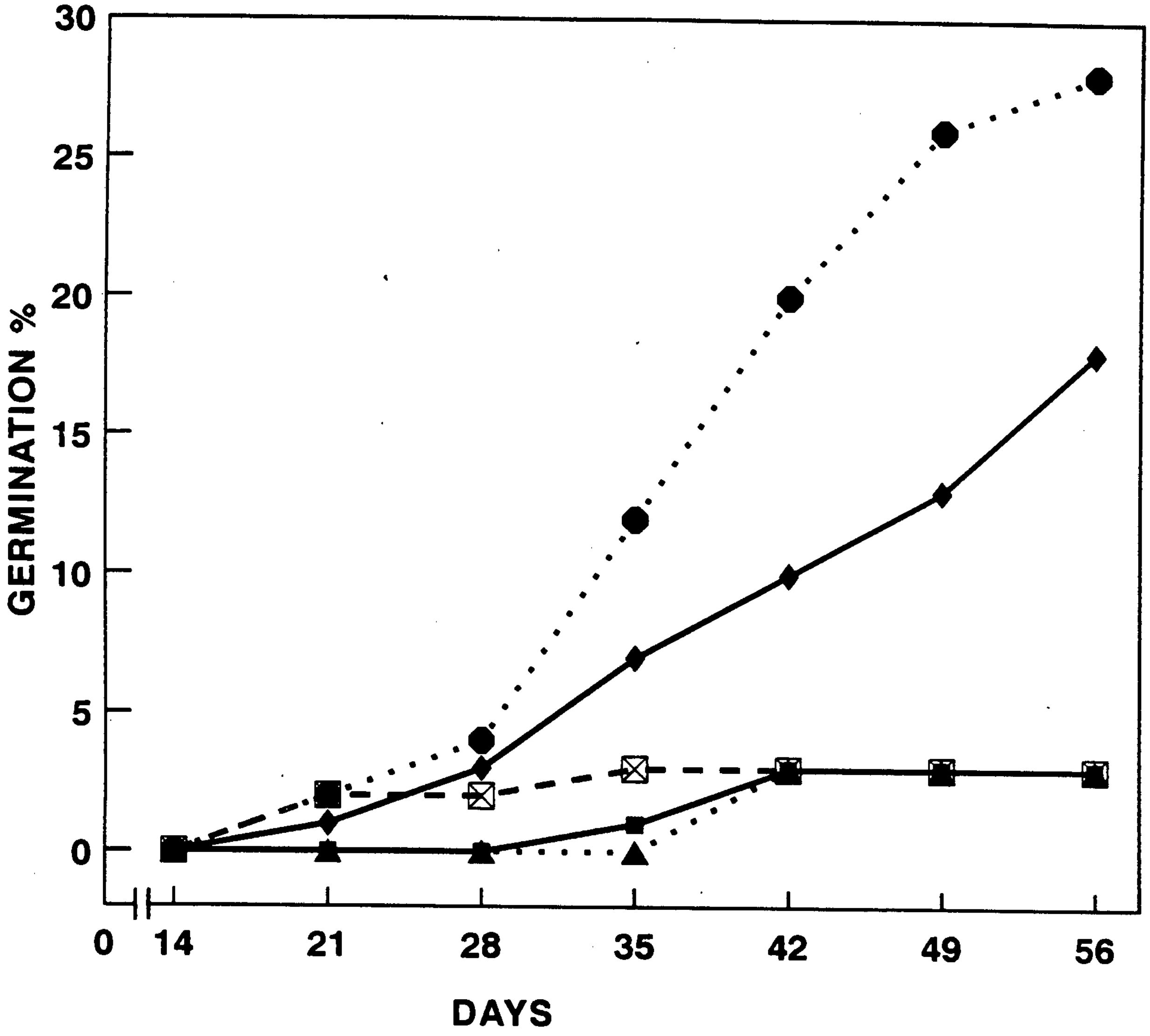

Figure 1. Germination response of *Leucospermum glabrum* scarified seeds incubated under different temperature regimes: 10x26 °C, estimated optimum (●· · · ·●); 10 °C (■———■); 26 °C (▲· · · ·▲); 18 °C, intermediate (□– – –□); and unscarified, 10x26 °C (◆—◆).

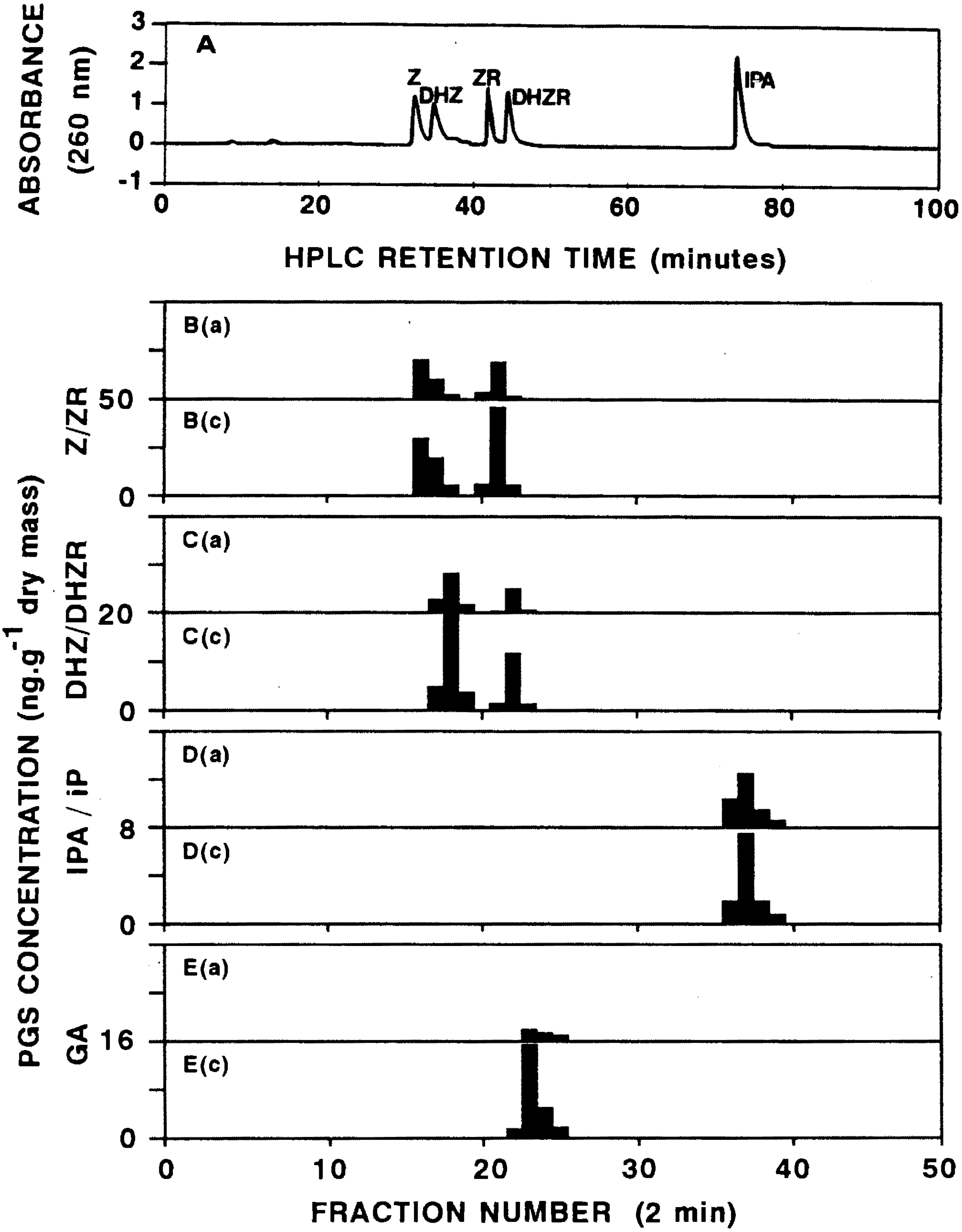

Figure 2. A: UV trace showing the retention times of authentic plant growth regulator standards after separation by HPLC; B-E: immunohistograms of Z and ZR; DHZ and DHZR; IPA; and GA_{1+3}, respectively, in *Leucospermum glabrum* seed axis (a) and cotyledon (c) extracts after 7 d incubation under optimum conditions.

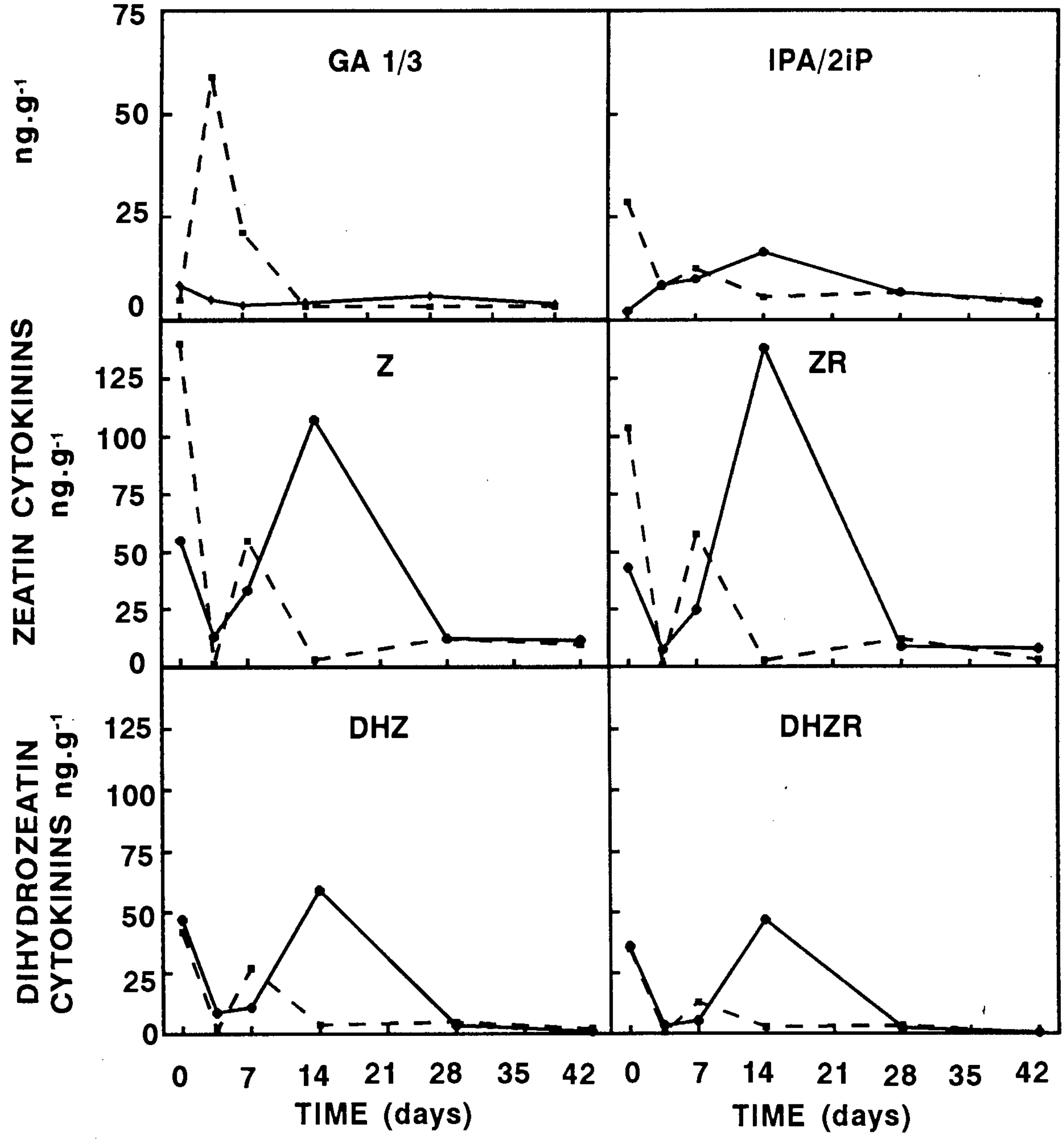

Figure 3. Plant growth substance concentrations in *Leucospermum glabrum* seed axis (●——●) and cotyledon (■– – –■) extracts from 6 incubation periods during normal germination under the optimum alternating 8 x 26 °C regime. Each point represents the total of 2-3 HPLC fractions covering the specific retention period of a PGS. Results were obtained by radioimmunoassay (three replicates).

The Z level in axes also dropped sharply after the first 3 d of incubation but then increased continuously to a maximum at d 14, corresponding in time with the minimal level attained in cotyledons and the onset of visible germination (Figure 3). Axis levels of Z again dropped after d 14, reaching similarly low levels as recorded in cotyledons. ZR levels followed a similar pattern to Z, except that the ZR level in cotyledons initially was markedly lower than that of Z, both falling to near-zero by d 3. In contrast, in the axis ZR rose to a maximum of almost 140 ng.g^{-1}, substantially higher than that attained by Z (Figure 3).

Dihydrogen derivatives DHZ and DHZR: DHZ levels followed similar patterns to that of Z, except that the initial levels in cotyledons were c. 1/3 of Z levels recorded, subsequently attaining c. 11 ng.g^{-1} by d 7, only 20% of the level attained by Z. Likewise maximum axis levels of DHZ rose to c. 59 ng.g^{-1}, substantially lower than that attained by Z (Figure 3). The levels of DHZ also fluctuated similarly to that of its riboside DHZR, the latter constantly measuring somewhat lower than DHZ levels. Thus DHZ and its riboside occurred at markedly lower levels than Z and ZR.

IPA/iP. The levels of these CKs remained relatively low in both axes and cotyledons (Figure 3).

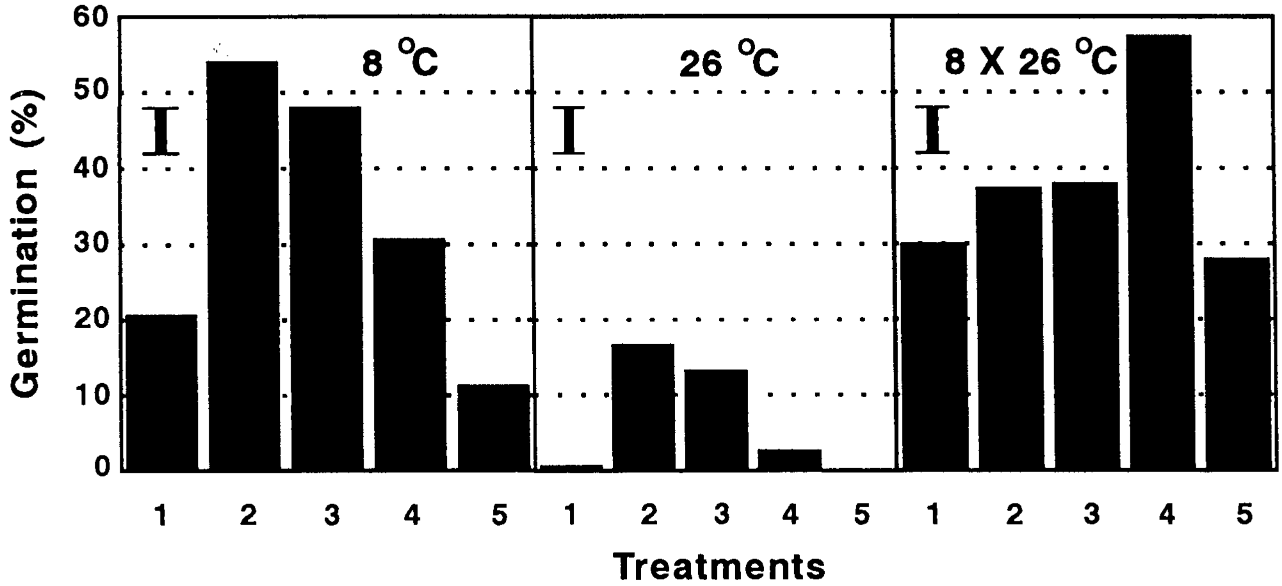

Figure 4. Germination percentage response of scarified *Leucospermum cordifolium* seeds under 3 incubation temperature regimes following treatment with BA (1), GA_{4+7} (2), BA + GA_{4+7} (3), control - scarified (4) and control - unscarified (5). Small bars represent LSD (5% level).

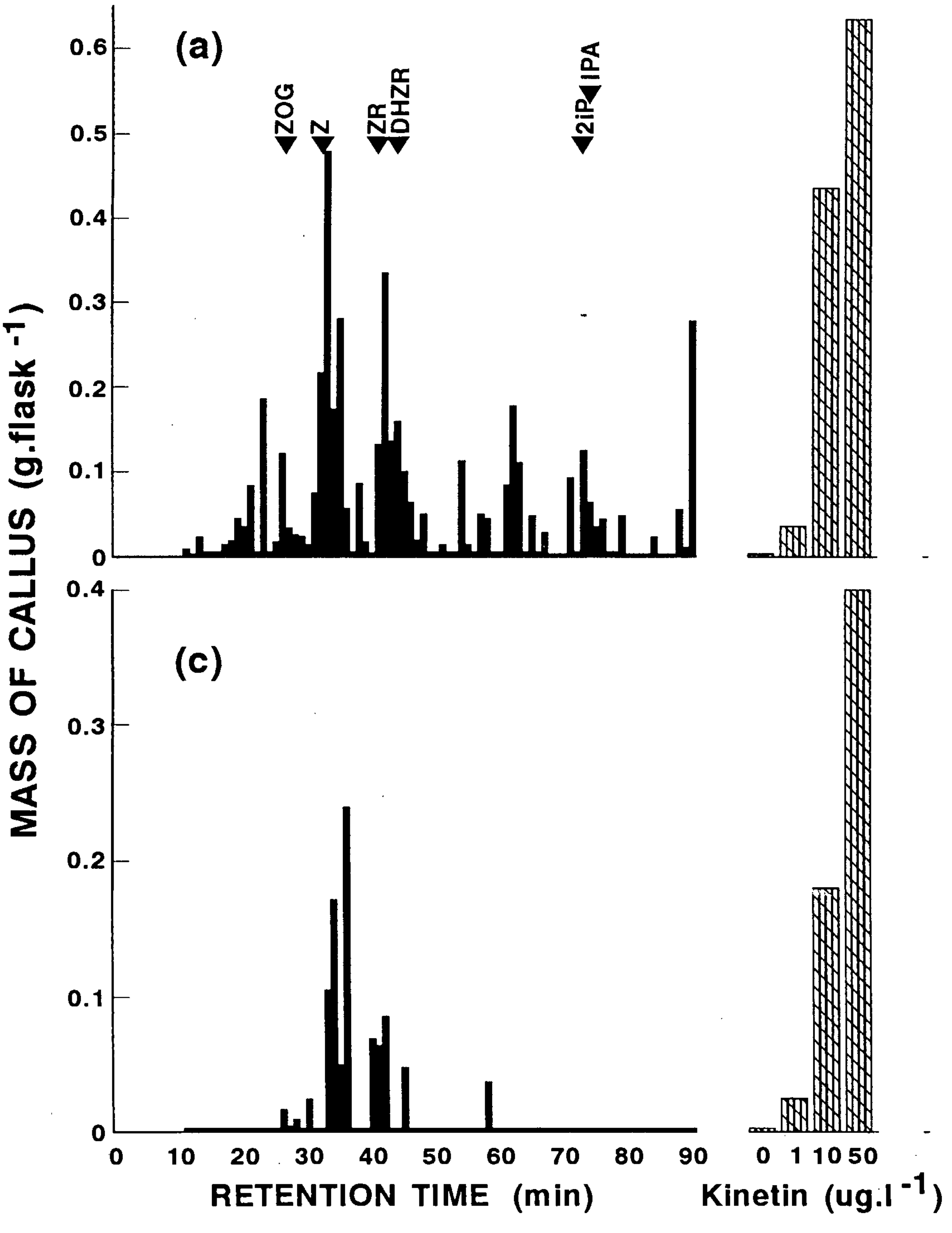

Figure 5. Distribution of endogenous cytokinin activity in non-imbibed *Leucospermum glabrum* axis (a) and cotyledon (c) extracts separated by HPLC in one-minute fractions, as reflected by the growth response of soybean callus. Each bar represents the mean of three replications. The HPLC retention times of authentic PGS standards are indicated (arrows): ZOG = zeatin-O-glucoside; Z = zeatin; ZR = zeatin riboside; DHZR = dihydro zeatin riboside; iP = isopentenyladenine; IPA = isopentenyladenosine.

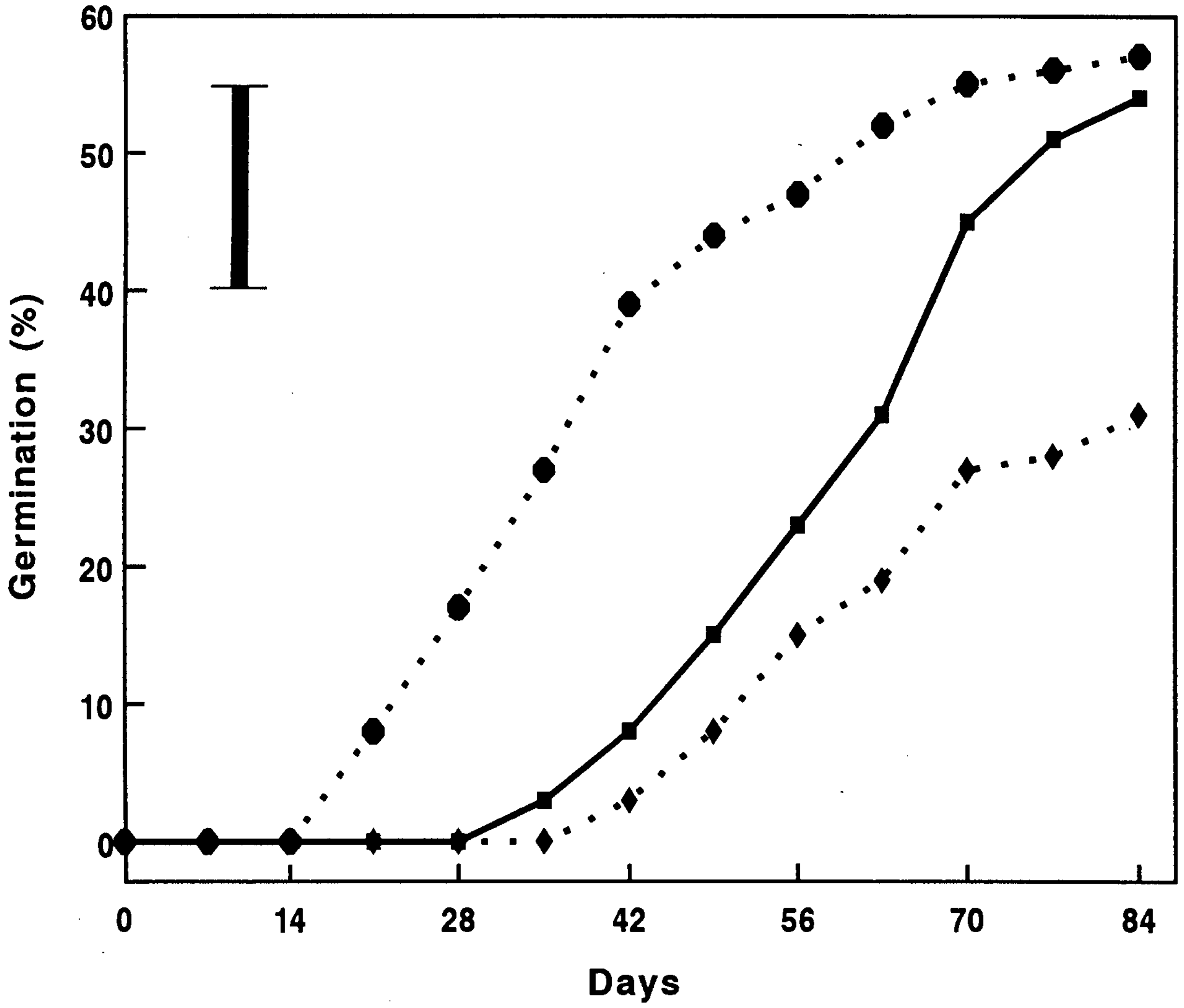

Figure 6. Germination response of *L. cordifolium* scarified seeds incubated under optimal 8x26 °C alternating regime without pretreatment (●····●); and under constant 8 °C without GA_{4+7} (◆····◆) and with GA_{4+7} pretreatment (■——■). Bar indicates LSD (5% level).

2. Bioassay of non-imbibed seeds

Z- and DHZ-like substances occurred at considerable levels in cotyledons and axes (Figure 5). In contrast to the radioimmunoassays (Figure 3) markedly higher concentrations were indicated in axes than in cotyledons.

Storage (conjugated) CKs (O-glucosides). Figure 5 also indicates the presence of a growth substance co-eluting with zeatin-O-glucoside. This occurred at a moderate to low level compared with that of free CKs.

3. Exogenous application.

BA clearly suppressed *L. cordifolium* germination under all temperature regimes (Figure 4) and scarified control seeds (treatment 4) germinated better than BA-alone (treatment 1) seeds under all temperature regimes, although statistical significance was reached only under 8 x 26 °C. In combination with GA, BA also tended to give lower germination percentages (treatment 3 *vs.* 2 - differences not statistically significant).

ABA - endogenous levels

ABA could not be detected in either axes or cotyledons.

Discussion

It is not clear why, under alternating 8x26 °C temperature (Figure 4), the degree of difference between exogenous PGS treatments and the scarified control (treatment 4) was marked ($P < 0.05$). Presumably the PGSs would not have been toxic to germinating embryos and at least an equivalent germination response to the control could have been expected.

Environmental requirements for germination

In *L. cordifolium* the consistently lower germination obtained in intact control seeds (Figure 4) emphasises the requirement for scarification of the exotesta, i.e. embryo oxygenation (relief of anoxia) in *Leucospermum* for germination to occur (Kelly *et al.*, 1992; Van Staden and Brown, 1973b) similarly as in *Leucadendron daphnoides* (Brits, 1986b; Brown and Van Staden, 1973c; Brown and Van Staden, 1975a; Van Staden and Brown, 1973a).

Constant high 26 °C temperature severely depressed germination in all treatments (Figure 4) and the average germination at constant 8 °C exceeded that at constant 26 °C by 26%, i.e. 4-fold. Although alternating temperatures are required for optimal germination in *Leucospermum* (Brits, 1986a; Chapter 4.4; Figure 1) low temperature is clearly the primary environmental condition required for germination.

The fact that under alternating temperature the scarified control response was the highest for the entire experiment (Figure 4) suggests that optimal conditions were supplied here, i.e. alternating high and low temperatures and air oxygen were the principal (or only) requirements for germination induction in *L. cordifolium*.

ABA

ABA appears to act as an antagonist or competitor of GA in seeds with primary embryo dormancy (Bradbeer, 1988; Karssen *et al.*, 1989; Le Page-Degivry, 1990). However, a physiological role for ABA in germination is not likely (Le Page-Degivry, 1990) and most mature non-dormant seeds contain no ABA or very low levels (Hilhorst and Karssen, 1992). ABA could not be detected endogenously in *L. glabrum* seed in this study. This is in agreement with Van Staden and Brown (1972) who could not detect ABA in embryos of *L. cordifolium.*

Gibberellins

Only two of the range of gibberellins were assayed in the endogenous hormone study and two others were used in the exogenous hormone application experiment. Putative roles for different GAs can therefore not be deduced from our data. However the four GAs focussed upon in this study are considered to be active in the seed germination of many perennial hardwood species.

The fact that, in *L. cordifolium*, the application of GA_{4+7} under constant low 8 °C temperature (treatment 2) increased germination to the same level as the control maximal germination response under 8x26 °C alternating temperature, treatment 4 (Figure 4, Figure 6), suggests that an essential substance for maximal germination could not be synthesized at constant 8 °C, and that this could be substituted for by exogenous GA. The strong treatment 2 response is echoed by treatment 3 (GA_{4+7} + BA - Figure 4). GA_{4+7} promoted germination markedly under both suppressive regimes of constant low and constant high temperature, which is consistent with the concept that GAs are the primary hormone group regulating the induction of germination in seeds in which dormancy is enforced by an unfavourable environment. In such seeds applied GA can replace the provision of the special environmental conditons needed for germination (Bewley and Black, 1982; Hilhorst and Karssen, 1992). It was shown unequivocally that the fulfilment of special environmental requirements in such seeds results in rising endogenous levels of GA which then induces germination (Hilhorst and Karssen, 1992; Karssen *et al.*, 1989). GA was proposed as the dormancy breaking hormone in *Protea compacta* R.Br. (Mitchell *et al.*, 1986) which requires low temperature for germination (Brits, 1986b; Mitchell *et al.*, 1986). In *L. cordifolium* GAs were proposed as by far the most active group of hormones in dormancy breaking (Brown *et al.*, 1986). In our study endogenous GA levels rose during the early incubation phase under optimal environmental conditions (Figure 3) which is consistent with the foregoing line of thought. Based on these combined considerations it is proposed, as a working hypothesis, that the inductive substance(s) in *Leucospermum* seed germination is one or more of the active GA forms.

In the exogenous hormone experiment the constant low 8 °C temperature regime differed from the alternating 8x26 °C regime, where maximal germination occurred, only in the omission of daily high temperature. Therefore the provision of high temperature may be inferred in either GA synthesis, GA interconversion from storage forms, or tissue sensitizing to GA (Trewavas, 1986) in *Leucospermum* seeds. However at constant high 26 °C, GA_{4+7} promoted germination relatively strongly over the control treatment 4, either as GA_{4+7} alone or as (GA_{4+7} + BA) (Figure 4). Mitchell *et al.* (1986) also found improvement of germination of *P. compacta* seeds at high temperature when seeds were treated with a commercial mixture of (GA_{4+7} + BA). This implies that GA could also not be synthesized at constant high temperature. In *Leucadendron daphnoides* seeds incubated at 25 °C endogenous GA levels remained low, accompanied by a very poor germination response (Brown and Van Staden, 1975a). This combined with the above evidence that GA synthesis could neither occur at constant low temperature without a period of daily high temperature, further indicate that only a combination of low and high diurnal temperature will induce GA synthesis, as is represented by the control maximal germination response under alternating 8x26 °C temperature (treatment 4, Figure 4) and within the context of rising GA levels under such conditions (Figure 3). In *L. daphnoides* high endogenous GA levels resulted from alternating 10 x 20 °C incubation of oxygenated seeds and were followed by a maximal germination response (Brown and Van Staden, 1975a). However at low temperature stratification GA levels did not increase, and germination response was only moderate. This suggests that in *L. daphnoides* the short period of high temperature experienced daily, in combination with low temperature, determined the increase in GA levels, which agrees with the above proposal that in *Leucospermum* the daily short pulse of high temperature induced GA synthesis.

In seeds of hazel (*Corylus avellana* L.) GA is rapidly synthesized under constant high regime of 20 °C following a temporally separate regime of constant low temperature pretreatment (Ross and Bradbeer, 1971). By contrast *Leucospermum* requires a single regime of daily alternating low and high temperature (Chapter 4.4) during which GA levels rise. Moreover the alternating temperature requirement in *Leucospermum* applies during the germination process itself (germination *sensu stricto*). In hazel the temperature shift applies to two distinct phases, i.e. the breaking of primary dormancy followed by germination *sensu stricto* (Bradbeer, 1988).

Leucospermum embryos in this study did therefore not show primary dormancy, as was also proposed for *Leucadendron daphnoides* (Mitchell, 1983). Rather the relief of enforced dormancy here is indicated by the absence of detectable endogenous ABA (cf. Hilhorst and Karssen, 1992); by requirements for special environmental conditions and a responsiveness of seeds to exogenous GA application. The fulfilment of these requirements results in a rise in endogenous GA levels and the commencement, without delay, of germination *sensu stricto*.

The continued absence of GA_1/GA_3 in the *L. glabrum* axis suggests that GA is not transported from this organ to the cotyledons. Therefore GA could be synthesized *de novo*, or be interconverted, within the cotyledons at an early stage. In germinating hazel cotyledons some GA is synthesized and some released from bound forms, although the axis is the main site of GA synthesis (Arias *et al.*, 1976). In *L. glabrum* the signal produced by GA must subsequently be translocated to the axis to permit germination to proceed. The axis is the seat where the main control is exerted, where reserve digestion in proteaceous seeds begins and where growth resumption later takes place. It is not clear how GA unlocks the germinative process from within the cotyledons and subsequently induces axis activity.

The early rise of endogenous GA_1/GA_3 in *L. glabrum* cotyledons suggests that GA may be involved in the mobilization of certain reserves (large-scale mobilization of reserves is ultrastructurally detectable only at a later stage (Mitchell, 1983)). The early mobilization of reserve food to the axis is especially evident in species where mature seeds contain an underdeveloped embryo (Karssen *et al.*, 1989). In *Leucospermum* the entire embryonic axis is rudimental, the plumule and radicle being present only as two undifferentiated meristematic zones (Chapter 7). The molecular mechanisms by which GA and other PGSs induce seed germination in dicotyledons are largely unknown (Bewley and Black, 1982; Napier and Venis, 1990).

Endogenous GA and CK levels in the *L. glabrum* embryo rose in two distinct phases during normal germination. This is in line with the suggestion by Karssen (1976) that a temporal relationship exists in the action of various hormones but are at variance with the findings of Brown and Van Staden (1975a) that GAs and CKs increased simultaneously during germination of *Leucadendron daphnoides*. The patterns of CK increase in the present study were similar to those in Brown and Van Staden (1975a), but not those of GAs. GA and CK levels increased in phases also in *Acer saccharum* Marsh (Webb *et al.*, 1973).

Cytokinins

The short-lived peaks of CKs evident in both axes and cotyledons of *L. glabrum* (Figure 3) are reminiscent of endogenous CK levels found in a number of dicotyledonous species, as in *Spergula arvensis* L. (Van Staden *et al.*, 1973) and *Carya illinoensis* K. Koch (Dimalla and Van Staden, 1977). The CK patterns in the germinating *Leucospermum* embryonic axis are also broadly similar to that in the axis of the chick-pea seed, which is non-dormant (Martin *et al.*, 1987a) although events in *Leucospermum* occur over a longer time scale.

All the CKs in *L. glabrum* peaked, and fell, in both axes and cotyledons well before any external signs of germination (Figure 1 *vs.* Figure 3). Very low CK levels were detected also in germinated *L. daphnoides* seedlings post-germination (Brown and Van Staden, 1973a; Mitchell, 1983) and it therefore appears that the CKs which accumulated prior to the completion of germination *sensu stricto* are rapidly metabolized once growth is resumed by the seedling.

Exogenous BA application in this study could not confirm a key role for cytokinins in *Leucospermum* seed germination, nor are the temperature requirements for CK synthesis/interconversion indicated. The suppression of *L. cordifolium* germination by applied BA was also found in several other studies on *L. cordifolium* (Brown and Drewes, 1991; Brown *et al.*, 1986) and on *Protea compacta* (Brown and Drewes, 1991; Mitchell *et al.*, 1986). It is possibly the fact that BA is not a naturally occurring compound (Van Staden *et al.*, 1982), or that the single, large pre-incubation dosage applied here, caused failure of BA to imitate the endogenous CK situation, inhibiting the metabolism in intact seeds, as happened in several studies with applied CKs (Gallego *et al.*, 1991; McGaw, 1987; Villalobos and Martin, 1992).

Endogenous free CKs in seeds of many species increase in response to low temperature incubation (Van Staden *et al.*, 1982). Brown and Van Staden (1975a) and Mitchell (1983) found that the endogenous levels of free CKs in *Leucadendron daphnoides* embryos rose markedly, to similar levels, under both constant low temperature (5/10 °C) and alternating temperatures. This suggests that low temperature only was required for CK interconversion/synthesis. Since low temperature is also the primary temperature requirement for *L. daphnoides* (Brown and Van Staden, 1975a), *L. tinctum* Williams (Brown and Dix, 1985) and *Leucospermum* germination (Brits and Van Niekerk, 1986), it is proposed that only low temperature is required for CK synthesis/interconversion in the germination of species of nut-fruited *Leucadendron* and in *Leucospermum*.

Cytokinin controlled processes in Leucospermum *seed germination.*

Earlier work on endogenous cytokinin levels indicated a primary regulatory role for this growth substance in the germination in Proteaceae (Brown and Van Staden, 1973c; Brown and Van Staden, 1975a). The latter authors found a stimulating effect of exogenous CKs on *Leucadendron daphnoides* germination, when applied at low concentrations.

Endogenous CK changes in this study occurred in three stages. These may be correlated with salient events in germinating proteaceous embryos (Mitchell, 1983; Van Staden and Brown, 1977). During *L. cordifolium*, *L. daphnoides* and *P. compacta*

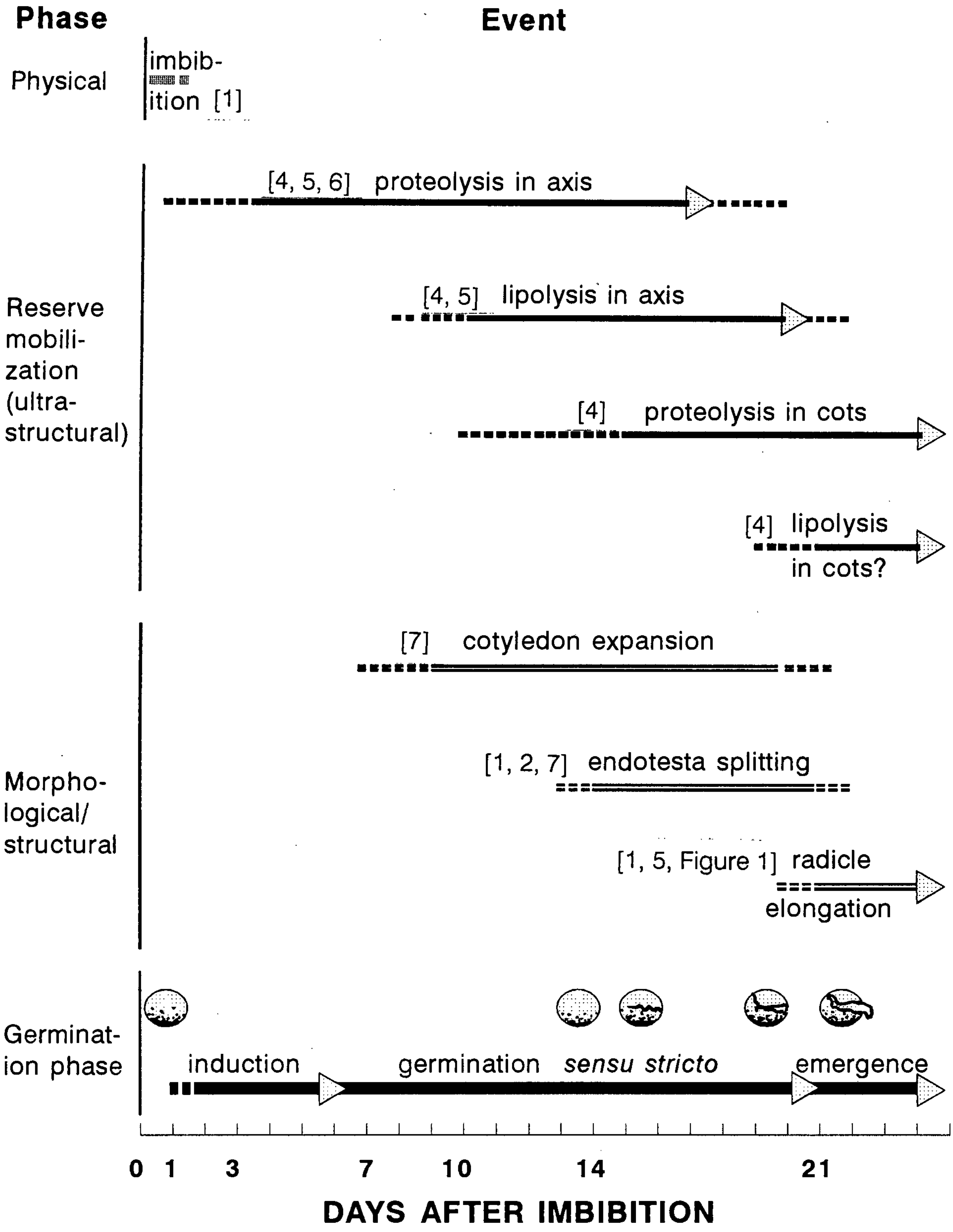

Figure 7. Diagram of known morphological, structural and ultrastructural events in Proteaceae seeds germinating under optimal conditions. Sources: [1] Brown and Van Staden, 1973a; [2] Brown and Dix, 1985; [3] Brown *et al.*, 1986; [4] Mitchell, 1983; [5] Van Staden and Brown, 1977; [6] Van Staden *et al.*, 1975; [7] Brits, unpublished.

germination the mobilization of storage proteins occurs before that of lipids, and first in the axis (Mitchell, 1983; Van Staden *et al.*, 1975). In cotyledons, protein and especially lipid breakdown is mainly a post-germinative event, in *Leucospermum* presumably to provide the considerably energy needed for seedling emergence from underground seed storage sites (Brits, 1987). The time scale of events in *Leucospermum* and other Proteaceae species during germination (Figure 7) applies roughly to *Leucospermum* in our experiment (Figure 6).

1. *First three d of incubation.* The considerable endogenous free CK content present in axes and cotyledons of freshly harvested *L. glabrum* seeds (Figures 3 and 5) falls rapidly. Our bioassay results support the RIA results that CKs occurred in marked quantities in freshly imbibed seeds, despite the discrepancy in the relative levels of CKs indicated in axes and cotyledons. Substantial levels of Z/ZR were also found in unimbibed *L. daphnoides* seeds (Mitchell, 1983). Whole *P. compacta* seeds incubated under optimal conditions gave a pattern for total butanol-soluble CKs (Brown and Van Staden, 1975b) reminiscent of Z/ZR in *L. glabrum*. In the *Leucospermum* axis the high initial free CK content (Figures 3 and 5) may mediate in early protein hydrolysis or enzyme synthesis (Figure 7). The mobilization of storage proteins in *L. cordifolium*, *L. daphnoides* and *P. compacta* occurs before that of lipids, and first in the axis (Mitchell, 1983; Van Staden *et al.*, 1975) which in *P. compacta* was observed ultrastructurally in the axis after 3 d of incubation (Van Staden *et al.*, 1975). Alternatively CKs may coordinate early with GA in inducing germination *sensu stricto* (Figure 7) by relieving enforced dormancy. Exogenous CKs when applied together with GAs act synergistically in relieving enforced dormancy in celery (*Apium graveolens* L.) seeds (Pressman and Shaked, 1991).

2. *c. day 7.* CK levels rose in the axis, but not pronouncedly (Figure 3). *De novo* CK synthesis or CK interconversion from storage forms at this stage was suggested for whole embryos of *Leucadendron daphnoides* and *L. cordifolium* (Mitchell, 1983). However during the germination period no synthesis of CKs occurs at any time in the cotyledons (Van Staden *et al.*, 1982). It is not clear whether *de novo* synthesis/interconversion occurs in the *L. glabrum* axis in this early germination phase, followed by polarized transport and accumulation in cotyledons, as was found in lupin (*Lupinus luteus* L.) (Nandi and Palni, 1989) and chick-pea (Martin *et al.*, 1987b).

Different active forms of CKs (Letham and Palni, 1983) regulate the mobilization of various classes of reserves. In *L. glabrum* Z and ZR levels were highest in our study, suggesting high activity in protein (and early lipid) breakdown in the axis during the phase of germination *sensu stricto* which has now been entered (Figure 3; Figure 7).

By d 7 a transient peak of free CKs occurred in cotyledons (Figure 3) immediately prior to the stages of cotyledon expansion resulting in endotesta splitting (Figure 7).

Germination is concluded by the radicle elongating through the split endotesta, a distinct stage occurring c. 3-6 d after the earliest signs of splitting (Figure 7 - Brits, unpublished). The rising levels of CKs in cotyledons at c. d 7 is thought to play an active role in their subsequent enlargement (cf. Brown and Van Staden, 1973a).

3. *c. day 14.* The peaking of axis CK levels by d 14 (Figure 3) immediately precedes visible radicle growth (d 21+ - Figures 1, 6, 7). This peak is presumably due to *in situ* biosynthesis and not to translocation from the cotyledons where CKs peaked shortly before. CKs were shown to be translocated from axes to cotyledons but not *vice versa* (Martin *et al.*, 1987b; Nandi and Palni, 1989; Villalobos and Martin, 1992).

The rise of axial free cytokinins at this stage suggests an active role in the subsequent stimulation of radicle growth in Proteaceae seeds (Van Staden and Brown, 1977). The involvement of free CKs in inducing embryo radicle growth has been suggested for many plant species (Van Staden *et al.*, 1982) and for *L. daphnoides* (Brown and Van Staden, 1973a) and *P. compacta* (Mitchell *et al.*, 1986) in Proteaceae.

Conclusion

Our results fit a pattern of CKs controlling key processes during germination *sensu stricto* following the induction of germination by GA, since the levels of the two GAs assayed here remained low during the main germinative period. This proposal is consistent with the dominant and varied mediating roles proposed for CKs over other PGSs, including GA, in the axial control of germination in dicotyledonous seeds (Ilan and Gepstein, 1980) following the induction of germination by GA (Hilhorst and Karssen, 1992). In *P. compacta* a dormancy breaking role for GA followed by growth resumption under CK control was suggested (Mitchell *et al.*, 1986). An example of results paralleling this model is the finding by Van Staden (1973), that dormant, light-requiring fruits of lettuce (*Lactuca sativa*) can be induced to germinate in the dark when incubated in a gibberellic acid solution and that subsequently extractable CK levels increased in germinating seeds. Thus strong parallels exist between widely divergent dicotyledonous species, including *Leucospermum*, which implicate CKs, once primary or enforced dormancy is relieved and the seed enters the main germination phase under optimal environmental conditions. However, correlation of different CKs and their peaks with specific functions in the *Leucospermum* embryo is provisional at present. The most likely direct linkage is that between high transient axial levels of free CKs by d 14 preceding the radicle elongation phase.

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CHAPTER 6: ASPECTS OF GERMINATION ECOLOGY

Contents

CHAPTER 6

ASPECTS OF GERMINATION ECOLOGY

PREFACE

Leucospermum seed ecology studies the relationships between the seed and its living and physical fynbos environments. Such interaction of seeds with their surroundings can be understood best when field work is complemented by laboratory study of seed structure and physiology (Angevine and Chabot, 1979). The latter aspects have been covered in previous chapters. "Field work" in this chapter is a series of habitat-simulating studies supported by laboratory work, e.g. the study of heat pulse influence on the testa by means of an oven (simulating the effects of fire). This is supplemented by seed bed studies under field conditions (simulating the fynbos soil environment after fire).

The environmental factors on which the present chapter focuses are primarily those that induce stress and risk to *Leucospermum* seeds in fynbos, such as fire, seasonal drought and unpredictable dry spells during the germinative season. The avoidance of such elements is the ultimate purpose of all environmentally regulated seed processes (Angevine and Chabot, 1979).

The chapter foremostly addresses the question - why is there a germination flush after fire? Previous work explains the flush in terms of diurnal temperature range but is there no more direct link to fire?

The role of smoke in germination

First explored is smoke as a possible germination stimulant. Smoke effects on seed germination of fynbos species are a subject of much interest at present. Preliminary investigation indicated no effect on *Leucospermum* seed germination by smoke, charred wood or ash. With improved control over treatment application this subject is reinvestigated.

During preparatory work it was discovered that the *Leucospermum* exo- and endotesta can be scarified (broken) by heat effects. Desiccation appeared to be the likely active principle in this process. However, Bond *et al.* (1990) found that *Leucospermum conocarpodendron* (L.) Buek seedling recruitment was strongly and positively correlated with fire intensity in fynbos. The question is therefore what is the connection between heat and desiccation in natural seed processes in fynbos? What is the nature of the observed heat effects and how may they relate to fire as a dormancy breaking cue:

Desiccation and heat effects on the testa - qualitative aspects

Firstly, does dry heat have a direct scarifying effect on the seed coat or is the effect indirect, via another principle such as desiccation?

Effects of heat pulsing, scarification and oxygenation on seed germination of three *Leucospermum* species

Based on the positive relationship found between heat-mediated desiccation and degree of scarification in the above study, the second question was asked: can heat effects, via desiccation, be quantified in both the exo- and endotesta?

Most important also was the question: can a positive effect be demonstrated of heat desiccation treatment on actual seed germination?

Related to these are the questions of whether dry heat directly stimulates the seed embryo (physiologically) and whether the effects of heat-desiccation can be distinguished from other forms of scarification and oxygenation.

Seed longevity and storage effects

Longevity in soil-stored seed banks is of pivotal importance in understanding *Leucospermum* seed ecology, yet almost no information (except circumstantial evidence) is available. Linked to this is the control and relief of severe dormancy in fynbos and the regulation of extended germination of seeds within the same and over several germination seasons. These factors are thought to play an important role in the short- and long-term survival of *Leucospermum* species. This chapter finally explores the germination behaviour of seeds under different storage conditions, including soil-storage, to test the new understanding of seed scarification through heat and its companion phenomenon, desiccation.

6.1 THE EFFECTS OF PLANT-DERIVED SMOKE ON *LEUCOSPERMUM* R.BR. SEED GERMINATION.

Introduction

Fire regimes play a major role in pyric succession in fynbos and other heathlands (Gill and Groves, 1981; Kruger, 1984; Cowling *et al.*, 1987). Fire-stimulated germination of seed has been reported for a wide variety of fynbos species (e.g. Levyns, 1935). Enhanced germination in a number of Californian chaparral species following exposure to charred wood (Keeley, 1986) and extracts of charred wood (Keeley and Pizzorno, 1986) have been reported. In the myrmecochorous (ant-dispersed) genus *Leucospermum* the existence of fire-related cue(s) for population recruitment has been postulated (Rourke, 1972; Bond *et al.*, 1990). Some of these have been identified, e.g. low and high temperature requirements during incubation (Brits, 1986; 1987), and desiccation-mediated scarification of the seed coat layers (Chapter 6.2). Extracts of post-fire ash were found not to exert any significant influence on the germination of *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade seeds (Pool, 1985).

L. cordifolium seeds germinate close to 100% of viable seeds using oxygenation treatment and incubating seeds under optimal alternating temperatures (Brits, 1986). However many other *Leucospermum* species do not (Chapter 4.3) and only two-thirds of *L. glabrum* seeds, for example, germinated when oxygenated and incubated under optimal alternating temperatures (Chapter 4.4). No indication could yet be found why a proportion of seeds remain dormant and the possibility of an unknown additional dormancy factor can not be ruled out. In view of the success with smoke treatment of a fynbos species by de Lange and Boucher (1990) it was logical to test smoke as a possible dormancy breaking cue in *Leucospermum* germination. Brown (1993) reported some success in stimulating germination of Proteaceae species with smoke. In this study it was decided to compare a "difficult" species such as *L. glabrum* with the known "standard", *L. cordifolium*. It was also important to simulate the natural soil-storage condition of seeds, i.e. seeds are pericarpless and the seed coat is either intact (newly dispersed seeds) or scarified (old seeds) (Chapter 6.4).

Materials and Methods

Experiment 1.

In the first of two experiments the pericarp around seeds was removed either by soaking in water at 40 °C for 2 h, rubbing and washing, resulting in an intact testa (Chapter 6.2) or by (c) H_2SO_4 scarification (Chapter 2). Pericarpless intact and acid scarified seed of

L. cordifolium and *L. glabrum* were dried at 20 °C for 24 h and planted 30 mm deep in fynbos topsoil in seedling trays. The trays were smoked using the method of de Lange and Boucher (1990). The soil was wetted and left to stand for 48 h, after which seeds were removed, washed in running water and air dried at 20 °C for 12 h to prepare them for inspection. Control seeds received the same treatment without smoke. The seed testas were inspected under a stereomicroscope, transferred to and incubated in petri dishes on a damp, single sheet of Whatman no. 1 filter paper under the optimal alternating temperature regime for *L. cordifolium* [8 °C (16 h) x 24 °C (8 h)] (Brits, 1986). Six replicates (Petri dishes) of 25 seeds per treatment were used. Seeds in which the radicle had protruded at least 1 mm were scored as germinated and germination was recorded weekly for 12 weeks.

Statistical treatment. The experiment was carried out once. The trial was a randomized blocks design which consisted of 8 treatment combinations with 6 replications (blocks, germination trays). The treatment combinations were 2 smoke treatments for each of 2 scarification treatments, for each of 2 species, resulting in an overall treatment structure of the form 2x2x2. One-way analysis of variance was performed on the LOG(X + 1) transformed data (for normality) of each variable using SAS statistical software version 6.04 (SAS Institute Inc., Cary, NC, USA). Means of interactions and main effects not involved in interactions were calculated. Student's t-Least Significant Difference was calculated to compare treatment means.

Experiment 2.

In the second experiment pericarpless intact and acid scarified seeds of *L. glabrum*, prepared as in Experiment 1, were soaked in a concentration series of smoke extract (De Lange and Boucher, 1990) in distilled water, of 0, 1/250, 1/50 and 1/10, for 24 h. Seeds were incubated in petri dishes under an alternating temperature regime suitable for *L. glabrum* [8 °C (16 h) x 26 °C (8 h)] (Chapter 4.4). Six replicates (Petri dishes) of 25 seeds per treatment were used and germination was recorded as in Experiment 1.

Statistical treatment. The experiment was carried out once. The trial was a randomized blocks design which consisted of 8 treatment combinations with 6 replications (blocks, germination trays). The treatment combinations were 3 smoke concentrations and 1 control for each of 2 scarification treatments, resulting in an overall treatment structure of the form (3 + 1)x2. One-way analysis of variance was performed on the LOG(X + 1) transformed data (for normality) of each variable using SAS statistical software version 6.04 (SAS Institute Inc., Cary, NC, USA). Means of interactions and main effects not involved in interactions were calculated. Student's t-Least Significant Difference was calculated to compare treatment means.

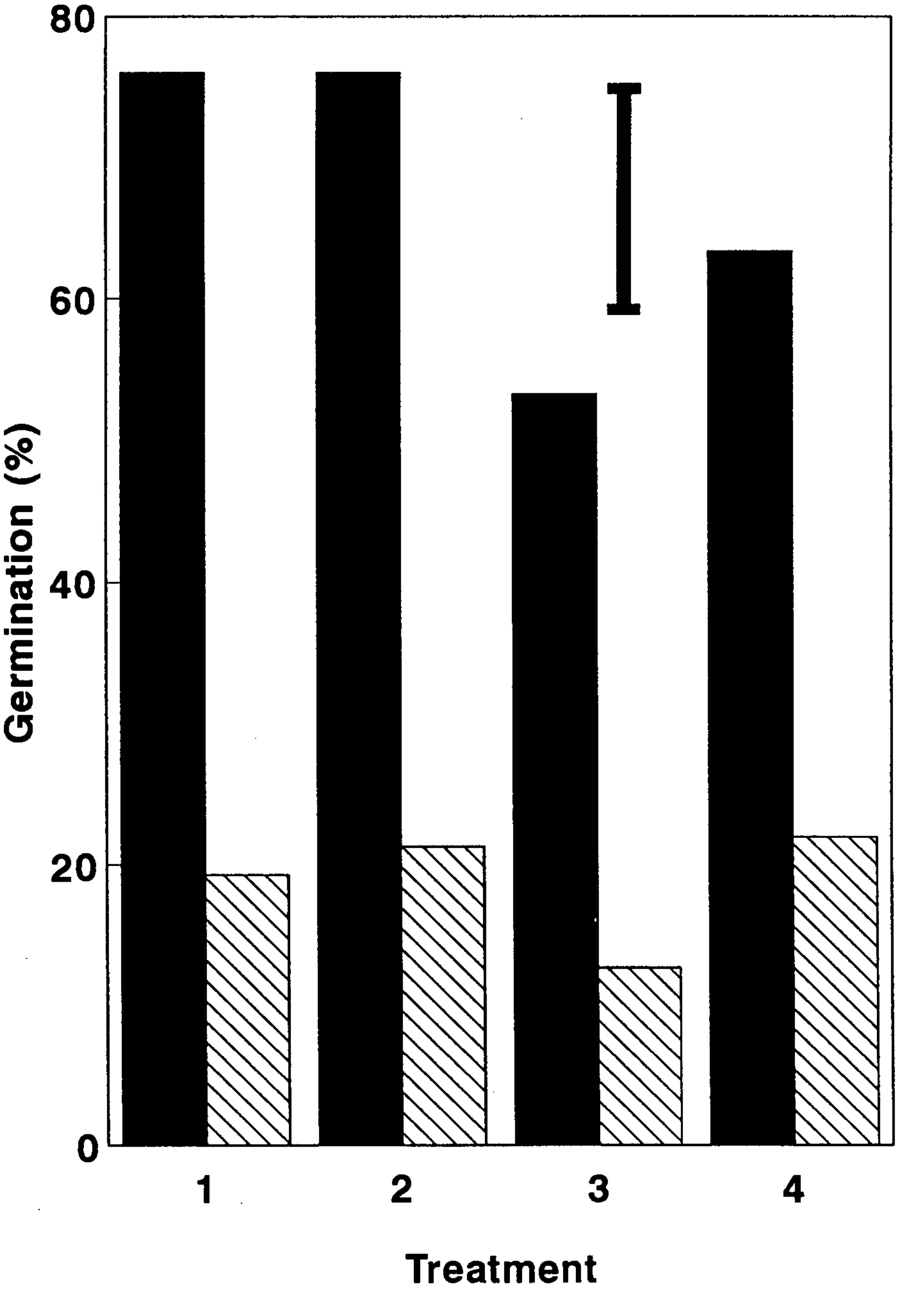


Figure 1. The effect of smoke treatment in the soil on the percentage germination of intact and scarified seeds of *Leucospermum cordifolium* (■) and *L. glabrum* (▨): (1), intact controls; (2), scarified control; (3) smoke-treated, intact seeds; (4), smoke-treated, scarified seeds; small bar represents LSD (P = 0,05).

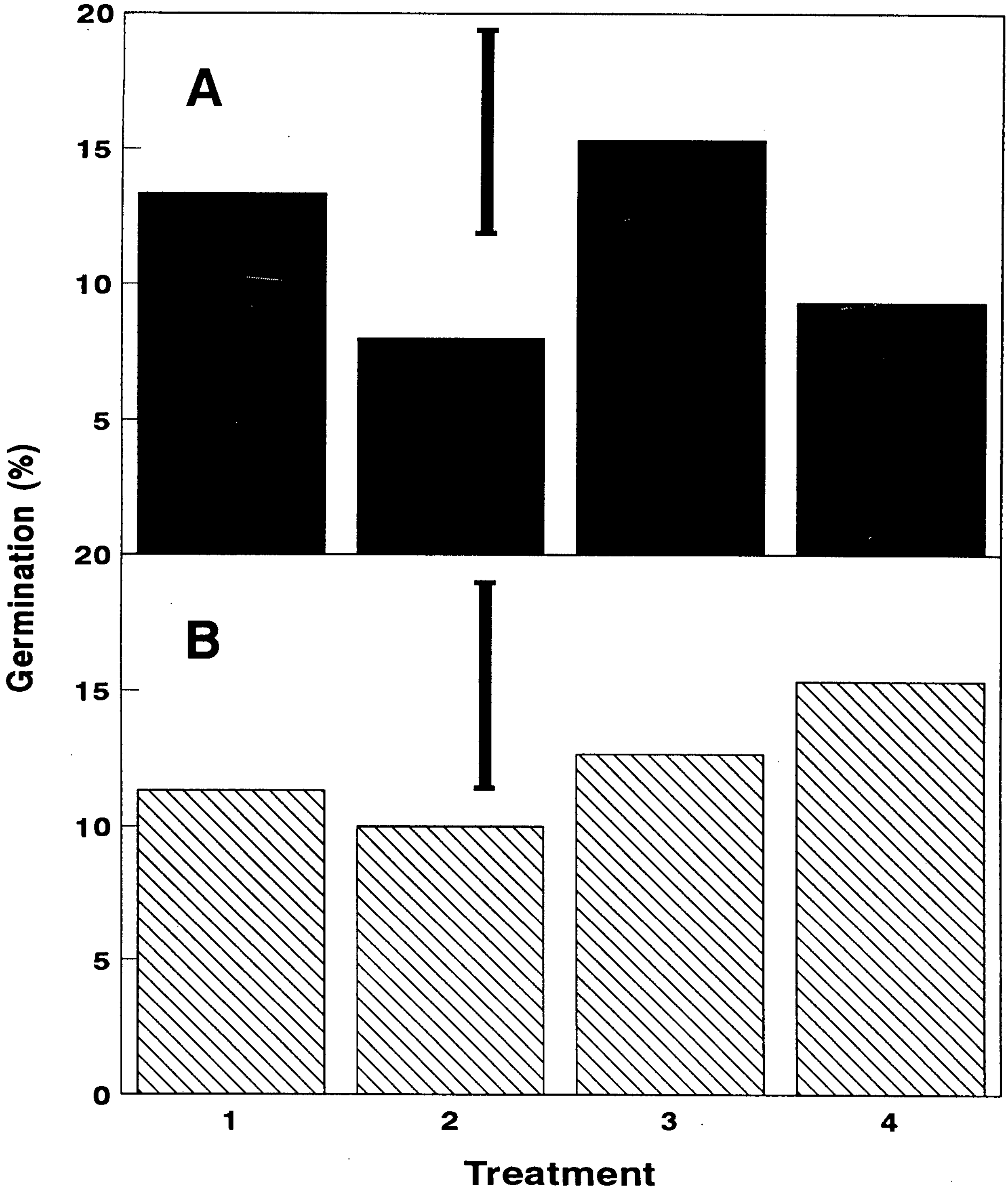


Figure 2. The effect of treatment with smoke extracts on the percentage germination of scarified (A) and intact (B) seeds of *Leucospermum glabrum*; seeds were soaked, prior to incubation, in (1) extract: water 1:10; (2) 1:50; (3) 1:250; or (4) water (control); small bar represents LSD (P = 0,05).

Results and Discussion

Experiment 1.

The testas of smoke treated and control seeds appeared not to differ, suggesting that smoke did not alter the testa mechanically.

Control seeds germinated significantly better than smoked seeds (Figure 1, $P < 0,01$). Increased fungal growth on smoked seeds suggested that seeds may have been damaged by smoke, causing leaching and diffusion of metabolites out of the seed and thus providing a growth substrate for fungi. *L. glabrum* seeds germinated markedly lower than the estimated viability (78%) but maximal *L. cordifolium* germination approached the viability estimate of 80%.

Experiment 2.

Results were variable (Figure 2) and the analysis of variance F-tests were not significant ($P >> 0,05$), indicating no significant reaction of germinating *L. glabrum* seeds to smoke extract treatment.

The negative/inconclusive results of both experiments suggest that smoke does not play a role in *Leucospermum* seed germination in nature.

6.2 DESICCATION AND HEAT EFFECTS ON THE *LEUCOSPERMUM* TESTA - QUALITATIVE ASPECTS

Introduction

The striking phenomenon in nature of *Leucospermum* seeds germinating uniformly *en masse* after fire (Rourke, 1972), is not yet fully explained. Bond, Le Roux and Erntzen (1990) found that *Leucospermum conocarpodendron* (L.) Buek seedling recruitment was strongly and positively correlated with fire intensity in fynbos. A mechanism for this has not been proposed. Smoke could not yet be shown to stimulate germination in *Leucospermum* (Chapter 6.1) as is the case with other fynbos species (Brown, 1993). This study reports on a new eco-physiological phenomenon in fynbos discovered in *Leucospermum*. It appears to be another fire-related stimulus of germination, by means of breaking of testa-imposed dormancy.

The *Leucospermum* "seed" is an achene, the layers of which consist of a papery pericarp surrounding a testa which comprises an exo- and endotesta (Figure 1). The endotesta is a hard shell which mechanically protects the embryo (Chapter 3). The endotesta can usually resist breaking by even strongly applied finger pressure. The embryo is oxygen deprived as a result of the oxygen impermeable exotesta and this strongly enforces seed dormancy in intact seeds (Van Staden and Brown, 1973; Chapter 4.3; Chapter 6.3). Natural soil scarification, such as microbial decomposition of the exotesta, could lead to oxygenation, and thus germination, of such seeds (Chapter 6.4).

In experimental work *Leucospermum* seed is routinely dried, following soaking in lukewarm water to remove the pericarp. During such work it was noticed that the exotesta showed extensive breaking and curling when oven dried at a moderately warm temperature of 40 °C. When seeds were transferred to water soon after oven drying, it was found that the hard endotesta started breaking as well. This phenomenon was investigated further.

Materials and Methods

The techniques used in this section distinguish between scarification effects on the two testa layers. The direct effects of heat and indirect desiccation effects mediated by heat are also distinguished.

Adapted from Brits *et al.* (1993)

Pericarp removal

Fresh, mature, hand sorted achenes (pericarp present) of *L. cordifolium* (Salisb. ex Knight) Fourcade were used in this study. Dried achenes were soaked for 2 h in water at 40 °C to soften the pericarp. Achenes were submerged completely to ensure wetting of all floating achenes. After soaking, the achene batch was rubbed gently in a cotton bag or individual achenes squeezed gently to remove seeds from their loose pericarps without damaging the exotesta. Seeds were dried lightly with paper towel and left to dry further at 22 °C at ambient relative humidity (RH) for 48 h. Seeds were then inspected with a stereomicroscope and only those with an intact (undamaged) exotesta selected for the following experiments.

Heat treatment

The seed source was mixed thoroughly and sorted into random samples of ten. These were transferred to small nylon gauze bags with mesh size ±2 mm. The nylon mesh bags were placed on a preheated revolving wire gauze platform (15 rpm) suspended in an oven with forced air circulation. At set time intervals bags were removed from the oven and immediately transferred to 50 ml Schott bottles, preheated to the same temperature to ensure equal RH. The lids were screwed down to form an airtight seal and the bottles left to equilibrate with room temperature (22 °C) for 2 h.

Recording of exotesta breakage

After cooling, seeds were removed from bottles and nylon bags and immediately observed under a stereomicroscope and scored individually for degree of exotesta breakage (Figure 2). This was expressed as the estimated degree of exposure of the underlying endotesta on a 0-4 scale of 0 - no exposure (exotesta intact) to 4 - endotesta fully exposed (i.e. exotesta fully removed from seed). These observations were completed within ten min and the subsequent effect of hydration on the testa structure tested by immediately transferring the seeds to water.

Recording of endotesta breakage

Seeds were transferred to water at 20 °C for 3-5 min, removed and dried lightly with a paper towel. The exotesta remnants were removed carefully with a scalpel. Seeds with a visibly broken endotesta were recorded individually by inspection under the stereomicroscope (Figures 2 and 3) and these seeds removed. To detect invisible hairline cracks in the endotesta the rest of the seeds were again transferred to water, for 3 min. Individual seeds of these were dried partially and re-examined under the microscope for hairline cracks (Figure 4) and cracked seeds recorded. The remainder of the seeds were firmly pressed between forefinger and thumb and those breaking were recorded. The scoring on an ordinal scale was as follows:

4, parts of endotesta broken out, exposing the embryo (Figure 3); 3, visibly cracked (Figure 2); 2, hairline crack when wetted (Figure 4); 1, no crack, but breaks if pressed between fingers; 0, does not break with finger-press test.

Experimental schedule

Preliminary test. Samples of 10 seeds with intact exotestas were randomly selected. In one set of treatments the seeds were scarified by hand removal of the exotesta before heat exposure. All samples were then exposed to 40 °C for 48 h and treated as follows (two replicates per treatment).

(a) Seeds heated, directly transferred to cold (20 °C) water.

(1) Samples with exotestas present (originally intact seeds) were transferred from heat to water.

(2) Samples with exotestas removed (scarified seeds) were treated likewise.

(b) Seeds heated, cooled prior to transfer to cold water.

(3) Exotesta present as in (1), seeds were transferred to airtight bottles, cooled for 2 h at room temperature and transferred to cold water.

(4) Exotesta removed, seeds treated as in (3).

(5) Exotesta present, seeds heated and cooled, followed by hand removal of the exotesta, then transferred to cold water.

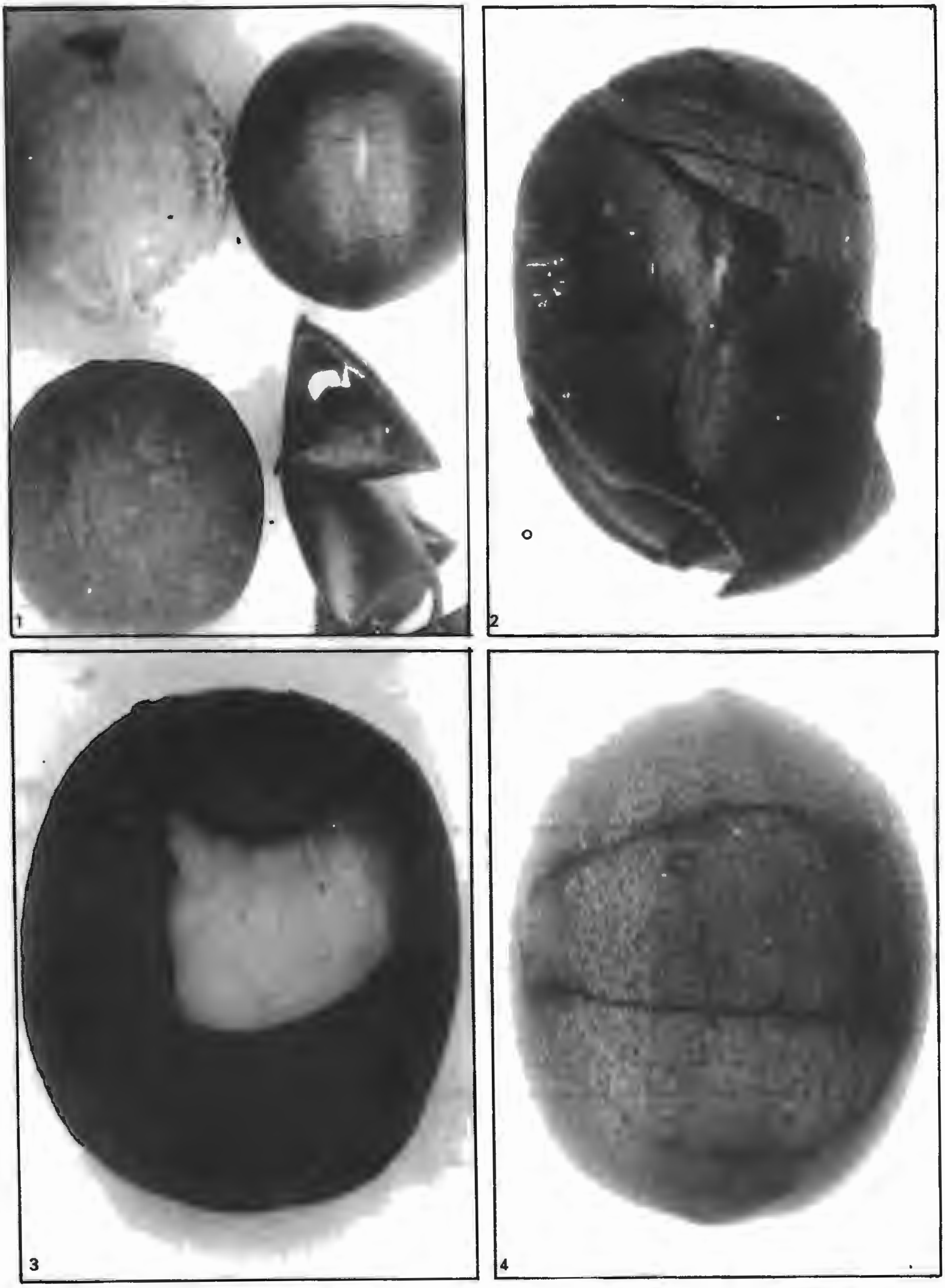
(c) Seeds heated, cooled, rehydrated, transferred to cold water:

(6) Seeds treated as in (5) but prior to transfer to cold water were allowed to hydrate slowly in air at room temperature for 24 h at ambient relative humidity (RH) (45%) and then at 100% RH for 24 h (suspended over water in a closed container).

Statistical trials

Trial 1. The pericarps of seeds were removed and seeds with intact exotestas selected. Seed samples (replicates) were exposed for varying periods to 40 °C (Figure 5) before removal from the oven and scoring for exo- and endotesta breakage. Seeds exposed for 6 h were rehydrated in moist air by exposure to 100% RH (seeds suspended over water in an airtight container) for 16 h at 22 °C before testing. Separate seed samples were suspended over preheated water at 40 °C for 6 h and scored for exotesta breakage.

Trial 2. In the second trial seeds were suspended over (c) H_2SO_4 or dried silica gel in airtight containers. Samples were removed from each container at 24 h intervals and scored for exo- and endotesta breakage. Two samples were rehydrated after 96 or 72 h desiccation over (c) H_2SO_4 or silica gel respectively, by exposure to 100% RH for 16 h at 22 °C before testing for endotesta breakage.



Figures 1-4. Stereomicroscope photographs of *Leucospermum cordifolium* achene/seeds. Figure 1. Achenes with different layers removed; top row: intact dried achene with papery pericarp present; pericarp removed, intact exotesta visible; bottom row: exotesta removed, intact endotesta visible; complete exotesta after removal from seed. Figure 2. Seed dried at 40 °C for 5 h, cooled, immersed in water and redried. Note broken exotesta and large crack in endotesta. Figure 3. Seed treated as in 2. and exotesta removed. Note exposed embryo where part of endotesta broke out. Figure 4. Seed treated as in Figure 3, returned to water and partly dried, causing three hairline cracks to become visible. Length of seed c. 6mm.

Statistical treatment. The two statistical trials were laid out as completely randomized designs with two replicates. Each experimental unit (replicate) consisted of a 10-seed sample. The first trial consisted of 14 treatments. These treatments consisted of a 5x2 factorial with 5 levels of time exposure (h) evenly spaced on a LOG scale and 2 breakage types (exotesta and endotesta) plus 2 control treatments and 2 hydration treatments. The second trial consisted of 22 treatments, consisting of a 3x2x2 factorial with 3 levels of time exposure evenly spaced on a linear scale, 2 breakage types (exotesta and endotesta) and 2 seed treatments (H_2SO_4 and silica) plus 2 additional 96 hour exposures with H_2SO_4 treatment, plus 4 control and 4 hydration treatments. For both trials the 10 seeds were rated individually on a 5-point ordinal scale.

The data (ordinal) were analysed using a general linear model (GLM) technique. The PC-PLUM program originally written by Peter McCullagh was used to analyse the data for both trials. Comparisons of interest were tested by means of analyses of deviances, Tables 2 and 3; Figures 5 and 6 show represented means on the underlying scale (McCullagh and Nelder, 1989).

Results and Discussion

Preliminary test

The preliminary non-statistical test was performed to answer an initial question: is the cracking of the endotesta when moistened following mild heat treatment due primarily to (a) the difference in temperature between seeds and water (e.g. as hot glass would react to cold water); (b) to period of exposure (indicating permanent heat-induced changes in the seed coat) or (c) to desiccation effects of heat treatment (temporary changes which are the product of heat intensity x duration of exposure)?

Treatments 1-5 resulted in extensive cracking of the endotesta, affecting all samples (data not presented). Where the exotesta was absent (scarified treatments 2, 4 and 5) seeds showed extensive breaking of the endotesta as well, leaving many embryos exposed (Figure 3); however with the exotesta present (treatments 1 and 3) little breaking of the endotesta with embryo exposure occurred. Rehydrated (treatment 6) seeds showed no cracking and seeds could also not be broken by finger pressure.

The fact that treatment 6 seed coats regained their mechanical strength after exposure to high RH in air suggests that the disintegration of the endotesta is due to wetting of the testa in the dehydrated state, irrespective of seed temperature. Endotesta breaking with embryo exposure was apparently due primarily to the fact that the exotesta, which forms a supporting covering around the endotesta, was absent during the breaking process. Seed temperature apparently did not affect the degree of endotesta cracking or breaking in treatments 1 vs. 3 or treatments 2 vs. 4, respectively.

Effects of desiccation on the exotesta and wettened endotesta - statistical trials

Considering the strong circumstancial evidence that heat intensity caused the observed heat pulse effects of natural fire on subsequent germination (Bond *et al.*, 1990) the above indication that desiccation was the active principle was surprising. This led us to further test desiccation of the testa layers in two statistical trials, using both "cool" techniques and heat desiccation treatments.

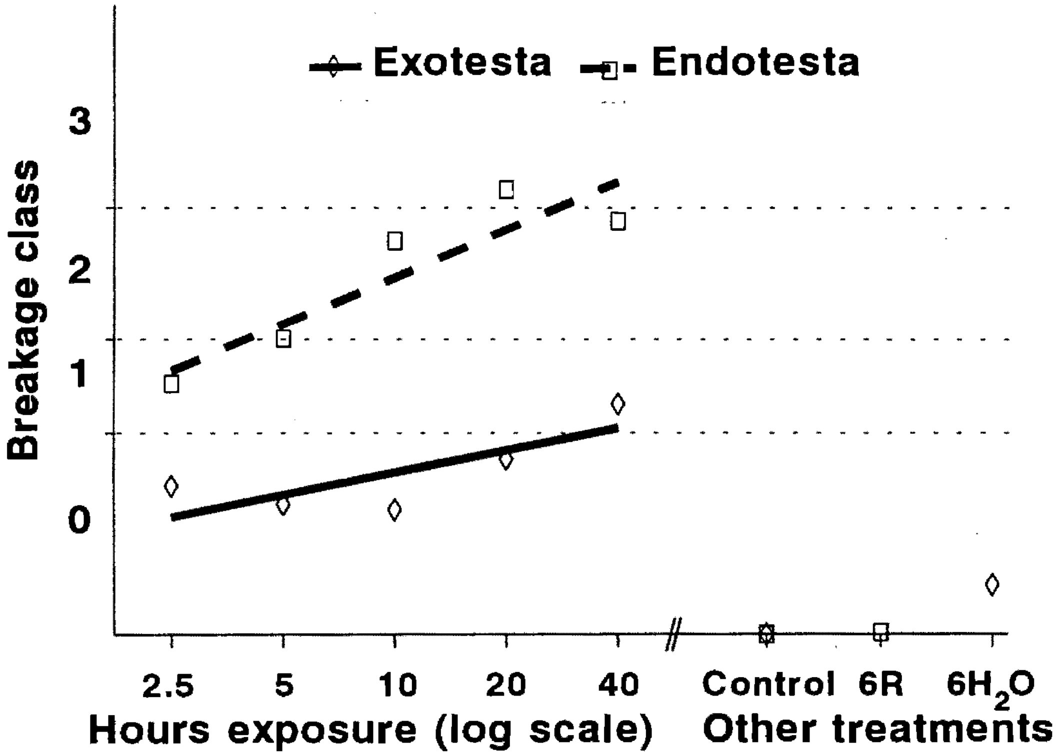


Figure 5. Mean degrees of exotesta and endotesta breakage of *Leucospermum cordifolium* seeds following heating at 40°C for different periods (endotesta breakage was recorded following immersion in water after cooling). Control, no exposure; 6R, seeds were rehydrated in moist air; 6H20, seeds were exposed to 100% RH for 6 h at 40 °C. Linear regressions were fitted on the values of the underlying scale; see explanation of methods and scales in text.

The results (Figure 5) show significant (Table 1) but relatively little differences in exotesta breakage with increasing period of exposure to 40 °C; endotesta breakage was severe and increased linearly with time (Table 1; Figure 5). There were no increasing trends over time for exo- (Figure 6a) and endotesta breakage (Figure 6b) in the range of exposures to both H_2SO_4 and silica gel (Table 2).

Table 1. Analysis of deviance on ordinal data of desiccation-induced testa breakage in *L. cordifolium* showing the source effects for Trial 1.

Source	d.f.	MDD	SL
Exotesta vs Endotesta	1	42.8921	<0.001
Within endotesta	6		
(2.5-40h exp) vs Control	1	19.6844	<0.001
(2.5-40h exp) vs 6R	1	12.2458	<0.001
(2.5-40h exp) Linear	1	10.7054	<0.001
(2.5-40h exp) Quadratic	1	1.4917	0.067
(2.5-40h exp) Cubic	1	0.8591	0.154
(2.5-40h exp) Quartic	1	0.0429	0.741
Within exotesta	6		
(2.5-40h exp) vs Control	1	13.0084	<0.001
(2.5-40h exp) vs 6H20	1	6.7866	<0.001
(2.5-40h exp) Linear	1	2.0082	0.037
(2.5-40h exp) Quadratic	1	1.1386	0.104
(2.5-40h exp) Cubic	1	0.0005	0.971
(2.5-40h exp) Quartic	1	0.0528	0.714
Experimental Error	14	0.3777	
Total corrected	27		

Abbreviations: d.f. = degrees of freedom,
MDD = Mean Differential Deviance,
SL = Significance level, h = hours, exp = exposure.

The levels of exotesta breakage were similar in heat- and H_2SO_4 treated seeds (Figure 5 vs. Figure 6a), the latter being significantly lower ($P=0,037$ - Table 2) than in the case of silica gel; once broken the damage to exotestas could not be corrected rehydration treatment (Figure 6a; Table 2). The level of endotesta breakage was similar in the case of the two "cool" desiccating agents, both causing more effective breaking than heat treatments (Figure 6b vs. Figure 5). Rehydration treatment completely prevented endotesta breakage in all treatments (Figures 5, 6b; Tables 1, 2). This and the fact that the "cool" desiccation treatments caused similar and in some cases stronger effects than heat treatment indicate that the breakage phenomena are desiccation effects and not caused directly by heat intensity. However, a low level of exotesta breakage ($P<0,001$ - Table 1) was apparent when seeds were heated at 40 °C under 100% RH (Figure 5).

Both the preliminary test and statistical trials thus confirm that strong exo- and endotesta breakage was effected by desiccation and not heat intensity *per se*. The results suggest that in nature desiccation by the heat of fire could break the exotesta. The endotesta could be broken as well if fire is followed soon by rain. Although the effects of desiccation-mediated scarification of the two testa layers on actual germination have not yet been determined, it is likely that both scarification processes will increase

Table 2. Analysis of deviance on ordinal data of desiccation-induced testa breakage in *L. cordifolium* showing the source effects for Trial 2.

Source	d.f.	MDD	SL
Exotesta vs Endotesta	1	81.1718	<0.001
Within exotesta	10		
H_2SO_4 vs Silica	1	4.3618	0.037
Within H_2SO_4	5		
(24-96h exp) vs Control	1	7.9795	<0.005
(24-96h exp) vs 96R	1	1.0622	0.303
(24-96h exp) Linear	1	0.2499	0.617
(24-96h exp) Quadratic	1	0.2771	0.599
(24-96h exp) Cubic	1	0.8138	0.367
Within Silica	4		
(24-72h exp) vs Control	1	5.0010	0.025
(24-72h exp) vs 72R	1	2.5231	0.112
(24-72h exp) Linear	1	0.4448	0.505
(24-72h exp) Quadratic	1	0.0025	0.960
Within endotesta	10		
H_2SO_4 vs Silica	1	0.0398	0.842
Within H_2SO_4	5		
(24-96h exp) vs Control	1	92.8978	<0.001
(24-96h exp) vs 96R	1	48.4786	<0.001
(24-96h exp) Linear	1	0.4188	0.518
(24-96h exp) Quadratic	1	0.0827	0.774
(24-96h exp) Cubic	1	0.0710	0.789
Within Silica	4		
(24-72h exp) vs Control	1	45.0989	<0.001
(24-72h exp) vs 72R	1	40.2479	<0.001
(24-72h exp) Linear	1	0.1329	0.715
(24-72h exp) Quadratic	1	0.0735	0.786
Experimental Error	22	0.2916	
Total corrected	43		

Abbreviations:
d.f. = degrees of freedom,
MDD = Mean Differential Deviance,
SL = Significance level, h = hours, exp = exposure.

oxygenation of embryos and thus could break the known oxygen exclusion dormancy in *Leucospermum*. Should this happen in nature, germination during the ensuing rainy winter will be promoted, thus contributing to the well-known phenomenon of massive emergence of seedlings following a summer fire. Importantly, the results also predict a positive correlation of seedling numbers with heat intensity of fynbos fires on account of differential desiccation effects determined by varying degrees of soil warming, thus supporting the results of Bond *et al.* (1990).

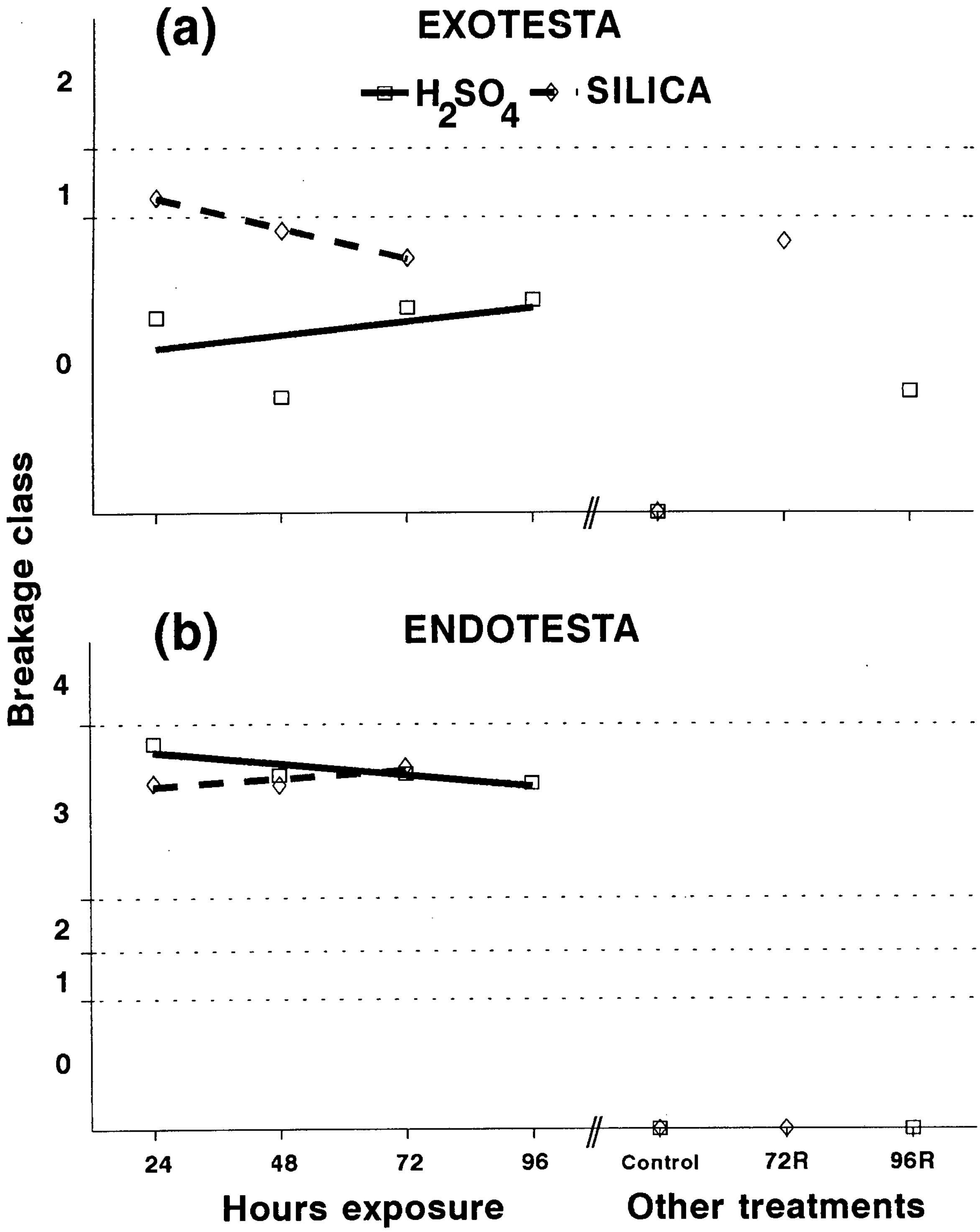


Figure 6. Mean degrees of exotesta (a) and endotesta (b) breakage of *Leucospermum cordifolium* seeds desiccated for various exposure periods over H_2SO_4 or silica gel at 22 °C; Control, no exposure; 72R and 96R, seeds were rehydrated in moist air after desiccation over H_2SO_4 or silica gel for 72 or 96 h respectively. Linear regressions were fitted on the values of the underlying scale; see explanation of methods and scales in text.

Endotesta cracking in the laboratory was observed following desiccation (and wetting) of seeds even at 25 °C. It is therefore proposed that desiccation-scarification could also result from the repeated cycles of dehydration/hydration caused by normal hot summer weather, followed by summer rainy spells, especially after exposure of the soil surface by fire, but also in unburnt fynbos during the interfire period. This can presumably contribute to a slower but progressive weakening of the hard endotesta. Thus the mechanical constraint by the hard endotesta on germinating embryos could be relieved. However during the interfire period dormancy would remain enforced by the non-optimal temperature regime prevailing in unburnt fynbos (Brits, 1986).

Our experimental conditions fall well within the normal limits of seed-environment interactions within fynbos. Summer air temperatures of 40 °C are often attained whilst fire-exposed soil temperatures in the open sun can average 55 °C maximum at 5 mm

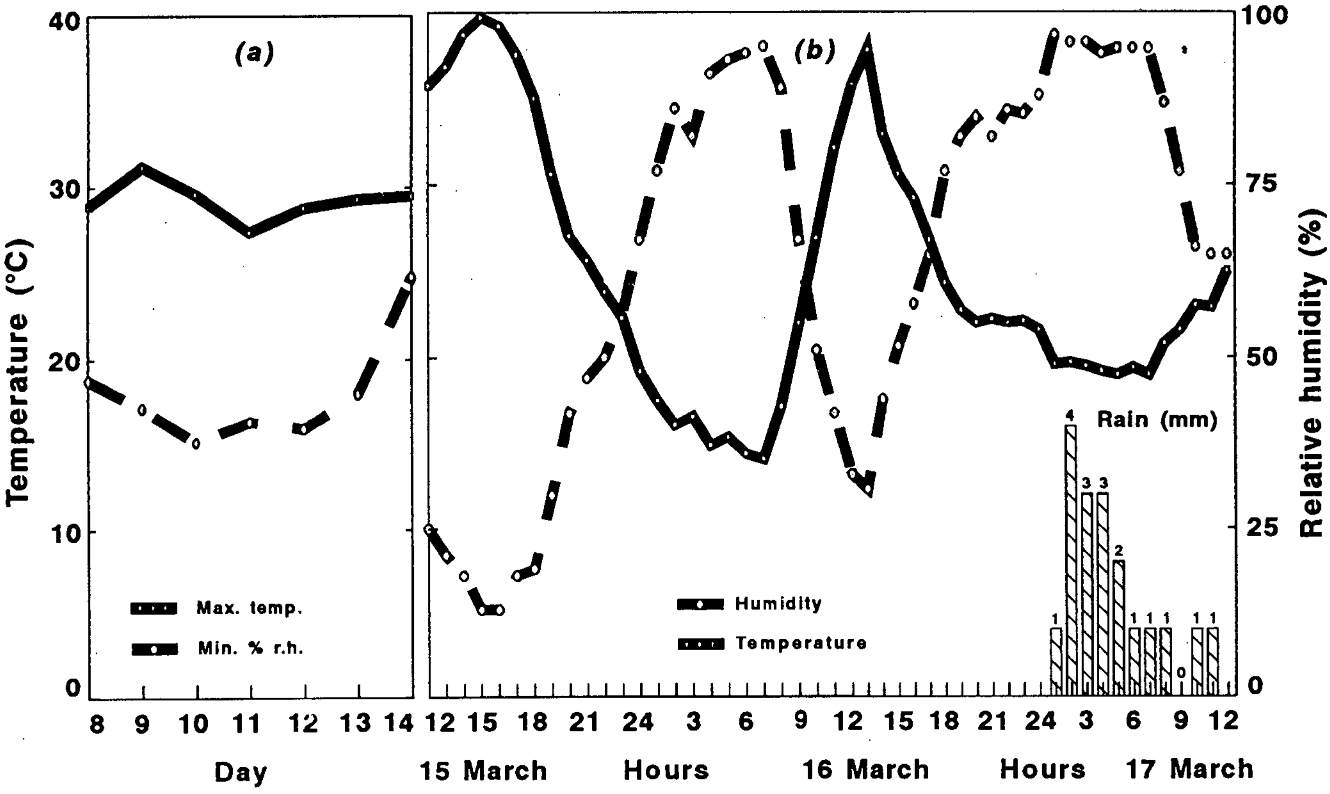


Figure 7. Typical "bergwind" conditions followed by rain in fynbos at Riviersonderend, South Africa (34°9'S, 19°54'E) during March 1992. (a) daily maximum temperature and minimum relative humidity for the week preceeding abnormally hot, dry conditions (b, hourly values), which was followed immediately by 18 mm of rainfall.

depth during January (Brits, 1986) and may reach 60 °C at the surface (J.H. de Lange, personal communication). Moreover, exceptionally hot, dry "bergwind" spells periodically occur in fynbos during summer and autumn. These conditions are typically caused by an eastward-moving coastal low-pressure system with cyclonic, offshore winds.

Bergwinds are commonly coupled with a cold front advecting rainy weather in from the Atlantic Ocean. Thus extremely dry conditions can alternate with rain in quick succession in fynbos (Figure 7) providing the conditions under which endotesta (and exotesta) desiccation-scarification could occur. During summer and autumn this pattern recurs sporadically, e.g. once monthly on average in the periods March - April 1991 and 1992.

The results on desiccation effects on the *L. cordifolium* testa suggest a mechanism for the apparent promotive effect of "heat pulse" by fire on seed germination and on natural scarification of the testa layers in the soil. The same phenomenon was also noted in other *Leucospermum* species. It is therefore proposed that this is another competition-avoidance syndrome in *Leucospermum*, i.e. a common strategy to pre-empt newly available growing space after fire.

6.3 HEAT DESICCATION AS A TESTA SCARIFYING AGENT IN THREE *LEUCOSPERMUM* SPECIES: QUANTIFICATION AND EFFECTS ON EMBRYO VIABILITY AND GERMINATION

Introduction

Although desiccation is the active principle in the scarification of *Leucospermum* R.Br. testal layers (Chapter 6.2), the primary desiccation agent in nature is heat. It was proposed that seeds of *Leucospermum* can be desiccation-scarified by the heat of fire, but also by solar radiation and high ambient summer air temperature, especially in soil exposed by fire. The relationship between heat intensity, duration, and the resulting degree of testa scarification has not been quantified over a range of moderate and high temperatures. However, the main question concerns the impact of heat-mediated scarification on actual germination response.

The sensitivity of *Leucospermum* seeds to heat is not known, but tests suggest that their viability is reduced at relatively low temperature and short exposure period (Brits, unpublished). The question therefore arises what possible damaging effects heat could have on the *Leucospermum* embryo during strong desiccation of the seed by fire. The seed embryo of fynbos species can, on the other hand, be stimulated directly by dry heat (Blommaert, 1972; Van de Venter and Esterhuizen, 1988; Musil and de Witt, 1991). It would therefore be important to distinguish between the physical scarifying effects on the seed coat and physiological effects of (non-damaging) heat on the embryo.

Although very little information has been gathered on the effects of "heat pulse" (DeBano *et al.*, 1979) on germination of fynbos species with soil-stored seed (c.f. Le Maitre and Midgley, 1992; Keeley, 1992) it has been shown to be an important germination cue for fire-recruiting taxa in California chaparral (Keeley, 1991). Heat pulse breaks innate dormancy in most of these species (Keeley, 1991), but little work has been done on the mechanisms involved. However, cracking of the water-impermeable testa by heat in heath- and shrubland species is well documented (Gill and Groves, 1981).

The scarification of seeds refers to any abrasive treatment of the testa surface to increase its permeability, usually to water but also to oxygen. The oxygen-impermeable testa in *Leucospermum* can effectively be scarified by various means, for example, with (c) H_2SO_4 (Brits and Van Niekerk, 1976). It was shown (Chapter 4.2) that near-complete germination of *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade seeds can presently be obtained by a combination of acid scarification, incubation in oxygen and thiram fungicide treatment. Acid scarification appears to approximate natural scarification during soil-storage (Chapter 7). These combined treatments were therefore used as a maximizing germination stimulus for the purpose of comparison in this study.

The null-hypotheses formulated for this study were:

1) Heat treatments of varied intensity and duration will not affect the degree of scarification of the testa layers.

2) Heat desiccation treatment will not affect the viability of *Leucospermum* embryos;

3) Testa breaking by means of heat desiccation treatment will not affect seed germination;

4) The effects of testa breaking by means of heat desiccation treatment on the germination of seeds, will not differ in degree from those of other known forms of scarification and oxygenation treatment.

Materials and methods

In the first of two experiments a series of heat treatments (intensity and duration) are applied in order to quantify the relationship between heat desiccation and progressive breakage of the testa layers. Experiment 2 determines the effects of the linked processes of heat desiccation, scarification and oxygenation on the germination process itself.

Experiment 1a. Effects of heat desiccation pulsing and rehydration on the testa and viability of Leucospermum cordifolium *seeds*

The methods below are given in Chapter 6.2. The pericarp of *Leucospermum cordifolium* seeds was softened by soaking seeds in lukewarm water and removed by rubbing, seeds were redried and those with an intact exotesta selected under the stereomicroscope. Intact seeds were subjected for varying periods (1,25; 2,5; 5; 10; 20; 40; 80; 160 min - Figure 1) to 30, 40, 60 and 80 °C in an oven with forced-air circulation; seeds were then cooled in airtight bottles and the degree of exotesta breakage recorded on a 0-10 scale, using a stereomicroscope. Seeds were immediately transferred to water, redried and the exotesta removed by hand. The degree of endotesta breakage was recorded on a 0-4 scale. Embryos were then isolated and treated with tetrazolium to establish the effect of heat treatments on seed viability (Chapter 2). Viability was scored as the proportion of each embryo coloured, on a 0-10 scale. In the 30 and 40 °C series only two samples were tested (Figure 1a) on account of small expected effects on viability.

Statistical treatment

The four heat-desiccation trials were laid out as completely randomized designs with two replicates. Each experimental unit consisted of 10 seeds. Seeds were treated with 5 levels of time exposure in hours evenly spaced on a log scale and 2 breakage types (exotesta and endotesta) plus control treatments (no time exposure). Seeds were rated individually on an ordinal scale and the data (ordinal) analysed as before (Chapter 6.2). Control treatments were compared to exposure time treatments within each temperature regime and polynomial regression coefficients were calculated as shown in the analyses of deviances, Table 2 (McCullagh and Nelder, 1989).

Experiment 1b. Effect on the exotesta of repeated heat desiccation alternated with high RH

Experimental conditions and statistical treatment apply as in Experiment 1a above. Seeds of *L. cordifolium* were prepared for study and exposed to 40 °C for 8 h daily, removed and allowed to cool for 10 min and then transferred to a 100% RH atmosphere at 10 °C for 16 h, by suspension over water in a closed container. The cycle was repeated for 10 d. Exotesta damage was scored daily after removal from high temperature. After analysis of deviance was performed the location values on the underlying scale were used to fit a non-linear inverse regression (Figure 3).

Experiment 2. Effects of heat desiccation, scarification and oxygenation treatments on germination of Leucospermum *seeds*

Non-desiccating heat was applied as parallel control ("background") seed treatments in this study. The pericarp of *Leucospermum* seeds was removed, seeds were redried and those with an intact exotesta selected, as in Experiment 1. Treatments 2, 4, 5 and 6 received similar "background" heat and moist air rehydration treatments (Table 1). All heat desiccation and rehydration treatments follow Brits *et al.* (1993) (Chapter 6.2).

1. Control 1: remove pericarp only - exotesta intact, no heat applied.
2. Control 2: As 1., treat at (55 °C x 100% RH x 7 min.) followed by (40 °C x 100% RH x 6 h); cool [giving similar background heat treatments as in 4, 5 and 6].
3. (c) H_2SO_4 scarification 8 min. (Chapter 2).
4. Hand remove exotesta completely (preheat 55 °C x 7 min. to break exotesta, facilitating removal) Rehydrate at (20 °C x 100% RH x 16 h) [i.e. cancel desiccation effects on endotesta].
5. Heat desiccate exotesta only: 55 °C x 7 min. i.e. partially remove exotesta; apply (40 °C x 6 h) as in 6 to equalise moderate heat effect; rehydrate at (20 °C x 100% RH x 16 h).
6. Heat desiccate exotesta as in 5 followed by heat desiccation of endotesta at (40 °C x 6 h); cool seeds (20 °C x 2 h) in airtight bottle, and transfer to water (20 °C x 3 min.) to break endotesta; dry seeds with paper towel.
7. Acid scarification 8 min; wash; incubate seeds in oxygen.

Table 1. The series of seed treatments in Experiment 2 receiving parallel heat treatments to equalise possible physiological effects of heat in seeds; as well as moist air rehydration treatment to cancel the desiccation effects of heat treatments (see Materials and Methods)

Treat -ment no.	Description	55 °C x 7 min treatment	40 °C x 6 h treatment	Rehydrate 100% RH x 16 h x 20 °C
2	"Control 2": pericarp removed, exotesta left intact; apply two moist heat treatments; cool seeds after treatment	**X** (100% RH)	**X** (100% RH)	-
4	Hand remove exotesta; apply dry heat pre-treatment; cool; rehydrate in moist air	**X** (dry heat)	-	**X**
5	Dry heat treatments, break exotesta only (55 °C); cool; rehydrate desiccated seeds	**X** (dry)	**X** (dry)	**X**
6	Dry heat treatments, break exotesta (55 °C); desiccate endotesta (40 °C); cool; transfer to water, break endotesta (+ rehydrate)	**X** (dry)	**X** (dry) + cold water	-

Following the above treatments seeds were soaked in distilled water for 24 h, dried lightly with paper towel and treated with thiram fungicide powder (Chapter 4.2), then incubated under a temperature regime of (25 °C x 8 h) + (10 °C x 16 h). Treatments 1-6 were incubated on a single sheet of Whatman no. 1 filter paper, kept damp with distilled water, in petri dishes (Brits 1986). Treatment 7 seeds were incubated in medical grade oxygen in 1 l flasks (Chapter 4.3). Seeds were scored as germinated when the radicle appeared.

Species:	*L. cordifolium*
	L. glabrum
	L. reflexum

Statistical treatment. Three trials were done, one on each species, each consisting of 7 treatments. All trials were laid out as completely randomized designs with 6 replicates. *L. cordifolium* and *L. glabrum* experimental units consisted of 25 seeds, *L. reflexum* of 20 seeds. Germination percentages and rates were calculated and data tested for normality. All variables were normally distributed therefore no transformation was carried out (Snedecor and Cochran, 1980). One-way analysis of variance was performed on percentage scores. Student's t-LSD's were calculated to compare treatment means. Comparisons of interest were tested as shown in the analysis of variance in Table 3. Germination rates were calculated by means of the formula of Heydecker (1973; Chapter 4.2).

Results and discussion

Experiment 1a and 1b

Tetrazolium scores. Tetrazolium scores varied between classes 8 and 9 (Figure 1a), i.e. 80-90% of the cotyledonary tissue respired, indicating high viability of embryos. The 30 and 40 °C series showed little deviation from control values but 60 °C and 80 °C results varied noticeably. Although the lowest and highest values within the 60 °C and 80 °C series differed statistically significantly, none of the slopes differed significantly from 0. Thus the highly variable scores preclude the detection of statistical trends (Figure 1a). It is, however, possible that viability began to decline at the longer 80 °C exposure (Figure 1a).

Exotesta. Heat intensity and duration correlated positively with the degree of breaking of the exotesta (Table 2). A typical response was the result of exposure to 60 °C (Figure 1c). At maximum average breakage the exotesta was 40-50% broken (percentage area of underlying endotesta exposed) (Figure 1c). The marked reaction of the exotesta to low levels of desiccation at 30 °C implies a continuous disintegration process in nature.

Endotesta. Heat pulse and subsequent wetting had a strong effect on the endotesta, i.e. most endotestas were visibly broken (class 3 breakage) at longer exposure periods (Figure 1b). Maximum endotesta breakage at 60 °C was reached after c. 40 min and at 80 °C after c. 20 min. Regression statistics for endotesta breakage show strongly positive, highly significant slopes, excepting the 30 °C series (Table 2). Slopes became steeper with increasing temperature intensity, except at 80 °C (differences statistically

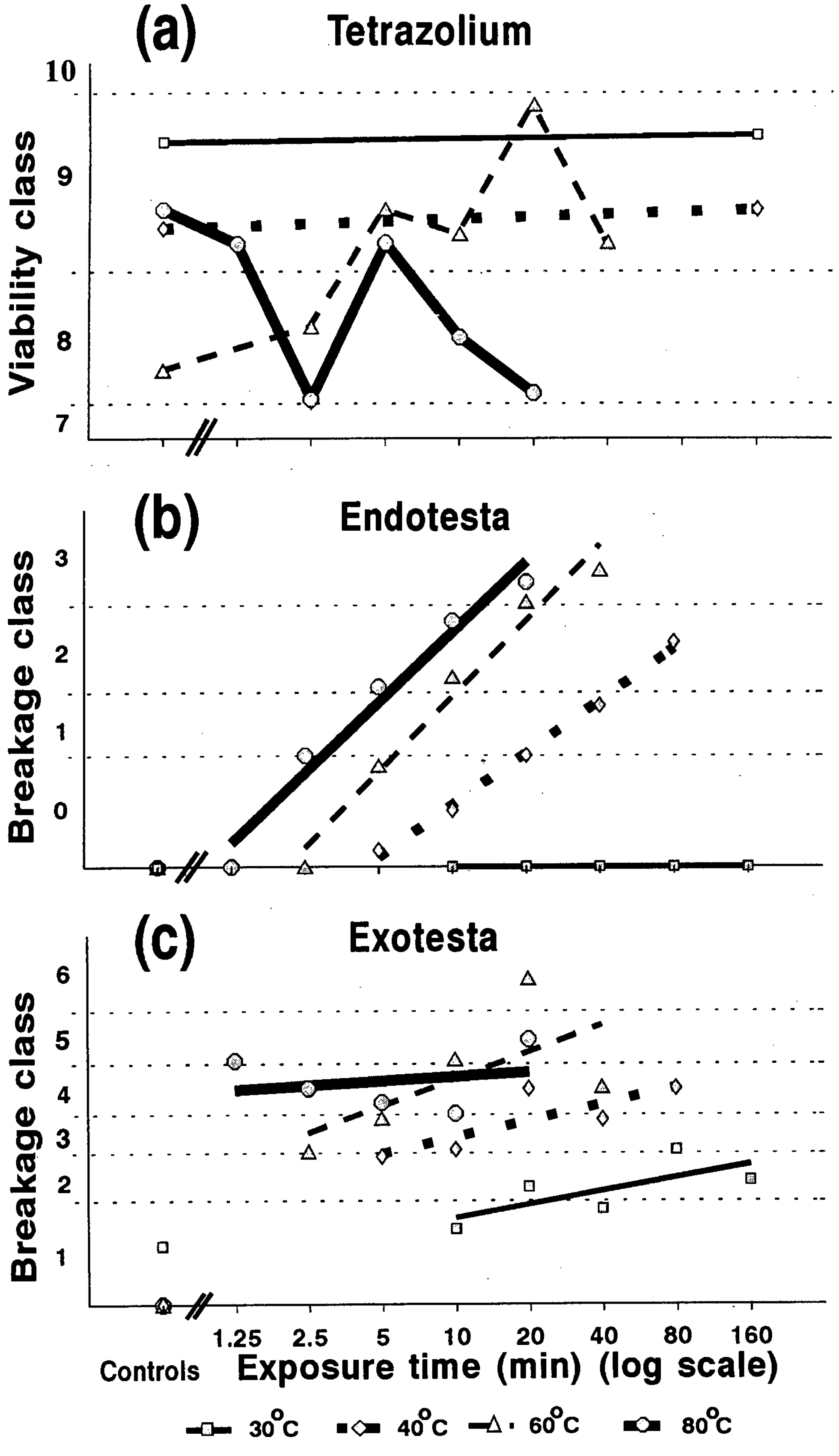


Figure 1a-1c. Mean degrees of exotesta (c) and endotesta (b) breakage of *L. cordifolium* seeds treated for various exposure periods at constant 30, 40, 60 and 80 °C (endotesta breakage was recorded following immersion in water after cooling) and mean tetrazolium viability scores (a) for treated seeds; Control = no exposure; see explanation of methods and scales in text. Each point represents the mean of two replicates of ten seeds.

Table 2. Regression coefficients for endo- and exotesta breakage under four temperature exposure series in Experiment 1a

Temperature	Exotesta			Endotesta		
series	Intercept (a)	Slope (b)	P	Intercept (a)	Slope (b)	P
30 °C	0,5962	0,1098	0,23	0,0000	0,0000^{x}	1,00
40 °C	1,0924	0,1363	0,14	-0,5928	0,7299^{y}	<0,01
60 °C	1,1818	0,2187	<0,05	-0,7918	1,0595^{z}	<0,01
80 °C	1,7143	0,0360	0,69	-0,6513	0,9838^{z}	<0,01
S.E.	0,2974	0,0897		0,2220	0,0669	
L.S.D. (5%)	0,859	0,259		0,641	0,193	
R^2	0,951			0,985		

Abbreviations:
S.E. = Standard error of intercept a or slope b
L.S.D. = Least significant difference
R^2 = Coefficient of determination
x,y,z: values followed by the same letter do not differ significantly
P = probability level for HO: b = 0

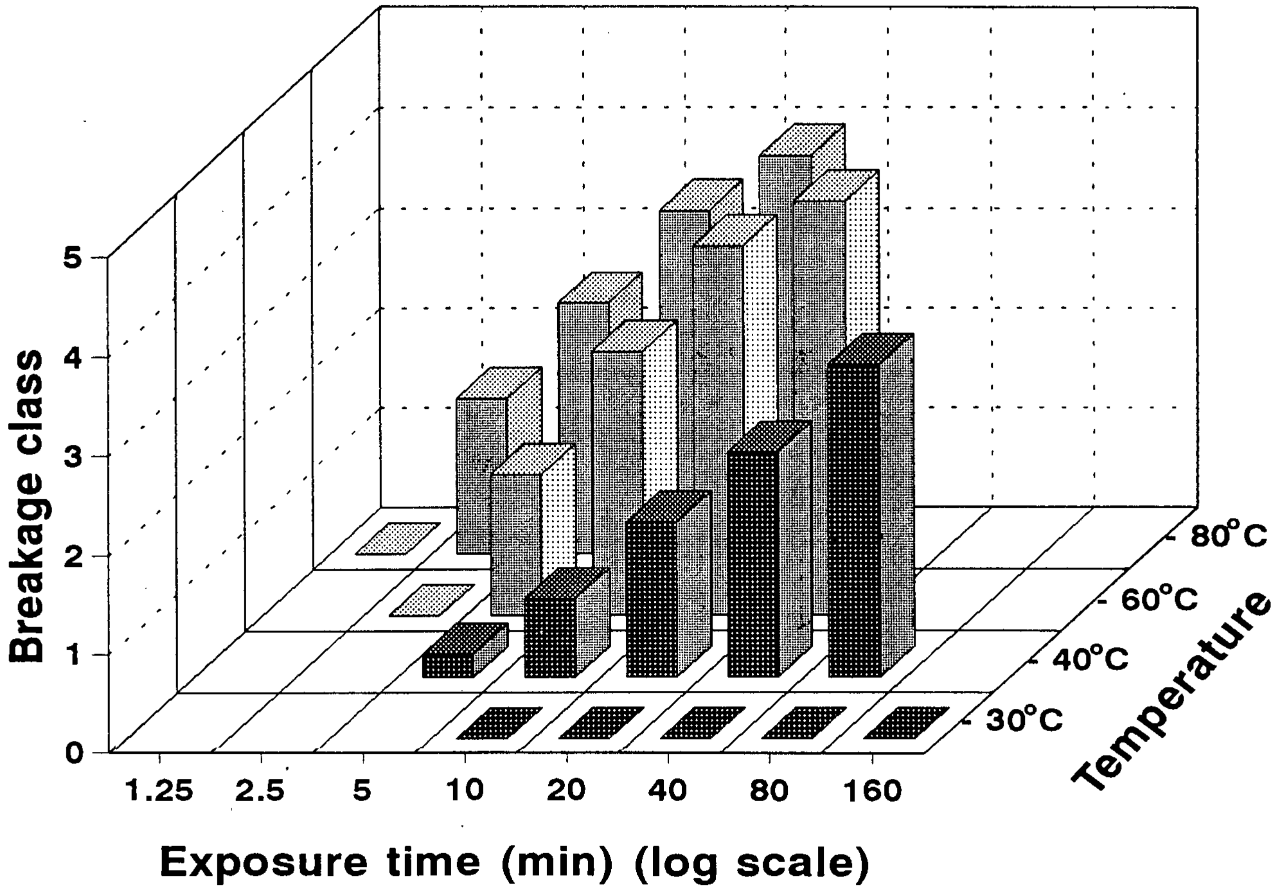


Figure 2. Endotesta data of Figure 1b represented 3-dimensionally on a linear Y-axis (breakage class) illustrating linearity of the 40 °C series and an asymptotic increase with increasing exposure time at 60 and 80 °C.

significant - Table 2). Although breakage increased linearly in most of the temperature series tested (Figure 1b) the effects of the longer experimental times in the 60 and 80 °C series may have levelled off (Figure 2), indicating the limits of treatment effects that could be expected.

Exo- vs. endotesta. Both exo- and endotesta were drastically scarified within the present range of treatment temperatures whilst maintaining high embryo viability.

At milder treatment levels the exotesta reacted more rapidly, and stronger, to heat pulse than the endotesta (Figure 1b *vs.* 1c - compare short exposure period effects in all series). Although the endotesta required a higher threshold temperature to react (no breakage occurred at 30 °C) than the exotesta, desiccation-scarification effects were severe in the endotesta in the range of 40-80 °C at longer exposure times. The regression slopes for the endotesta were much steeper than for the exotesta (Table 2).

Exotesta reaction to temperature exposure time was much more variable than for the endotesta, as is reflected in the probability levels for exotesta slopes (Table 2). The high R^2 values (Table 2) reflect good descriptions of data distributions by the regressions, especially those of the endotesta.

Very little information on fynbos soil temperature during fire is available (cf. Pierce, 1987; Cowling, 1992) despite numerous hypotheses involving both soil-stored seed banks and fire in fynbos (e.g. in Le Maitre and Midgley, 1992). By contrast the relationships between heat intensity and soil temperature have been reasonably well investigated with respect to chaparral (e.g. DeBano *et al.*, 1979) and Australian shrubland (e.g. Humphries and Craig, 1981). Typical temperature maxima and durations in these heathlands varied between 100-550 or 700 °C for 1-10 min at the soil surface; 60-120 °C for 10-40 min at 2,5 cm depth and 35-70 °C for 15 min to 2 h at 5 cm depth. Although fynbos fires are generally cooler than those in chaparral (Keeley, 1992) the ranges of experimental temperatures in this study clearly fall within the expected limits of natural fire in fynbos. The observations by de Lange and Boucher (1993) on soil temperature during cool fires in two fynbos localities, support this conclusion. Since *Leucospermum* seeds are buried by ants at a typical depth of 30-45 mm in the soil (Brits, 1987) it is likely that a large proportion of intact (freshly dispersed) soil-stored seeds could be scarified by means of heat-desiccation pulse during fire.

The sensitivities of both the exo- and endotesta thus fall well within the expected ranges of soil temperatures during fynbos fires, the summer climatic regime in burnt-exposed soil (De Lange and Boucher, 1993; Chapter 6.2) and even in shaded soil under pre-burn conditions during summer (Brits 1986).

Effect on the exotesta of repeated heat desiccation alternated with high RH

The exotesta disintegrated progressively, leveling off at c. breakage class 4, i.e. with 40% of the underlying endotesta exposed (Figure 3). This pattern suggests that in nature desiccation-scarification progresses continuously. In this study the exotesta was destroyed to a considerable degree using "dry" means only (no wetting). It may be speculated that the effects in nature of prolonged seasonal cycles of wetting coupled with microbial decomposition would add to the above effects. Advanced deterioration of the exotesta was actually observed in seeds buried for 4 years in a soil-storage experiment (Chapter 7).

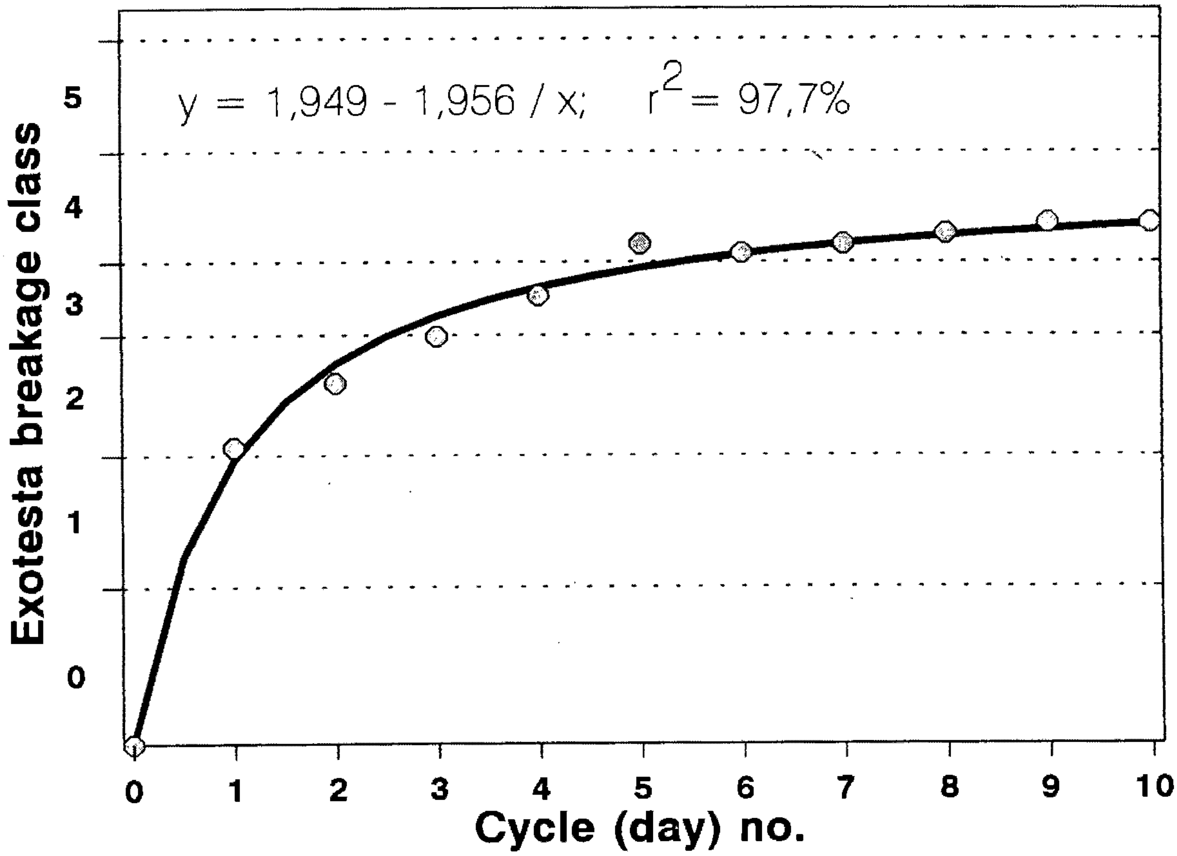


Figure 3. Effect of repeated 40 °C heat desiccation treatment (8 h daily) alternated with a 100% RH atmosphere at 10 °C (16 h daily) on the exotesta of *L. cordifolium*.

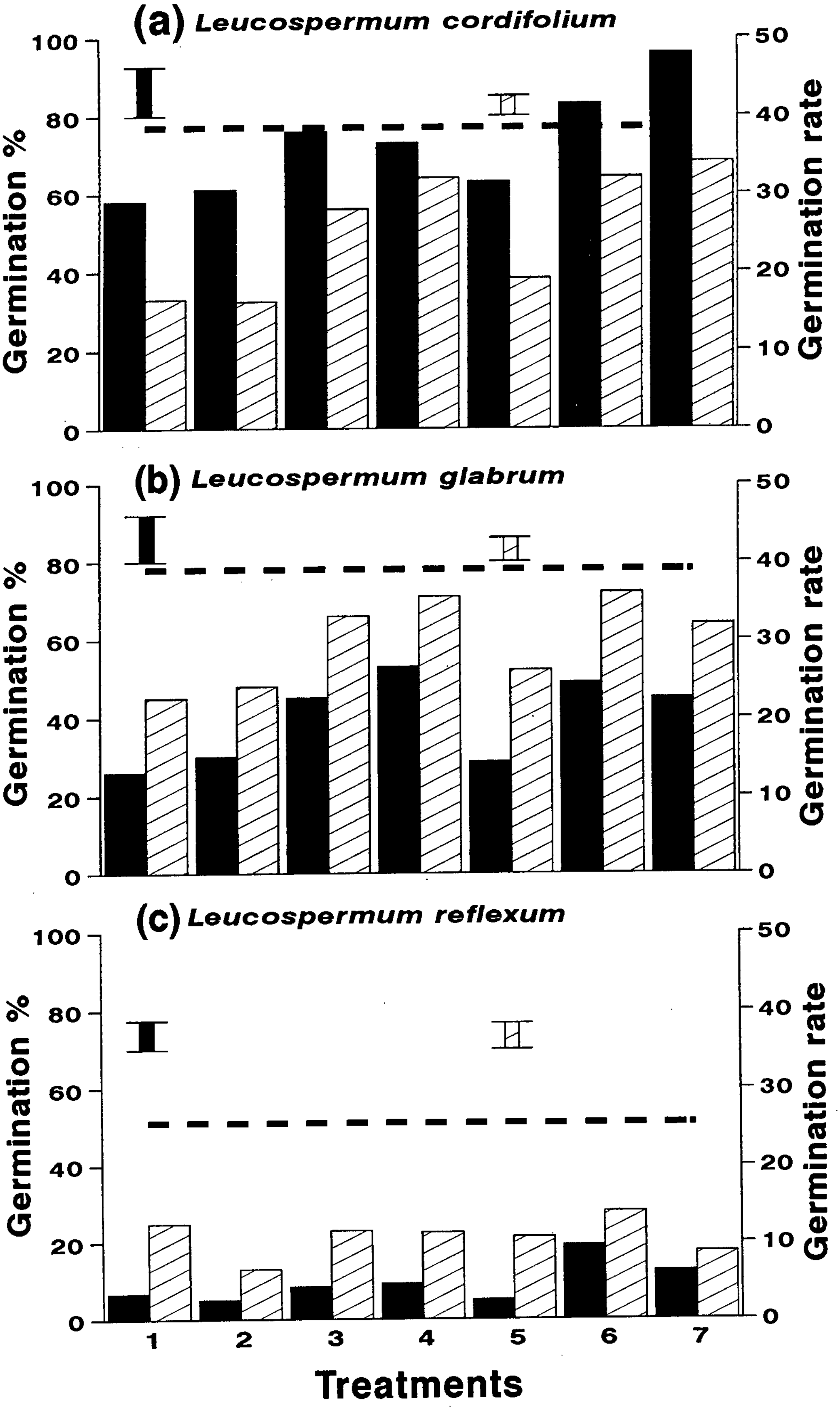


Figure 4a-4c. Mean seed germination percentage (■) and germination rate (▨) for three *Leucospermum* species subjected to 7 treatments; small bars represent least significant differences (5% level); ---- tetrazolium viability estimates. T1: control 1 (no heat), T2: control 2 (heated), T3: H_2SO_4 scarified, T4: exotesta hand removed, T5: exotesta desiccation-scarified, T6: exo- + endotesta desiccation-scarified, T7: H_2SO_4 scarified + O_2 incubated.

Experiment 2

The germination percentage and rate results are summarized in Figure 4. Germination rates generally follow similar trends as percentages (Figure 4) and thus underscore the primary variable of percentage germination in this experiment. For this reason only an analysis of variance of percentages is given (Table 3). Germination response in *L. reflexum* was extremely poor (Figure 4c), which was apparently due to both a low viability of the seed source and a supra-optimal incubation temperature regime (Chapter 4.4).

The parity found for germination percentages and rates between treatment 1 (untreated seeds) and treatment 2 (preheated control) was typical for the three species (Figure 4 - differences are statistically not significant, except germination rate in *L. reflexum*). Germination percentage and rate of the two controls as a group were highly significantly less ($P<0.001$) than for the rest of the treatments [except in *L. reflexum*, germination percentage ($P<0,05$) and rate ($P=0.36$)].

Hand removal of the exotesta (treatment 4) had a marked effect over control treatments 1 and 2, and gave a response on a par with acid scarification (treatment 3), the latter difference not being statistically significant (Table 3). Additional oxygenation

Table 3. Analysis of variance showing the source effects (heat-desiccation treatments) for *Leucospermum* germination percentages in Experiment 2

		L. cordifolium		*L. glabrum*		*L. reflexum*	
Source	d.f.	MS	SL	MS	SL	MS	SL
Treatments	6	1118,02	<0,001	742,48	<0,001	152,38	<0,01
(C1+C2 +T5) vs. (T3+T4+T6+T7)	1	4725,95	<0,001	4156,30	<0,001	466,72	<0,01
(C1+C2) vs. T5	1	46,32	0,53	3,26	0,86	2,78	0,80
C1 vs. C2	1	19,17	0,69	37,34	0,55	8,33	0,66
(T3 + T4) vs. (T6 + T7)	1	1350,00	<0,01	32,67	0,58	301,04	0,01
T3 vs. T4	1	33,33	0,59	192,00	0,18	2,08	0,82
T6 vs. T7	1	533,33	0,04	33,33	0,58	133,33	0,08
Experimental Error	35	115,13	-	104,40	-	41,31	-
Total corrected	41						

Abbreviations: C = Control
T = Treatment no.
MS = Mean square
d.f. = Degrees of freedom
SL = Significance level

by incubation in pure oxygen (treatment 7) did not increase germination any further in *L. glabrum* and *L. reflexum* (Figure 4b, c).

Heat mediated desiccation-scarification of the exotesta alone (treatment 5) had little effect (Table 3), but desiccation-scarification of both exo- and endotesta (treatment 6) resulted in significant increases over controls (Figure 4).

Treatment 6 seeds (desiccation-scarification of exo- plus endotesta) responded even more strongly than treatment 4 seeds (hand removal of exotesta), except in *L. glabrum*, the difference being not significant (Figure 4). In *L. reflexum* treatment 6 increased germination response by a factor of 50% over treatment 7 where seeds were incubated in oxygen.

In all species treatments 3, 4, 6 and 7 formed a group of outstanding germination responses, relative to controls (differences highly statistically significant) in both percentages (Table 3) and rates (excepting *L. reflexum* germination rate), with these treatments mostly not differing significantly within species (Figure 4).

Maximum germination in *L. cordifolium* exceeded the tetrazolium viability score by c. 20%, however in the other two species tetrazolium scores considerably overestimated the realized maximal germination response (Figure 4). Although these discrepancies suggest limitations to the use of the tetrazolium technique it is likely that a considerable proportion of seeds of the latter two species remained dormant from unknown causes. The relatively high control values for *L. cordifolium* (Figure 4a) could possibly be ascribed to selection pressure for non-dormancy in this semi-domesticated species (Chapter 4.3).

The similar percentages and rates found in treatments 1 (untreated control) and 2 (preheated control) (Figure 4) indicate that the effects of heat are probably not related to physiological changes caused by high temperature.

The fact that hand removal of the exotesta (treatment 4) gave a similar response to acid scarification (treatment 3 - Figure 4a-c) suggests that the exotesta plays an important role in oxygen exclusion from the embryo. Brits and Van Niekerk (1976) proposed near-complete germination in *L. cordifolium* seeds resulting from (c) H_2SO_4 scarification. Additional oxygenation in this study by incubation in pure oxygen increased germination further only in *L. cordifolium* (Figure 4a). The strong effect of treatment 4 over treatments 1 and 2 indeed indicates that the exotesta is the primary oxygen barrier in the seed. However treatment 5 (heat desiccation of the exotesta alone) as test of this hypothesis, by means of desiccation-scarification failed, although it gave slightly better results than both controls in most cases, these being not statistically significant (Figure 4).

The exotesta is strongly hygroscopic and curls up or spreads out when dehydrated or hydrated respectively. It was observed that the broken exotesta spread out over the seed surface upon wetting in treatment 5. This covered most of the seed surface, thus isolating the seed once more. This may have been the reason for the weak effect in treatment 5. In nature this process would however be different :

1) Firstly, there would be continuous (daily) treatment of this order during the first summer after fire; or over consecutive summers in unburnt vegetation. It was indeed found that repeated desiccation treatment led to a progressive increase in extotesta breaking (Figure 3). Thus the exotesta in nature could be scarified repeatedly as opposed to the same, basically once-applied, treatment here.

2) The additional effect of microbial digestion of the exotesta in the soil. Microbial destruction of the exotesta progresses with increasing period of soil-storage (Chapter 7). It is therefore likely that the effect of one-time treatment 5 here, is strongly amplified in nature, leading on towards treatment 4 levels of effect.

The stronger response of treatment 6 than treatment 4 in *L. cordifolium* and *L. reflexum* indicates that endotesta breaking can also contribute substantially to germination in nature. Cracks in the endotesta could lead to improved oxygenation and to reduced mechanical constraint by the seed coat. Evidence that mechanical constraint by the seed coat was reduced significantly can be seen in *L. reflexum*, where treatment 6 had the strongest effect by far. This *L. reflexum* seed source, although fresh, was observed to contain a majority of poorly developed (smaller, shrunken) embryos, in contrast to the other two sources. The seed was probably harvested prematurely (Brits and Van Niekerk, 1986; Chapter 4.3) which also would have determined its low viability (51% - Figure 4c). This would also have manifested as low germination vigour (Matthews, 1981) i.e. a reduced capability of embryos to emerge through the seed coat. The hard seed coat in *Leucospermum* must necessarily be split by the expanding cotyledons before radicle elongation can occur (Brown and Dix, 1985; Brown *et al.*, 1986; Chapter 5). Although mechanical constraint has a minor influence in vigorous, fresh, fully matured seed (Brits and Van Niekerk, 1976) it is very likely that the breaking of the seed coat by an exterior mechanism would physically augment the emergence of under-developed or weak embryos. It is therefore likely that older seeds in the soil-stored seed bank, in which germination vigour is deteriorating, would benefit mechanically by endotesta fracturing or breaking.

The uniformly outstanding response of treatments 3, 4, 6 and 7 within species (Figure 4a-c) may point to a common denominator, oxygenation. Stereomicroscopic inspection confirmed that acid scarification (treatments 3, 7) caused breakage of both the exotesta and the endotesta. Hairline cracks in the endotesta could be seen in acid scarified seeds; in some seeds parts of the endotesta broke out, exposing the embryo and giving apparently the same reaction as in desiccated, wetted seeds (Chapter 6.2). Moreover acid scarification characteristically increases the brittleness of the tough endotesta, causing it to break easily by means of finger pressure (Brits and Van Niekerk 1976). This is very similar to the effect found after wetting the heat desiccated endotesta

(Chapter 6.2), brittleness indicating the presence of fractures, i.e. an improved oxygenation potential in the endotestal shell.

Conclusions

High temperature *per se* has been ruled out as a factor in the breakage/scarification of the testa layers in *Leucospermum* (desiccation being the active principle - Chapter 6.2) and also as a physiological factor (this study). Exotesta as well as endotesta scarification is therefore caused only by desiccation effects (excepting microbial scarification). The mechanism of the desiccation-scarification germination stimulus is primarily increased oxygenation. In effecting this the exotesta is the primary instrument, and to a lesser degree the endotesta.

The combined results suggest that in nature desiccation-scarification of soil-stored seeds could occur through a single high-intensity heat pulse of short duration, such as fire, or through repeated cycles of desiccation/hydration caused by a pattern of recurring moderate heat. Thus even in unburnt fynbos ambient summer temperatures alternated with high atmospheric RH or wetting by rain would cause scarification. In a study of seeds stored experimentally in the soil in unburnt fynbos both the exo- and endotesta were indeed scarified extensively over a four-year period (Chapter 6.4), supporting this conclusion.

Desiccation-scarification of *Leucospermum* soil-stored seed may thus be caused by either one, or a combination, of three heat phenomena in fynbos:

1) direct heat desiccation of soil by fire;
2) daily strong warming of fire-exposed soil by the sun during midsummer - an indirect effect of fire;
3) high ambient summer temperature combined with the prevailing strong south-easterly wind (De Lange and Boucher, 1993) and especially periodic hot, dry bergwind conditions, which further strongly amplify normal summer desiccative conditions; especially when, as is a frequent pattern, bergwind conditions are succeeded by rainy weather (Chapter 6.2).

The present results underline the high degree of adaptedness in *Leucospermum* seeds to fynbos stress and disturbance factors. Investigation of the effects of heat intensity of fire on subsoil temperature and soil moisture content in fynbos are needed to test the hypotheses above.

6.4 EFFECTS OF STORAGE UNDER LOW TEMPERATURE, ROOM TEMPERATURE AND IN THE SOIL ON VIABILITY AND VIGOUR OF *LEUCOSPERMUM CORDIFOLIUM* SEEDS

Introduction

Dormancy and its control imply long-living seeds. Thus desiccation-scarification patterns and the stimulative effects of scarification / oxygenation on seed germination, all relating to dormancy control (Chapters 6.2 and 6.3) make little sense without understanding longevity of *Leucospermum* seeds during soil storage. Storage and longevity of *Leucospermum* seeds in subterranean seed banks in fynbos are therefore important subjects about which, however, almost no information (except circumstantial evidence) is available. Coupled to seed longevity is the role of strategically extended germination ("germination polymorphism" *sensu lato* - Cavers and Harper, 1966; Keeley, 1991) of seeds within the same (Brits, 1987) and over several germination seasons. These seed characters and their ecological relationships are critical for short- and longterm survival of species populations in mediterranean-type vegetations (Keeley, 1991; Bell *et al.*, 1993) and generally in moisture-stressed, unpredictable environments (Gutterman, 1993).

Longevity and polymorphic germination have also not yet been studied in any detail in other fynbos genera with soil-stored seeds (Pierce and Cowling, 1991). Attrition of buried seed samples of 6 non-proteaceous fynbos species during soil storage in fynbos was rapid over 6-30 months, including those of myrmecochorous seeds (Pierce and Cowling, 1991). In California chaparral, soil-stored seed banks decay continuously, including those of fire-recruiting species (Parker and Kelly, 1989). Losses during soil storage are thought to be caused by pathogens (Cook, 1980). Germination during storage in unburnt vegetation (Cook, 1980; Pierce and Cowling, 1991) and seed ageing (Cavers, 1983) also lead to seedling loss. Soil-stored seed banks of four chaparral shrub species were found not to accumulate steadily in the soil (Keeley, 1977). However, some species have seeds with a soil storage life exceeding 20-100 years (Keeley, 1991).

Observations of erratic germination of many Proteaceae species with nut-fruited seeds within the first germination season and even over subsequent seasons are well documented (e.g. Knight, 1809; Van Staden and Brown, 1973). However this has never been studied systematically. In Australian mediterranean-type vegetation this strategy in soil-stored Proteaceae have likewise been researched scantily (Lamont *et al.*, 1985).

In the present study storage under simulated fynbos seed bank conditions was compared with the accepted commercial method of open-shelf storage at ambient temperature (R. Parsley, personal communication) and a potentially maximum-longevity regime. Van Staden (1978) stored seeds of *Protea neriifolia* R.Br. for three years, with minimal loss of viability, at 5 °C and in nitrogen gas at 20 °C. A combination of 3 °C in nitrogen was used in this study in an attempt to maximize the storage life of *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade seed.

To test survival capacity of stored seeds the criteria of viability and vigour were used. The viability of a seed population may be defined as the percentage seed that gives rise to normal seedlings under optimal incubation conditions in a standard laboratory germination test (International Seed Testing Association [ISTA], 1985). The biochemical tetrazolium-formazan reduction colouring test is regarded as the "most significant advance in seed viability testing technology of this century" (Grabe, 1970) and may be used as an alternative viability test (ISTA, 1985). Disinfected seeds of *L. cordifolium* which are incubated in oxygen at the optimum alternating temperature regime (Brits, 1986a) could lead to near-complete germination of the viable seed fraction, as compared against the tetrazolium colouring test ("laboratory" germination - Chapter 4). Dormancy is thus broken completely in such seeds. Seed vigour may be defined as those seed qualities which determine rapid, uniform emergence of normal seedlings under a wide range of field conditions (McDonald, 1980). The vigour of dormant seeds ageing in the soil-stored seed bank would be an important parameter in the study of natural seedling recruitment.

It is believed that complex ecological-physiological interaction occur in seeds under field, as opposed to laboratory, conditions (Keeley *et al.*, 1989). It is therefore essential to simulate the fynbos germinative environment as closely as possible to augment laboratory studies. In this study the germination of seeds in an open seed bed adjacent to fynbos, under late autumn conditions (Vogts, 1976) was assumed to approximate the seedling emergence regime of the post-fire habitat. The seed bed could therefore give emergence behaviour similar to that of naturaly recruiting *Leucospermum* populations (Brits and Van Niekerk, 1976; Chapter 4.1). A similar seed bed regime was used successfully in the study of seasonal temperature effects on *L. cordifolium* seed germination (Brits and Van Niekerk, 1986).

To maximize seedling emergence in the seed bed for testing vigour, seeds may be oxygenated with 1% H_2O_2 before sowing (Brits and Van Niekerk, 1976). Seeds scarified in (c) H_2SO_4 can likewise improve oxygenation of seeds in the seed bed, leading to near-complete emergence as verified by tetrazolium testing (Brits and Van Niekerk, 1976). Tetrazolium tests are used to also verify field performance (vigour/seedling emergence) tests (Moore, 1973). Another method of stimulating all viable seeds to germinate is the use of growth regulators in addition to an optimum environment. Cytokinin, for example, was found to enhance Proteaceae seed germination when applied at a low concentration (Brown and Van Staden, 1973). In this study cytokinin was applied as the commercial preparation benzyladenine.

Materials and methods

Seed supply

Seeds of *L. cordifolium* were harvested from cultivated plants (Brits, 1987; Chapter 2) under controlled conditions after natural release in December. "Seeds" were harvested as achenes, the true dispersal units, with a mature, turgid pericarp (elaiosome) present, and were dried for 6 w under summer conditions in the shade. Seeds were hand sorted to ensure the use of fully developed embryos (Vogts 1976; Van Staden and Brown, 1973) and thoroughly mixed.

1. Seed storage experiment

Storage treatments. Seeds were divided into lots and stored under four storage regimes during late summer:

1. Control seeds were sown in a seed bed, following two months of dry storage at ambient temperature and humidity (Year 0).
2. Soil-storage (simulating ant dispersal and pre-fire soil seed bank conditions): two hundred intact seeds were placed in a bag of glass-fibre gauze (1,5 mm mesh size), covered in addition by an outer layer of galvanized wire gauze (1 cm mesh size) to exclude possible seed eaters. Three such sub-lots were prepared and buried in fynbos soil. Seeds were buried in a typical *Leucospermum cuneiforme* (Burm. f.) Rourke habitat in mesic fynbos (Brits, 1987), unburnt for eight years, in the zone where *L. cuneiforme* seeds are normally dispersed by ants (Brits, 1987). A spot was chosen 75 cm south of each of three *L. cuneiforme* plants (reference plants) in shade of average density (Brits, 1986a) of the reference and other plants such as *Erica* spp. A spade was inserted parallel with the soil surface at c. 3 cm depth in the direction of the reference plant so as to minimally disturb the soil and root mass, the spade was lifted slightly and the flat bag with *L. cordifolium* seeds guided into place after which the soil mass (damp from recent rain) was replaced as before.

Soil-storage treatments were limited to a single seed sub-lot (replicate) per storage period on account of availability of reference plants and especially the need to leave soil-stored seeds in an undisturbed condition in each storage site. Thus sub-samples were not removed from seed bags during the storage period.

The seed bag sub-lot from each reference plant was removed after one, two or four years of undisturbed burial and the seeds examined under a stereomicroscope for signs of possible damage and mortality. Empty, broken testas were assumed to indicate germinated seeds and were counted. The seed coat was inspected for signs of scarification brought about by soil factors such as microbial decomposition. Unbroken seeds were removed and cleaned slightly, taking care not to add to the degree of scarification already present.

3. Warm-storage (dry storage under ambient conditions): Seeds were stored in groups of 33 in porous plastic bags placed in a 1 l glass flask of which the opening was covered with a single layer of filter paper. The container flask was placed on a laboratory shelf in shade and at designated intervals seed bags were removed randomly from the flask for germination tests.

4. Cold-storage (dry storage at 3 °C in nitrogen): Seeds were stored in groups as above in a 1 l glass flask in nitrogen and closed airtight with a screw-on lid sealed with petroleum jelly. The flask was flushed at six-monthly intervals. At designated intervals seed bags were taken randomly from the flask for germination tests after standing the flask at room temperature to attain temperature equilibrium.

Seed preparation and sowing. Seeds were germinated in March-April in the designated years. Seeds were soaked in distilled water and in non-soil stored seeds the pericarp was then removed (Chapter 6.2). All seeds were dried lightly with paper towel until the seed coat contained no free water and then were treated with thiram (75% a.i.) w.p. (Benic, 1986). Care was taken not to disturb the generally fragile condition of soil-stored seeds.

Germination regimes. 1. Laboratory germination. Seeds were incubated in air or medical grade oxygen in 1 l flasks (Chapter 4.3) under the optimum 8 x 24 °C temperature regime for *L. cordifolium* (Brits, 1986a).

A tetrazolium viability test was carried out in Year 0 on a sample of seeds to estimate viability of the fresh seed source (Chapter 2). Six replications of 33 seeds were treated, scored and the mean tetrazolium colouring values calculated.

2. Seed bed germination. Seeds were germinated in standard seed beds fully exposed to sunlight (Vogts, 1976) following disinfection. Disinfection was considered necessary because regularly used seed beds can accumulate pre- and post-emergence seed(ling) pathogens (Benic, 1986). Disinfection consisted of spray-watering the sand-medium in the bed with a 3% formalin solution at an application rate of 10 l solution.m^{-2}.

To obtain an indication of the germination vigour of the fresh seed source, the average total emergence scores of the two H_2O_2 oxygenated treatments in the "extended germination" experiment below was used. The same seed source was used and the two experiments commenced on the same date.

Microscopy. Soil-stored seeds for SEM examination was freeze-fractured in liquid nitrogen, mounted onto aluminium stubs and sputter coated with gold-palladium for viewing in a Cambridge S200 at 10kV.

Observations and statistical treatment. Germination in Experiment 1 was recorded weekly for 12 weeks. Laboratory incubated seeds were scored as germinated when the radicle had protruded at least 1 mm. In the seed bed successful emergence was recorded when the seedling cotyledons had become effectively exposed to daylight. Germination percentages and rates were calculated, the latter by means of the formula of Heydecker (1973; Chapter 4.2) from the 4th week after sowing.

The statistical layout was a completely randomized design with 24 treatments, 3 replications and 20-33 seeds per experimental unit (replication). The treatments consisted of a 2 x 3 x 4 factorial structure with factors: 2 regimes (laboratory and seed bed); 3 treatments (soil-, warm-, cold-stored); and 4 years (0 = start; 1, 2, 4 years later).

Percentage data tested positively for normality (univariate procedure) and analysis of variance was carried out on germination percentage values and germination rates. Student's t-LSD's were calculated to compare treatment means.

2. *Extended germination of* Leucospermum cordifolium *seed*

The second experiment was designed to study polymorphic germination under simulated fynbos (seed bed) conditions. Fresh, intact seeds of *L. cordifolium*, using the same seed source as above, were disinfected in hot water at 50 °C for 30 min. (Benic, 1986). Seeds were soaked in 1% H_2O_2 for 24 h (Chapter 2) or in a 200 mg l^{-1} benzyladenine (BA) solution (Mitchell *et al.*, 1986) or in a combination of BA and H_2O_2. Controls were soaked in water, one disinfected, not receiving H_2O_2 oxygenation or BA treatment, and another non-disinfected and similarly treated. The softened pericarps were rubbed off, seeds were washed briefly in running water and then allowed to dry off until the seed coat contained no free water. Seeds were then treated with thiram fungicide powder and sown in autumn as in the storage experiment. Six replicates of 33 seeds per treatment were sown in a randomized block design. Seed bed emergence was recorded weekly throughout the winter (20 weeks) in the first and in four successive winters; observations of weeks 8-20 were pooled in each season. Seed bed irrigation was discontinued during the summer months and the seed bed was weeded at the start of each germination season to obtain a sun-exposed soil surface. Analysis of variance was performed on germination (emergence) percentage and rates as in the storage experiment.

Results

1. *Seed storage experiment*

Viability and vigour estimates. The tetrazolium viability estimate for the seed source at the start of the study was 81% (Figure 1a). In contrast H_2O_2 oxygenated seeds in the seed bed averaged 57% emergence in Year 0 plus a further 13% emergence extending over the following 4 years, totalling 70%. The latter figure was used as an estimate of the vigour of the seed source (Figure 1b).

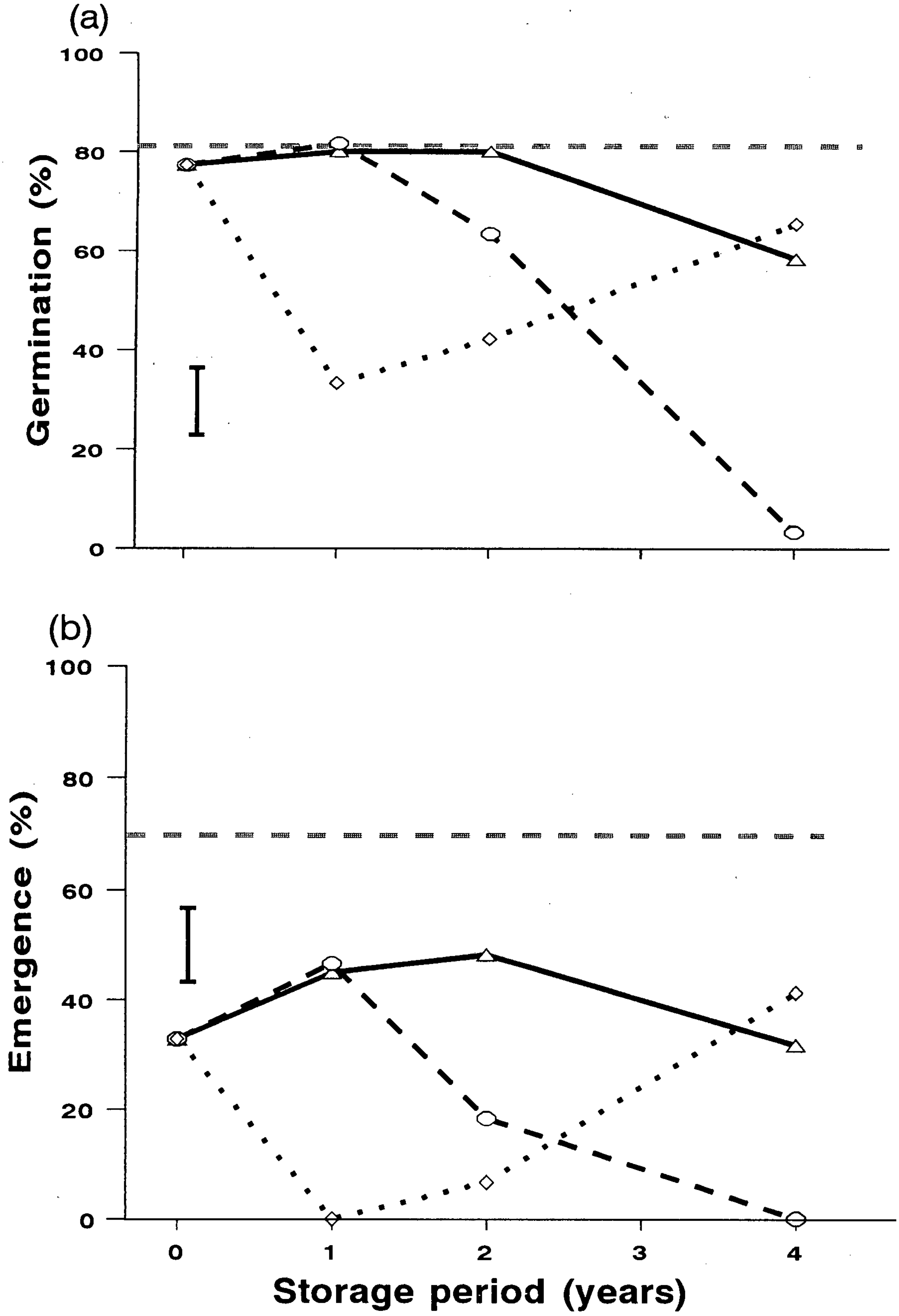


Figure 1. Laboratory gemination (a) and seed bed emergence (b) percentages of *L. cordifolium* seeds stored in the soil (◇), under ambient temperature (- - o - -) and at 3 °C in nitrogen (—△—) for various periods; ▬ tetrazolium viability estimate (a) or seed bed vigour estimate (b). Bar represents LSD (P = 0.05).

Table 1. Analysis of variance of *L. cordifolium* seed germination rates and percentages under different storage treatments and germination regimes.

		Germination Rate		Germination Percentage	
Source	d.f.	MS	SL	MS	SL
Regime	1	3527,623	0,0001	17520,335	0,0001
Treatment	2	3615,490	0,0001	2848,021	0,0001
Reg*Treat	2	401,811	0,0239	22,582	0,6952
Year	3	2500,759	0,0001	1260,876	0,0001
Year*Reg	3	235,010	0,0823	499,801	0,0002
Year*Treat	6	1291,732	0,0001	2878,270	0,0001
Year*Reg*Treat	6	284,100	0,0190	174,777	0,0207
Error	42	98,369	-	61,582	-
Total	65	-	-	-	-

Abbreviations:
MS = Mean square
d.f. = Degrees of freedom
SL = Significance level

Percentage germination. Seed bed regime emergence was generally lower than laboratory regime germination, the difference totalling 33% (Figure 1; Table 1) with the two regimes showing similar treatment patterns, i.e. with low statistical interaction (Regime x treatment - Table 1).

Cold-stored seeds germinated maximally over Years 1 and 2, close to the level of the tetrazolium viability estimate and the initial value of unstored seed in Year 0 (Figure 1a). This treatment maintained relatively high average levels of germination and emergence of 72 and 41% respectively over the experimental period (Figure 1a, 1b). Seed bed emergence of cold-stored seeds rose markedly from Year 0 (fresh seed vigour estimate) to Years 1 and 2 (following cold storage) the latter difference being statistically significant ($P < 0.05$ - Figure 1b). This is in contrast to the relatively uniform values obtained in the laboratory (Figure 1a). The increase in the seed bed over time would not have been due to after-ripening (Brits and Van Niekerk, 1986) and probably reflects seasonal effects operating in the seed bed, but not in the laboratory. The decline of cold-stored seed bed emergence in Year 4 however corresponded with a decrease in laboratory germination, both changes being statistically significant ($P < 0.05$).

In soil-stored seeds both germination and emergence fell strongly in Years 1 and 2, then increased to near original levels in Year 4, the latter differences in the case of seed bed emergence values not being statistically significant (Figure 1). Since viability declines continuously in the soil-stored seed bank (Cavers, 1983) the low values in Years 1 and 2 relative to Year 4 are probably due to unknown experimental artefact. Soil germinated seeds (broken seed coats) amounted to 6% (Year 1), 6% (Year 2) and 12 % (Year 4), therefore seeds were not lost in the soil in large numbers through "pre-fire" germination due to a lack of dormancy. A limitation of this study was that time trends in soil-stored seeds could not be determined, however the burial treatments represent single-sample estimates of year effects on viability of soil-stored seeds. Thus the significantly higher performance of buried seeds in Year 4 than in other storage years, although coincidental, represents an unbiased estimate of burial effects after 4 years. Under both regimes the Year 4 values exceeded the performance of cold-stored seeds, although the difference was not statistically significant (Figure 1). The Year 4 soil-storage values represent c. 80% of the original tetrazolium and germination viability estimates (Figure 1a) and c. 60% of the emergence vigour estimate.

Storage at ambient temperature and humidity led to a much faster decrease in germination percentages than low temperature storage (Figure 1). Thus in Year 1 warm-stored seeds germinated and emerged on a par with Year 0 and cold-stored seeds but surprisingly soon, in Year 2, germination fell markedly ($P < 0.05$ - Figure 1). Seed germination occurred only at extremely low levels in both the laboratory and seed bed regimes after four years.

Germination rate. Germination and emergence rates are presented in Figure 2 and Table 1. Laboratory regime germination rates were markedly higher than seed bed regime emergence rates (Figure 2, $P < 0{,}001$ - Table 1). In the seed bed seedlings were observed to emerge at least 1-2 w slower than seeds germinating in the laboratory. This reflects the additional time required for seedlings to emerge above soil level, compared with radicle protrusion only for germination.

Buried seeds germinated much faster than non-soil stored seeds especially after two and four years of soil storage (Figure 2). The rates in non-soil stored seeds, sown with intact testas, did not differ significantly within either the seed bed or the laboratory, except in cold-stored seeds of Year 2 in the laboratory regime (Figure 1a). Progressive

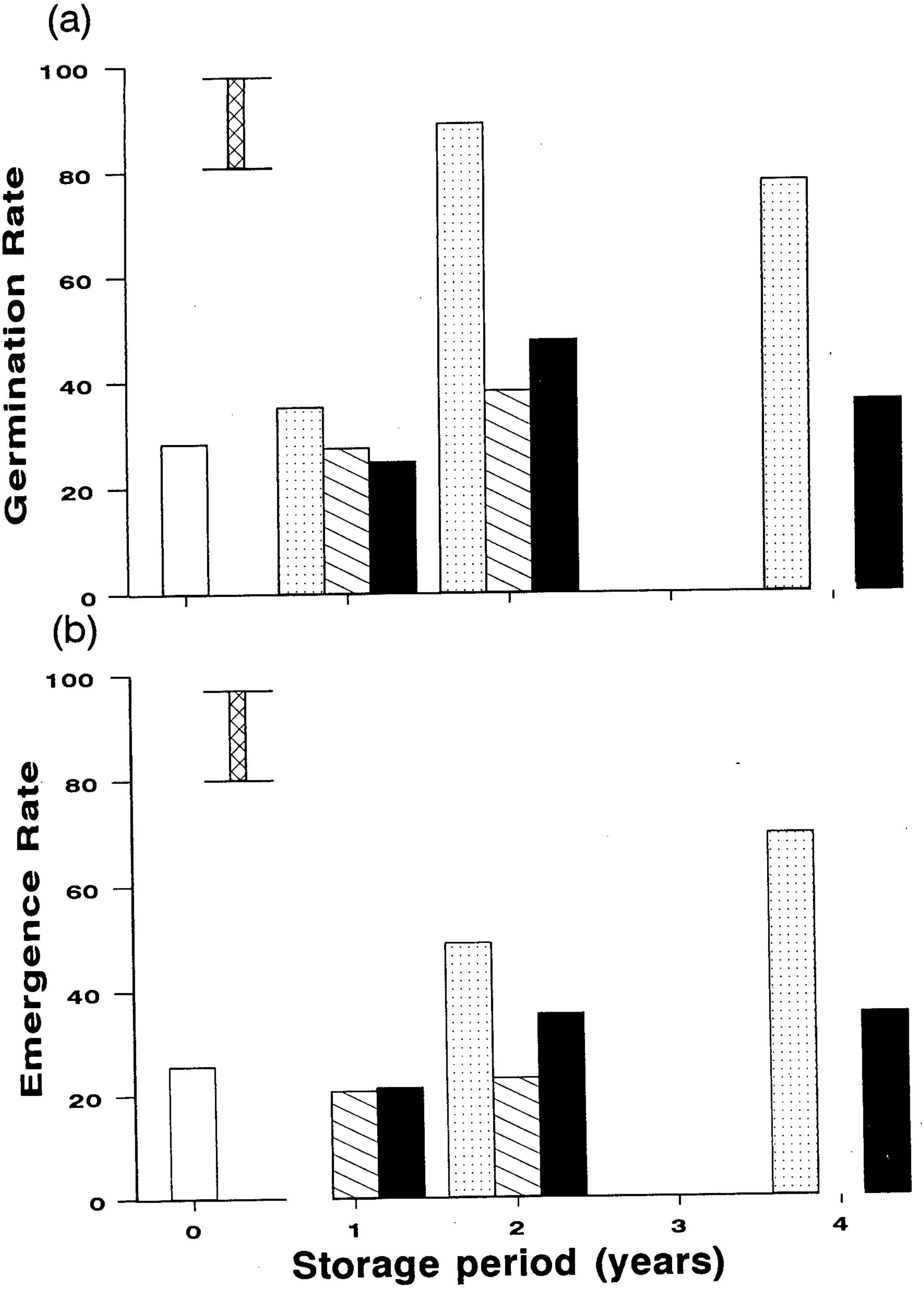


Figure 2. Rates of laboratory germination (a) and seed bed emergence (b) of *L. cordifolium* seeds stored in the soil (░), under ambient temperature (▧) and at 3 °C in nitrogen (■) for various periods; small bar represents LSD (P = 0.05). Missing bars are due to low germination / emergence values.

scarification of the testa during soil storage is suggested by the positive slopes of both laboratory germination (b = 13,261; $P<0,05$ - Figure 3) and seed bed emergence (b = 10,579; $P<0.01$ - Figure 3) in the regression of rate on period of soil storage. Effective scarification was also confirmed by inspection (Figure 5).

2. *Extended germination of* Leucospermum cordifolium *seed*

Benzyladenine treatment did not have an effect on seedling emergence (Figure 4), therefore the +BA and -BA treatments are treated as two replicates of the other factors, H_2O_2 oxygenation and disinfection. Brown *et al.* (1986) and Brits *et al.* (1995) found a suppressive effect of BA on laboratory germinated *L. cordifolium* seeds. Disinfected seeds emerged significantly better ($P<0,05$) in the first germination season and maintained a significant increase over the control up to the 5th germination season (Figure 4). It is not known whether the suppression of pathogens, or possibly an

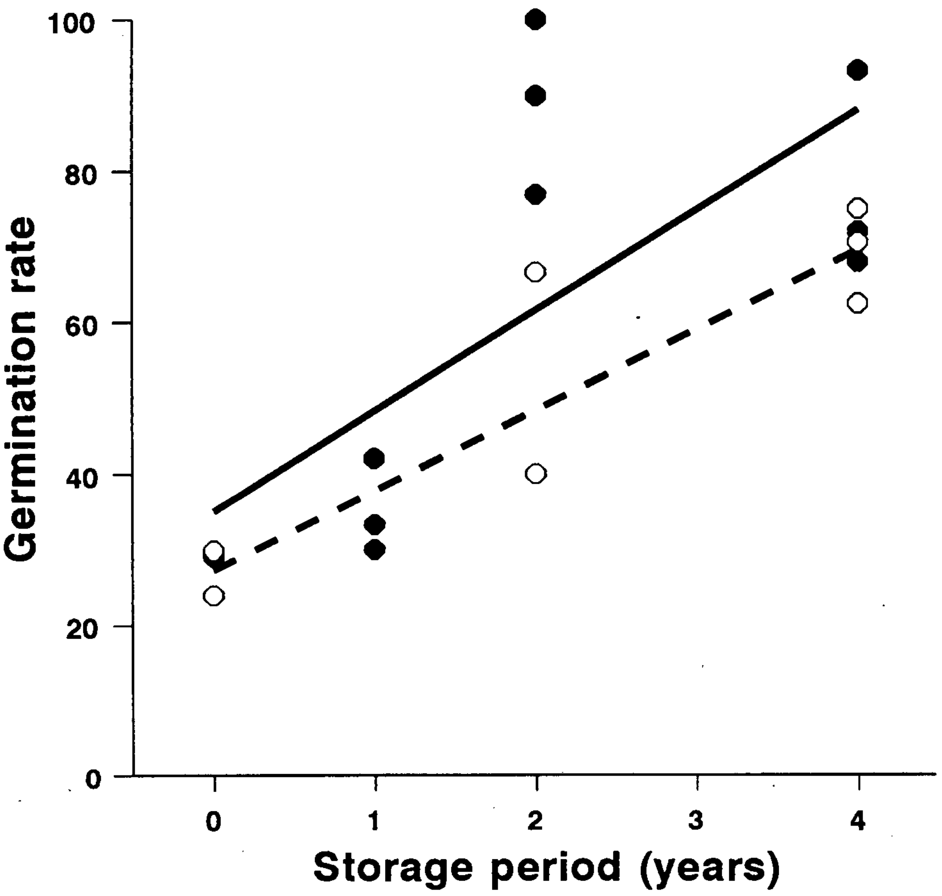


Figure 3. Regressions of laboratory germination rate (●,——) and seed bed emergence rate (o, - -) on period of soil storage in *L. cordifolium*. Germination rate: Y = 35,104X + 13,261; R^2 = 0,51 (laboratory). Emergence rate: Y = 27,274X + 10,579; R^2 = 0,79 (seed bed).

unknown treatment artefact, for example direct heat stimulation of the embryo during disinfection (cf. Blommaert, 1972) caused the difference.

H_2O_2 oxygenated seeds gave a much stronger germination response than control seeds (Figure 4). Seedling emergence percentages of oxygenated seeds accumulated close to the tetrazolium viability estimate by the 5th year, suggesting near-complete emergence of vigorous seeds after the 5th season (cf. Brits and Van Niekerk, 1976). Control seeds gave the typical erratic, extended pattern, and low total emergence, of intact non-oxygenated seeds (Brits and Van Niekerk, 1976).

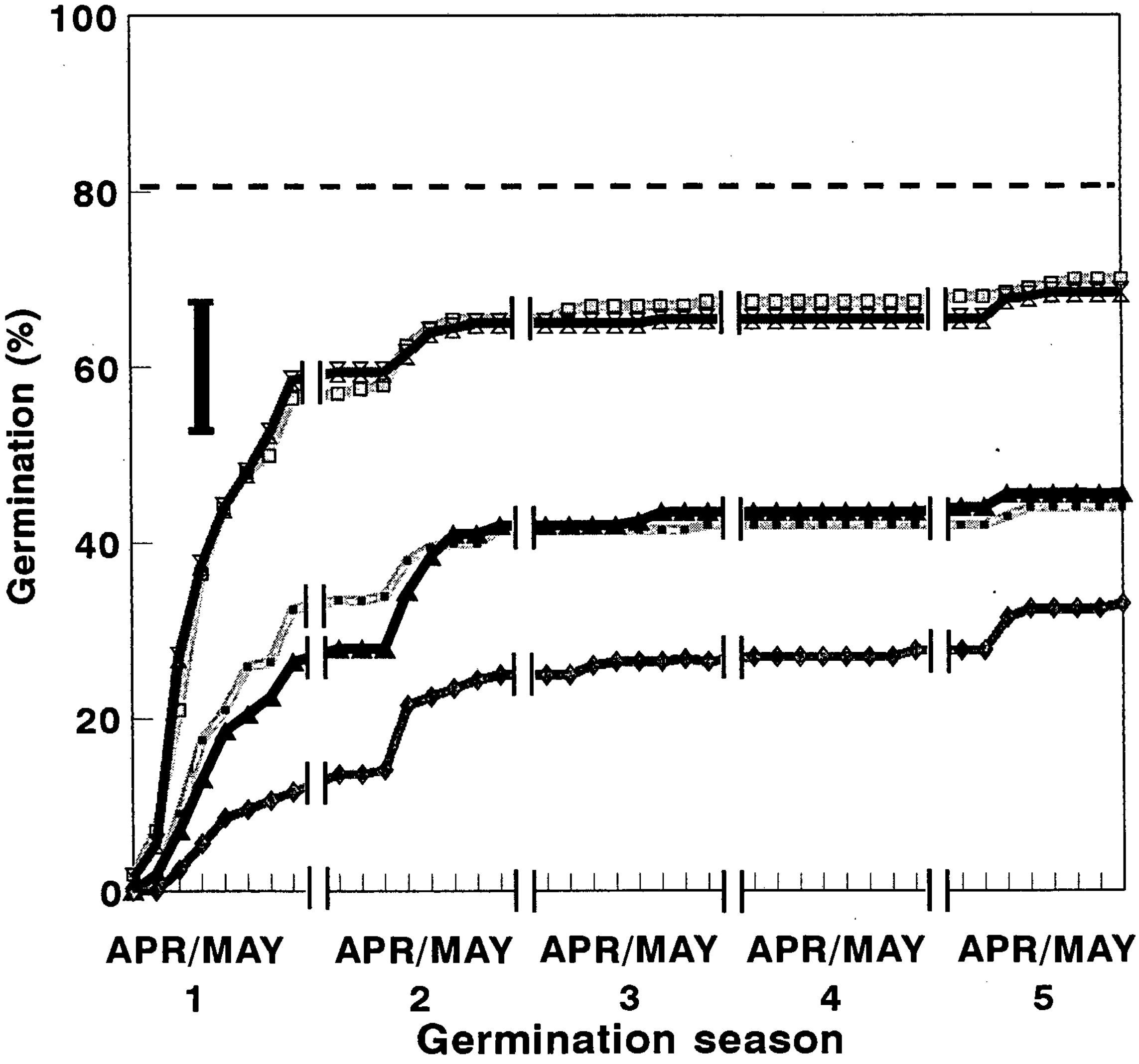


Figure 4. Seed bed emergence of *L. cordifolium* seeds extending over 5 germination seasons;—□— H_2O_2 oxygenated;—x— H_2O_2 + benzyladenine (BA) treated;—▲— disinfected + BA; ▪ disinfected;—◇— control; ----- tetrazolium viability estimate (ex storage experiment); bar represents LSD (P=0,05). Emergence was recorded weekly; the last data points in each germination season represent the total emergence for weeks 8-20.

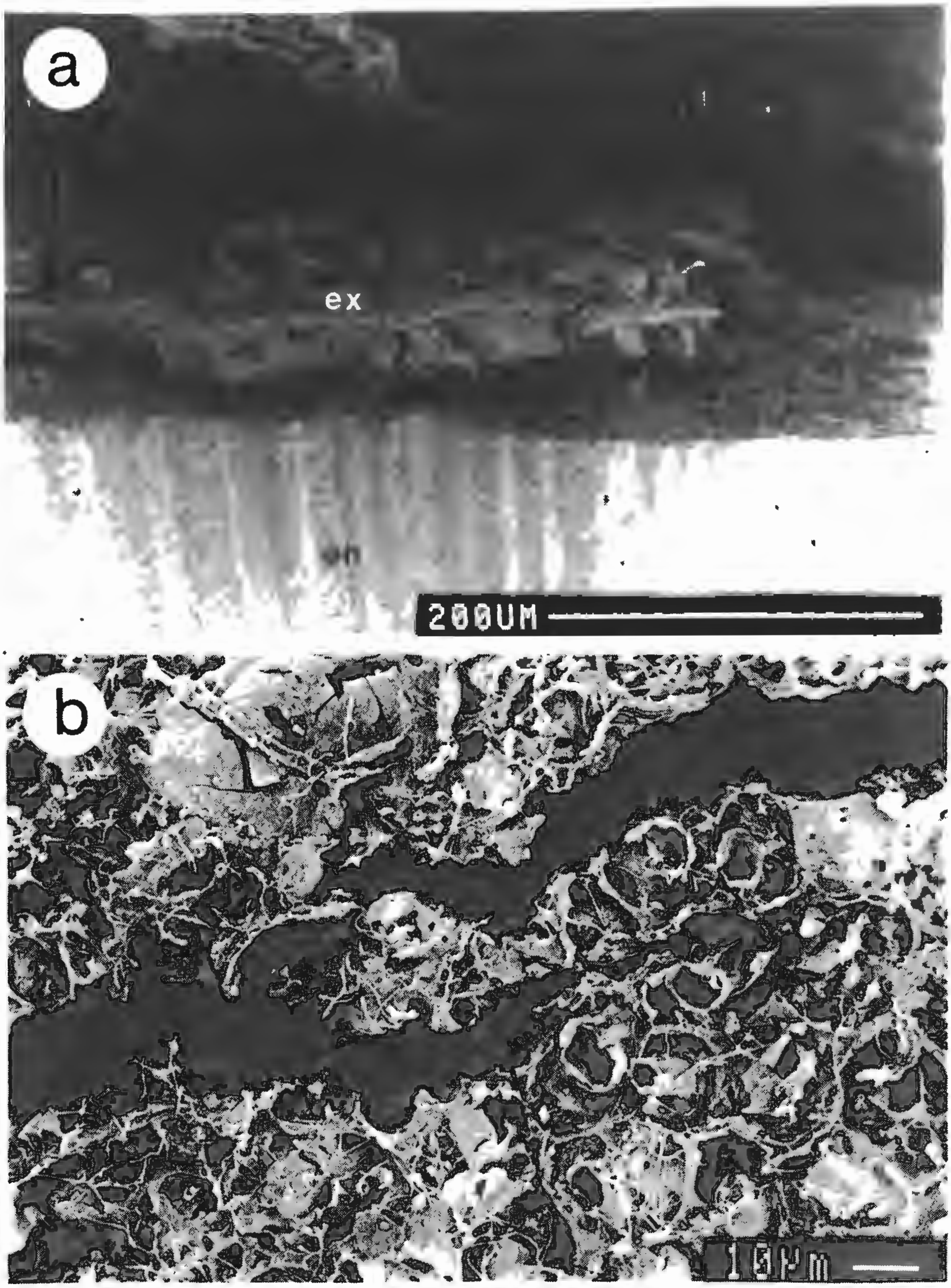


Figure 5. SEM micrographs of testa scarification in *L. cordifolium* seeds after 4 years of soil storage; a: exotesta, ruptured extensively, exposing the endotesta; overlying pericarp completely decomposed. b: surface view of endotesta, showing hairline cracks which contribute to a brittle seed coat texture. en - endotesta (palisade cells); ex - exotesta

Emergence extended over five germination seasons in all treatments and seeds emerged only during autumn and early winter each year; in controls germination extended much stronger than in oxygenated treatments (Figure 4). Thus control seeds gave only 34% emergence in the first year of the final portion emerged (by the end of the 5th season), amounting to only 16% of the vigorous seed fraction (i.e. of the cumulative total of H_2O_2 oxygenated seeds emerged by the end of the 5th season - Figure 4). H_2O_2 oxygenated seeds, by contrast, gave 83% emergence (average of two H_2O_2 treatments) in the first germination season, increasing to 100% of vigorous seeds by the fifth season.

Discussion

Seed viability vs. *vigour*

The constantly lower performances in both germination percentage and rate of seeds in the seed bed as opposed to the laboratory reflect the difference between seed vigour and seed viability measurement respectively. Germination viability estimates over Years 0 and 1 for non-soil stored seeds corresponded closely with the tetrazolium viability estimate (Figure 1a) validating the use of the laboratory germination regime as a measure of viability. In the seed bed, on the other hand, considerable embryo and seedling vigour was required in addition to radicle protrusion (definition of a viable seed) to a) establish an initial root system, b) displace a considerable weight of soil above the seedling and c) to expand the cotyledons at the soil surface. Marked differences may occur between seed viability and vigour estimates, especially in seed populations with poor viability (Moore, 1973; Hegarty, 1974).

Seed bed emergence also declined faster than laboratory germination, as is reflected by Year 2 values of warm-stored seeds. This illustrates that vigour of a seed populaton declines faster than does its viability (Delouche and Caldwell, 1960). Thus seed bed emergence gave an indication of population recruitment in nature, which is primarily a function of the vigour of soil-stored seeds.

Responses of soil-stored seeds

Inspection revealed that buried seeds contained no remnants of the pericarp/elaiosome. The exotesta (Chapter 3) was apparently thoroughly scarified and clearly showed progressive deterioration with time (Figure 5). The endotesta surprisingly also became brittle and easily breakable with finger pressure, which likewise indicates strong scarification, caused by cracking (Figure 5). Mechanically and visually the testa after 2 and 4 years of soil storage more or less resembled acid scarified seeds (Brits and Van Niekerk, 1976). Testa imposed dormancy as a result of oxygen exclusion would thus have been broken in older soil-stored seeds. Significantly the embryos in soil-stored seeds appeared white, plump and healthy-looking, including those stored for 4 years. Thus attrition of the four-year seed sample was remarkably low. The Year 4 estimate is probably the most reliable indication of potential seed longevity during soil storage, assuming that the experimental soil-storage regime would be suboptimal compared to the regime adapted to in nature.

The high germination rate of buried seed was probably contributed to by effective scarification of the testa. Scarified seeds would thus have been oxygenated naturally and optimally during incubation, as opposed to non-soil stored seeds in which the exotesta remained intact. The scarified, brittle state of the endotesta would in addition have permitted rapid emergence of the radicle. Alternatively seeds may have completed and accumulated the initial stages of germination during repeated wet periods in nature, shortening the final period of continuously favourable conditions required for germination (Lush *et al.*, 1984). This aspect of *Leucospermum* seed ecology deserves more study.

Seed bed vs. soil storage conditions

The results demonstrate that a seed batch can survive in fynbos for a minimum of 4 years and that seeds become strongly scarified during soil storage. Viability and vigour was maintained at a high level in the 4-year seed batch (Figure 1). Moreover the uniform, vigorous, undelayed germination following sowing in the seed bed indicates that seed dormancy must have been enforced effectively in the reference plant soil zone. Conversely, the seed bed represents the conditions for dormancy relief.

The factors that could possibly have contributed to dormancy breaking in the seed bed are 1) removal from allelopathic suppression of germination by the reference (and other) plants (enforced dormancy - Keeley and Keeley, 1989); 2) removal from oxygen deprivation by respiring roots and soil microflora (Brits, 1986b); 3) most likely, the strongly increased diurnal temperature amplitude in the exposed seed bed surface soil (Brits 1986a). Smoke as a germination stimulus in *Leucospermum* is not implicated here.

Decline in germinability of non-soil stored seeds.

The germination and emergence of warm-stored seeds declined markedly after 2 years and drastically after 4 years. Even storage at low temperature in a nitrogen atmosphere did not prevent decline after four years, as is shown by both laboratory and seed bed results. Bass (1980) concluded that seed storage under inert gases is not effective and that the main determinants of longevity are humidity and temperature. It is noteworthy that, despite circumstantial evidence for high longevity of *Leucospermum* seeds (Rourke, 1972) and despite the combination of two treatments which separately maintained a high viability in *P. neriifolia* seeds over 3 years (low temperature and storage under nitrogen - Van Staden, 1978) these treatments failed to prevent gradual loss of viability in *L.*

cordifolium by Year 4. Storage of *Leucospermum* seed under ambient conditions for longer than 1-2 years, and under low, non-freezing temperature for longer than 2-4 years, can therefore not be recommended.

Van Staden (1978) and Van Staden *et al.* (1981) reported roughly similar deterioration patterns of *P. neriifolia* (R. Br.) seeds during longterm dry storage. Even when stored at -10 °C seeds showed signs of deterioration. Although germination percentage after 6 years of low temperature storage did not fall appreciably below control values there was a marked reduction in germination rate (Van Staden *et al.*, 1981).

Ageing *P. neriifolia* seeds stored at 20 and 26 °C show primarily lipid body and lipid membrane degeneration (Van Staden, 1978; Van Staden *et al.*, 1981). Protein bodies also showed signs of degeneration, as did nuclear material. Damage to the nuclear material affects glyoxysome production and thus lipid utilisation for energy release (Van Staden *et al.*, 1976).

L. cordifolium seeds were observed to contain protein and lipid as principal storage reserves (Mitchell, 1983) as was also shown for *P. neriifolia* (Van Staden *et al.*, 1981); *P. compacta* (Van Staden *et al.*, 1975; Van Staden *et al.*, 1976); *Leucadendron daphnoides* (Thunb.) Meisn. and *P. compacta* (Mitchell, 1983). It seems likely that seed ageing would follow comparable biochemical and physiological patterns in these species. Stored lipids are susceptible to autoxidation, particularly at high temperature: autoxidation of unsaturated fatty acids results in the formation of free radicals and hydro-peroxides, which in turn sustain the process of lipid denaturation. This leads to the inactivation of enzymes, membrane injury and histone denaturation, causing loss of seed viability (Koostra and Harrington, 1969).

In soybeans, which also contain a relatively large amount of storage lipid, the loss of both seed viability and vigour was slowed markedly when seeds were hydrated, then dehydrated, during mid-term storage (Saha *et al.*, 1990). The proposed mechanism for this phenomenon is the activation of the cellular repair system and counteraction of free radical and lipid peroxidation reactions during hydration. Clearly the soil- and warm-stored *L. cordifolium* seeds in this experiment would have been subjected to a roughly similar temperature regime in the field and on the laboratory shelf. The major difference in storage conditions would therefore have been the numerous hydration/dehydration cycles to which soil-stored seeds were subjected in nature over four years.

L. cordifolium seeds contain more storage lipid than *P compacta* and *L. daphnoides* (Mitchell, 1983). It is therefore tempting to infer lipid autoxidation as the major degenerating factor in the surprisingly short shelf life of *L. cordifolium* seeds stored dry at ambient temperature; and in nature, cellular repair during periods of hydration as the basis for potentially high longevity of seeds in soil. However, non-viable *P. compacta* seeds characteristically have primarily lost the ability to digest available lipid reserves and thus are unable to germinate (Van Staden *et al.*, 1975; Van Staden *et al.*, 1976). This may also be the case in *L. cordifolium*.

Canopy- vs. artificial storage in serotinous species

An interesting parallel exists in natural vs. artificial seed storage in some serotinous Proteaceae species. Although *P. neriifolia* seeds rapidly deteriorated when stored dry at ambient temperature (Van Staden *et al.*, 1981) seeds of this species when stored on the plant for three years and longer apparently lose very little viability and vigour, except for a slight decline in germination rate (Le Maitre, 1990). Essentially the same result was found for 4-year and older canopy-stored seed of two other, closely-related species, *Protea susannae* Phill. and *P. obtusifolia* Buek ek Meisn., and two serotinous *Leucadendron* R.Br species (Mustart and Cowling, 1991). These species typically gave germination percentages of >95% of 4-year and older seeds. Although roughly comparable temperature regimes applied in all cases, the major difference in storage conditions was the fact that all serotinous Proteaceae seeds were stored in nature in a turgid condition within the infructescenses on the parent plants (unpublished) as opposed to the artificial dry storage regime used by Van Staden *et al.* (1981) for *P. neriifolia.* Van Staden and Gilliland (1976) proposed that the inability of ageing *P. compacta* seeds to utilize lipid was due to chromosome damage in the dying nuclei. Chromosomal damage in ageing lettuce seeds was found to be much less when seeds were maintained at a relatively high moisture content of 18.1%, as opposed to dry storage at low moisture content (Rao and Roberts, 1990). At high moisture content the cellular repair mechanisms are probably activated, a process sustained by respiration in the presence of oxygen (Rao and Roberts, 1990; Murdoch and Ellis, 1992). Thus maintenance of longevity during soil storage, mediated by periodic hydration, remains a distinct possibility in *L. cordifolium*, a parallel for which apparently exists in serotinous Proteaceae seeds. This aspect of seed longevity, and the implied potentially improved seed storage technology for Proteaceae, deserves further study.

Extended germination

Extended germination (Figure 4) could have survival value for a species population regenerating from seed. Thus a pattern of seedling cohorts emerging in succession over one or several germination seasons could result in the extant gene pool escaping possible total destruction by disasters, e.g. predation (Cavers, 1983). Such extended emergence patterns were observed in recruiting *Leucospermum cordifolium* and *L. cuneiforme* seedling populations in fynbos (Brits, 1987). These were limited to a minority of seedlings, in contrast to a large preponderance of older seedlings of c. the same age which emerged early in the germination season (Brits, 1987).

Germination in this study resumed only during autumn in successive years. This demonstrates the narrow requirements for alternating germination temperatures in *Leucospermum* (Chapter 4.4).

Conclusions

The present results confirm, for the first time, that in *Leucospermum*:

1) seeds are long-lived and may retain high viability and vigour for up to at least four years of soil storage;

2) conditions in unburnt fynbos enforce dormancy; apparently only oxygenation and alternating temperatures are required to subsequently break dormancy;

3) the seed coat is effectively scarified during soil storage and this implies germination mediated by embryo oxygenation in air when favourable temperature and moisture regimes co-occur after fire. This suggests that after fire the larger portion of the seed bank will germinate rapidly and synchronously during autumn in a first, major wave of recruitment, with some seeds remaining dormant;

4) seeds in nature can tolerate repeated hydration and desiccation whilst maintaining their viability. Regular hydration/dehydration cycles may indeed be the key to maintaining high seed viability. It is proposed that this feature of soil storage may play an important part in maintaining longevity, possibly by activating cellular repair mechanisms;

5) older scarified seeds in soil storage germinate much faster than young, intact seeds. The principle of partial completion of the early germination stages during storage could play a role and this phenomenon deserves futher investigation.

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CHAPTER 7: SEED STRUCTURE AND FUNCTION

CONTENTS:

[Results and Discussion:]

CHAPTER 7

SEED STRUCTURE AND FUNCTION

PREFACE

In the final study seed coat development is related to structures that have known ecological function. The ontogeny, and subsequent fate of these structures during soil storage, is followed. On the basis of newly-found seed-environment relationships a working model of the dynamics of the *Leucospermum* myrmecochore seed bank in fynbos is developed. Finally the implications of proposed seed functions and population dynamics are considered in evolutionary context: how do the advantages for the species of stress- and risk-negotiating characters weigh up against the price of adaptation?

7. EVOLVING *LEUCOSPERMUM* R.BR. (PROTEACEAE) SEED STRUCTURE AND PHYSIOLOGY IN FYNBOS

Introduction

Seed structure and function in relation to ecology have been little studied in fynbos (Le Maitre and Midgley, 1992). The abundance of *Leucospermum* seed studies (see review by Van Staden and Brown, 1977; Brown *et al.*, 1986) offers an opportunity to investigate this aspect in myrmecochorous Cape Proteaceae. Such study is of special interest because of the severe and complex dormancy found in especially the "nut-fruited" Proteaceae seeds (achenes technically) in fynbos (Kelly *et al.*, 1992). "Complex" refers to multiple dormancy characters in which internal regulatory mechanisms interact with external, environmental factors and also within the seed (Chapter 1). Dormancy has also been characterised as complex in *Cyperus inflexus*, in which multiple environment-seed interactions occur (Gutterman, 1993, quoting studies by Baskin and Baskin) but otherwise the multiple-mechanism process to break dormancy in seeds is little understood and few published reports exist (Bell *et al.*, 1993). Keeley (1991) discusses germination cues in chaparral which are required in addition to charred wood and heat shock to break seed dormancy, however the ecophysiology of these multiple mechanisms are unknown. The most challenging aspect of the study of dormancy in the nut-fruited Proteaceae, especially in *Leucospermum* (Brown *et al.*, 1986) is to relate its regulatory mechanisms to ecological function ("environmental factors" - Van Staden and Brown 1977).

Leucospermum species form a closely-knit group with respect to their seed reproductive strategies (Rourke, 1972; Chapter 4.3). The same applies to their seed anatomy, which has been studied in *Leucospermum conocarpodendron* (L.) Buek (as *L. conocarpum* R.Br. - Jordaan, 1945) and in *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade (Chapter 3). Seed dormancy and germination in *Leucospermum* have been well characterised. Dormancy is of both the primary and enforced types (Chapter 1), i.e. dormancy is present in the mature seed, imposed by the oxygen-impermeable coat (Chapters 3, 6) and is enforced during soil storage by environmental requirements for two temperature levels (Chapter 4.4). Germination of the embryo ensues as soon as anoxia is removed and suitable temperatures are provided (Chapter 5).

The model developed in this study applies to the above-mentioned complement of fynbos *Leucospermum* species with roughly similar seed ecologies and with similar germination strategies. This group occurs mainly in the areas of greatest species concentration and excludes the non-fynbos members of the ancestral Section Crassicaudex (Rourke, 1972).

The fynbos environment may be characterized as harsh in that it subjects seeds to multiple and severe stresses and disturbances. The main influences are extremely nutrient-deficient soils (Deacon *et al.*, 1992); summer drought (Kruger, 1979) resulting in some conditions which parallel deserts (Gutterman, 1993); recurrent canopy-destroying fire (Le Maitre and Midgley, 1992); severe inter- and intra-specific spatial competition in dense plant communities (Kruger, 1979; Bond *et al.*, 1992; Yeaton and Bond, 1991); high predation pressure in the nutrient-poor environment (Kuo *et al.*, 1982) of fynbos, by granivores especially (Le Maitre, 1988); and the occurrence of short-lived (false), episodic favourable germination conditions of high moisture availability during summer (Stock *et al.*, 1992) and its converse: unpredictable, unfavourable conditions (e.g. drought) during the germinative winter season (Brits, 1987).

The *Leucospermum* seed has responded with multiple adaptations (Kelly *et al.*, 1992). It is proposed that the *Leucospermum* seed responds with at least one adaptive strategy to each stress or disturbance factor operating in nature. Some of these adaptations appear to have lateral (or interwoven) functions in the seed.

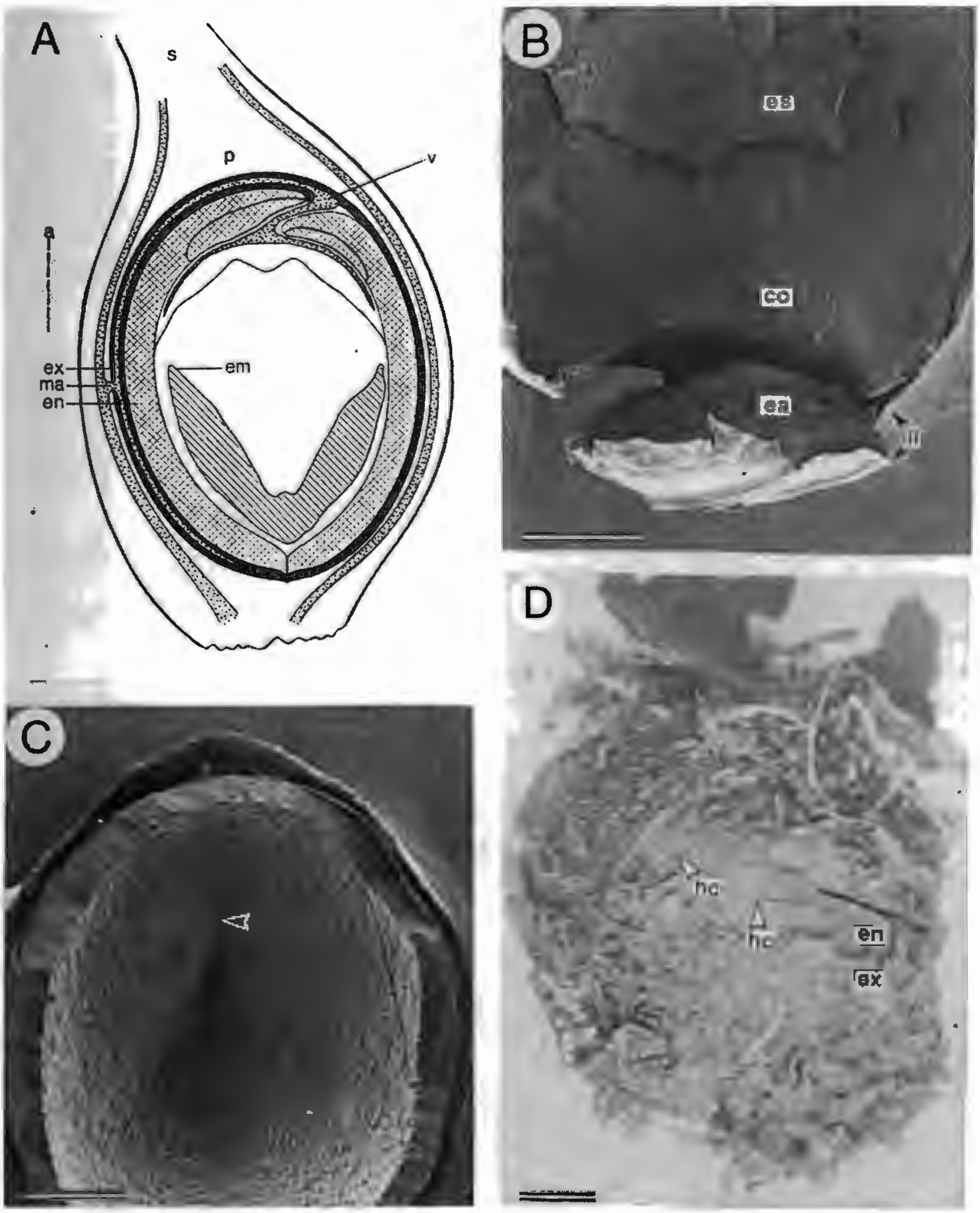
Despite the availability of the above data curious aspects of *Leucospermum* seed biology in fynbos require clarification:

- seeds have been shown to be long-lived and to remain dormant in the soil-stored seed bank for many years (Chapter 6) yet the acquisition and breaking of dormancy by the coat structures involved, on the cellular level, have not been studied;
- although seeds germinate *en masse* only after fire (Rourke, 1972), a small proportion of seeds will delay their germination, spreading emergence over the entire favourable post-fire season (Brits, 1987). The mechanism(s) involved, their importance and whether or not the seed bank is partitioned to this end are unknown.

Successful adaptation of a plant to a range of environmental factors requires sufficient genetic variability to develop a reproductive strategy with multiple elements (Stebbins, 1974). The question arises of how extreme the specialization in *Leucospermum* characters is, i.e. what is the status of adaptive processes in the evolutionary seed ecology of *Leucospermum*.

Developmental studies are essential in studying seed dormancy mechanisms which are regulated by embryo envelopes (Kelly *et al.*, 1992) and although the literature on dormancy is vast, information concerning the structure, histochemistry and function of the relevant envelopes is meagre (Werker, 1980). Thus the integration of seed structure and function, and even its evolution, can be understood only by considering the seed's multidimensional nature during development (Van Staden *et al.*, 1989). This study focuses on aspects of testal anatomy during seed development, from ovule to maturation, which in the dispersed propagule are involved in interaction with environmental factors. In addition the fates of these structures during prolonged underground storage, and subsequent germination, are also followed.

Figure 1. General features of the developing and dispersed *Leucospermum* achene: A: Diagram of developing achene at 5 w after anthesis, showing embryo envelopes, vascularisation and position within the infructescense (not drawn to scale). B: Mature cotyledon and isolated embryonic axis. The axis consists of only undifferentiated meristems. C: Endotesta with micropylar seam crack originating at the closure line of the outer integument (arrow) in the dispersed seed (w 13). D: *Leucospermum* seed after 4 years of burial in unburnt fynbos soil. The elaiosome (pericarp) has disappeared and the exotesta is broken, loosened from the endotesta and overgrown with hair roots; large and hairline cracks have formed in the endotesta. Scale bars = 1 mm. a, inflorescence axis; co, cotyledon; ea, embryonic axis; em, embryo; en, endotesta; es, endosperm remnant; ex, exotesta; hc, hairline crack; ii, inner integument / nucellus / endosperm remnant (seed pellicle); ma, medial attachment; p, pericarp; s, style; v, vascular bundle.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Materials and Methods

Production of seed

Seeds of *L. cordifolium* were produced from cultivated stands (Chapter 2) and harvested under controlled conditions after natural release in December. Diaspores were harvested as achenes, the true dispersal units, with a mature, turgid pericarp (elaiosome) present, and were dried for 6 w under summer conditions in the shade.

Microscopy

Inflorescences of two cultivars of *Leucospermum cordifolium* were tagged on one day in peak flowering time and open florets were removed. Developing fruits from tagged inflorescences of each cultivar were collected directly into FPA at weekly intervals. Microscopy techniques are those of Chapter 3 (Manning and Brits, 1993). Material for SEM examination was freeze-fractured in liquid nitrogen, mounted onto aluminium stubs and sputter coated with gold-palladium for viewing in Cambridge S200 and Jeol 6100 instruments at 10kV and 5 kV respectively.

Scarification

Sulphuric acid scarification. One sample of fruits was scarified in (c) H_2SO_4, washed in tap water (Chapter 2) and dried.

Manual and desiccation scarification. The pericarps of fruits were removed (Chapter 6.2) and seeds with intact exotestas selected stereomicroscopically. The exotesta was removed manually in one sample of seeds. In two other samples the exo- and endotesta layers were scarified by exposure to 40 °C for 6 h followed by cooling and immersion in water, or by drying over silica-gel for 24 h followed by immersion in water (Chapter 6.2).

Soil scarification. The methods in this section are those of of the soil-storage experiment in Chapter 6.4. The purpose of summer burial in the shaded root zone of mature *Leucospermum cuneiforme* (Burm. f.) Rourke plants at c. 3 cm depth, was to simulate natural dispersal (Brits, 1987). Seeds were sampled after 0 (control), 1, 2 and 4 years of storage and carefully cleaned. Seeds were tested for viability (Chapter 6.4).

Seed coat morphogenesis

The unilocular ovary has parietal placentation with a single sessile ovule, attached medially to an adaxial placenta; attachment of the ovule is hemitropic (Figure 1A). The ovule is adnate to the ovary wall except at the micropyle (Figure 2A). The embryo envelopes attain their mature dimensions at c. 5 w after anthesis and the tissue of the pericarp develops as a fleshy parenchyma which is thicker at both seed poles (Figure 1A). The outer epidermis of the pericarp develops a thick waxy cuticle.

On account of the relatively thin, rapidly drying pericarp covering the large seed, the fruit in *Leucospermum* is regarded as an achene (Chapter 3). Achenes are dispersed at c. w 12 - w 13 (Figure 3E, F).

Pericarp

The pericarp remains attached to the exotesta. The pericarp inner epidermis has a densely staining cytoplasm (Figures 3A, E; 5B) and it is this layer which effects adnation. Although the pericarp can be easily removed by hand from the turgid seed at maturity it remains tightly connected until c. w 11. In contrast mature, dried achenes when wetted have a gelatinous, easily removable pericarp. The inner tangential surfaces of the pericarp epidermis remain attached to the seed after removing the pericarp by hand (Figures 3E; 4D; 5F). This transparent layer (Figure 4A) technically forms the true outer covering of the seed and completely obscures the underlying cuticular ridges in SEM (Figure 4D).

Exotesta

This consists, at maturity, of a thick cuticle, a thickened outer cell wall of the outer epidermis and 0 to several tanniniferous subepidermal cell layers (Figure 3). The cuticle is covered with raised cuticular ridges (Figure 3E, F). The ridges spread out at the top, enlarging the ridge-top surface by c. 50% and resulting in canal-like depressions between the ridges. They thus form a prominent, finger print-like pattern on the exotesta (Figure 4) from the 5th week of development, measuring 7 - 10 µm wide at the top, c. 5 µm across the base and c. 3 µm high, in the mature seed (Figures 3, 4). Ridges appear dark in unstained sections (Figure 3B, D, E).

Figure 2. Early development of salient embryo coverings, orientation as in Figure 1A. A: Embryo and micropyle at 1 w post-fertilization, arrow indicates entering pollen tube. The future testa (outer integument) is not adnate to the ovary wall and overgrows the inner integument at the micropylar opening, to form an endotestal seam at maturity (C), inner integument is seen degenerating; x 250. B: Chalazal pole at 5 w (mature dimensions), showing double palisade and vascular bundle travelling through the exotesta, leaving the raphe to traverse the endotesta. Abundant tanniniferous cells are present in the exotesta but absent in the parenchymatous pericarp elaiosome tissue; x 40. C: Micropylar pole at 5 w showing fully elongated endotestal cells forming the testal palisade and seam; x 100. em, embryo; en, endotesta; ex, exotesta; ie, inner epidermis of oi; ii, inner integument; o, ovary wall; oe, outer epidermis of oi; oi, outer integument; p, pericarp; se, seam; t, tanniniferous cells; v, vascular bundle.

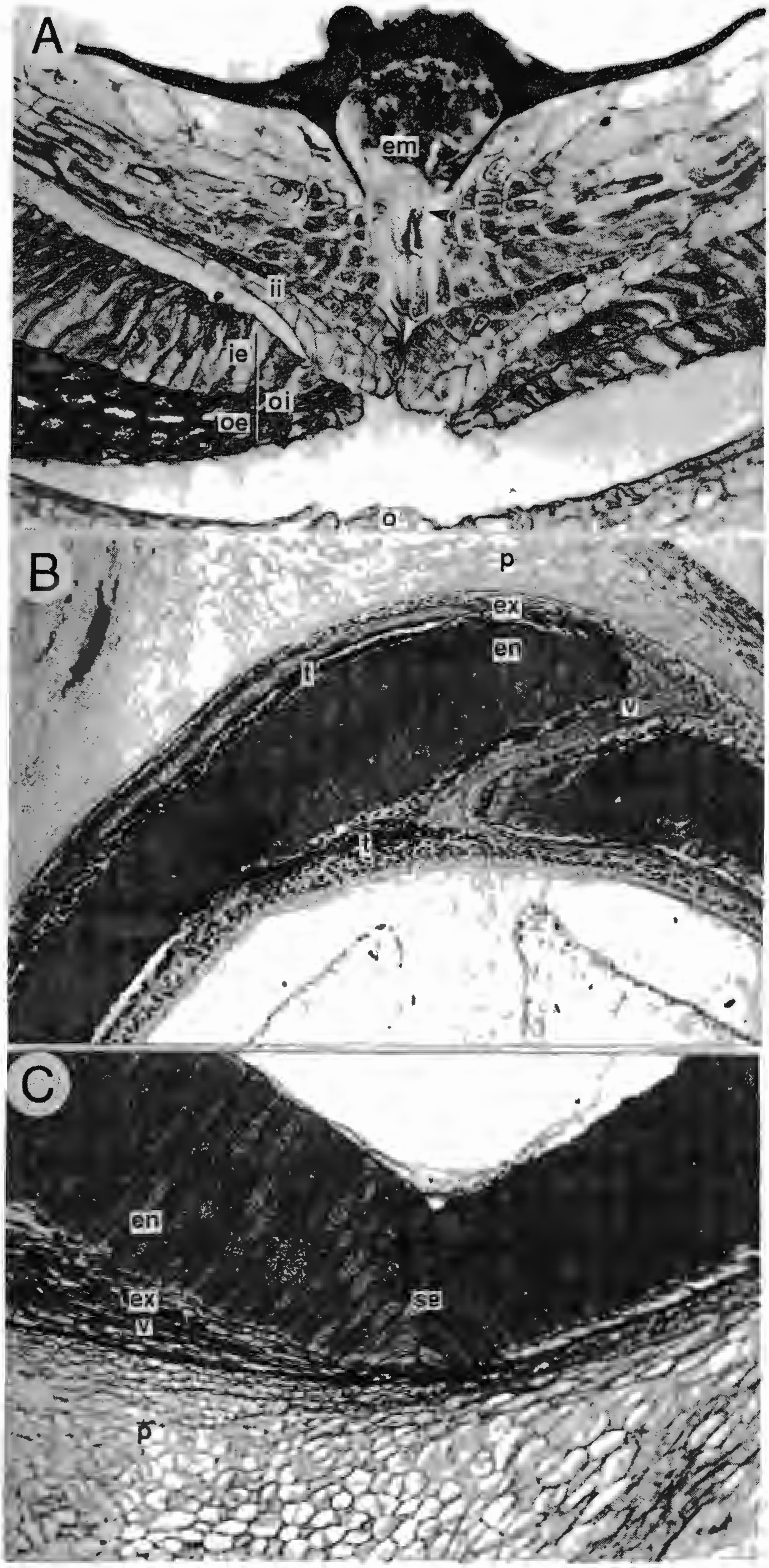
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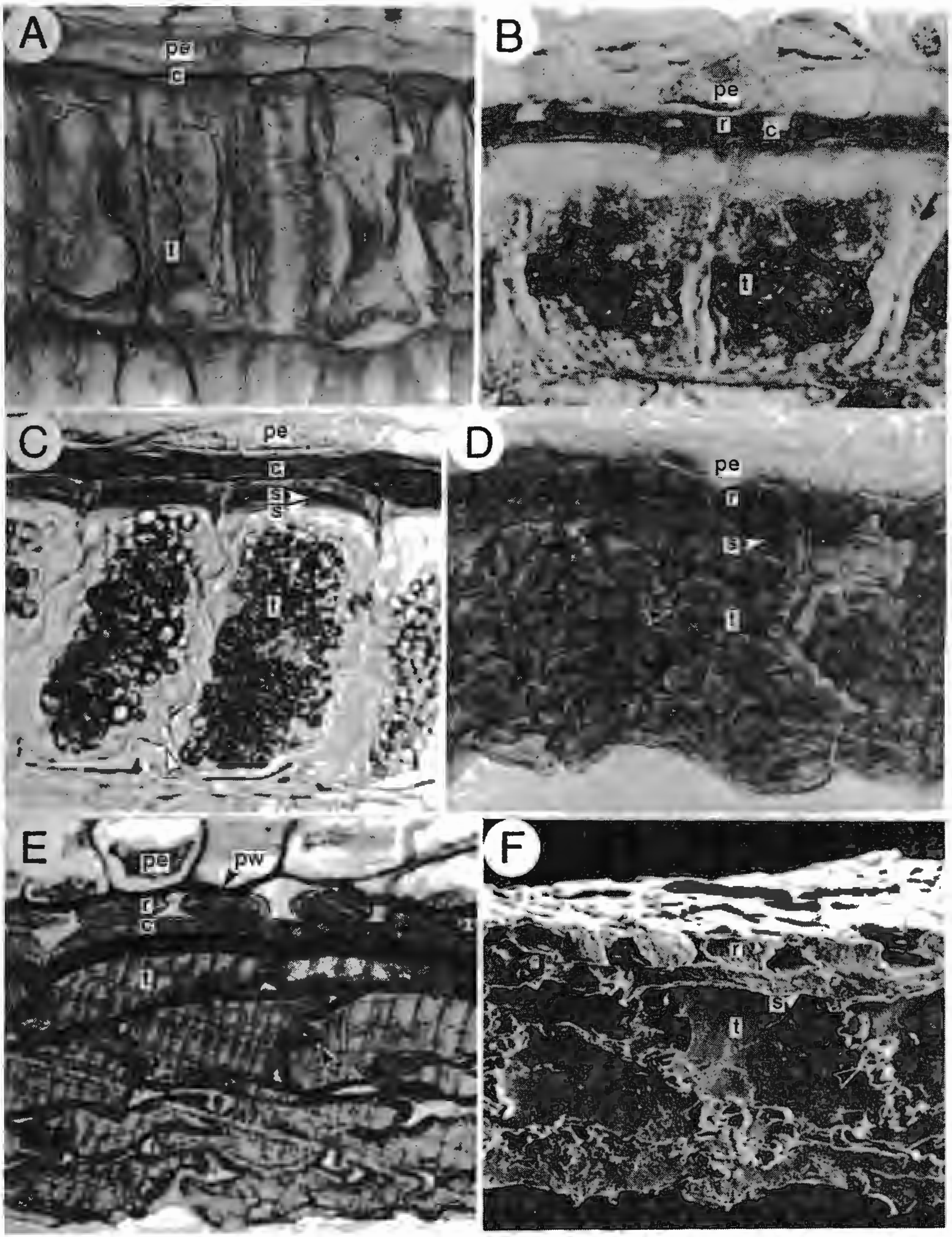
Figure 3. Exotesta and cuticle development in relation to the pericarp: A: 2 w; B: 5 w; C: 7 w, unstained; D: 9 w, unstained; E: 11 w, unstained; F: 13 w, SEM of fractured testa of dispersed seed, the pericarp is shed. The cuticle is prominent at w 2 (A) and shows early ridge development at w 5 (B); outer cell wall secondary thickening is present at w 5 (B) which becomes striated from w 7 (C) onwards; tannin depositing occurs at w 2 (A), tannin becoming granular (w 5, 7 - B, C) and finally occluding the cell lumina (w 9, D, onward); cellular compression and strongly undulating cell walls (arrows) are seen from w 7 (C) onwards; the lower epidermis of the pericarp has a densely staining cytoplasm (A, E) and thickened outer walls (E). All figures x 1250. c, cuticle of exotesta; en, endotesta; ex, exotesta; pe, pericarp epidermis; pw, thickened epidermal cell walls; r, cuticular ridges; s, secondary wall thickening; t, tannin.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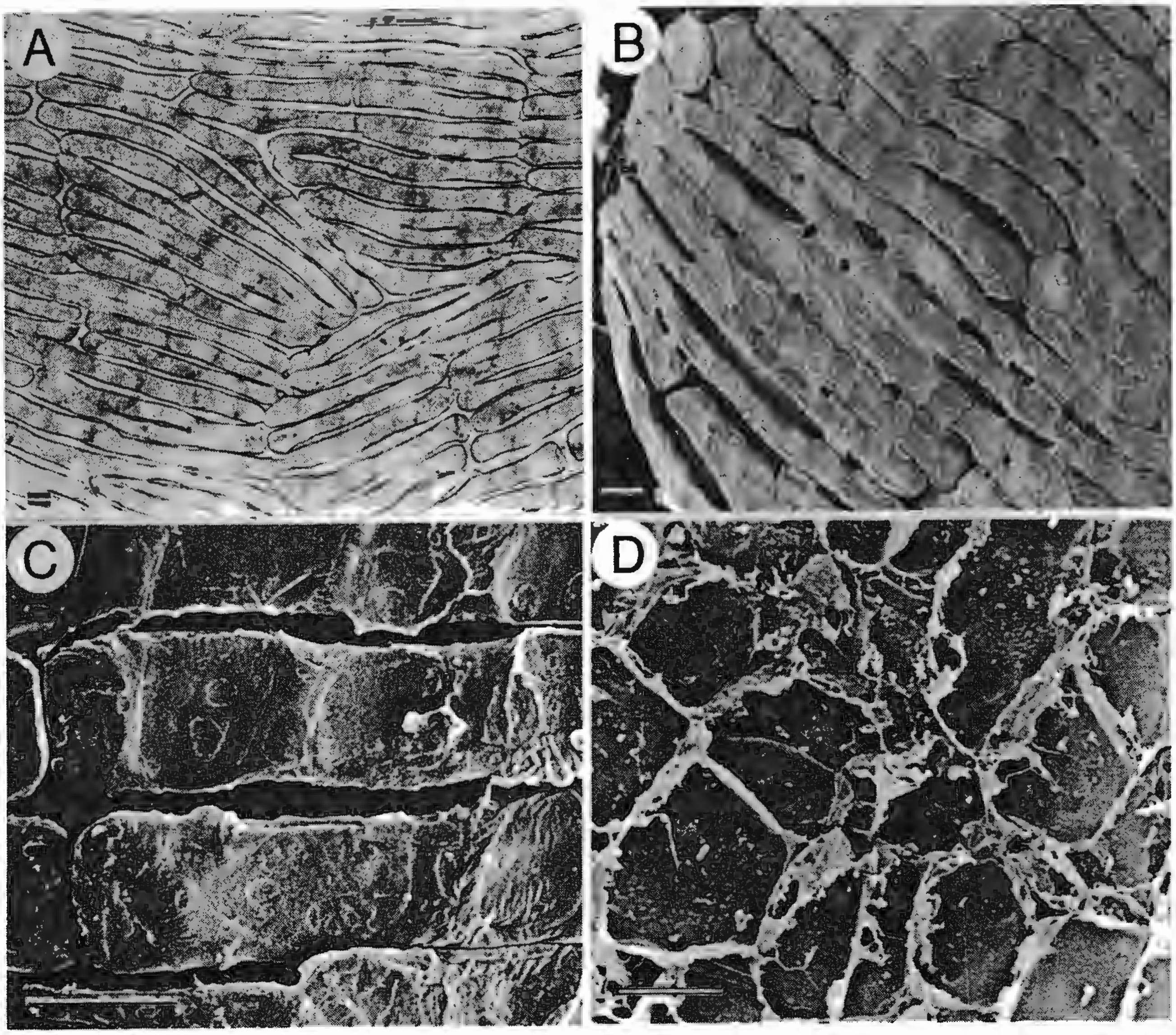

Figure 4. Cuticular ridges of the exotesta. A: Light micrograph of finger print-like pattern. B: SEM of partly exposed ridges showing relation to the cuticular base. C: Detail of ridges. D: Persistent pericarp lower epidermal cell walls normally overlying ridges. Scale bars = 10 μm.

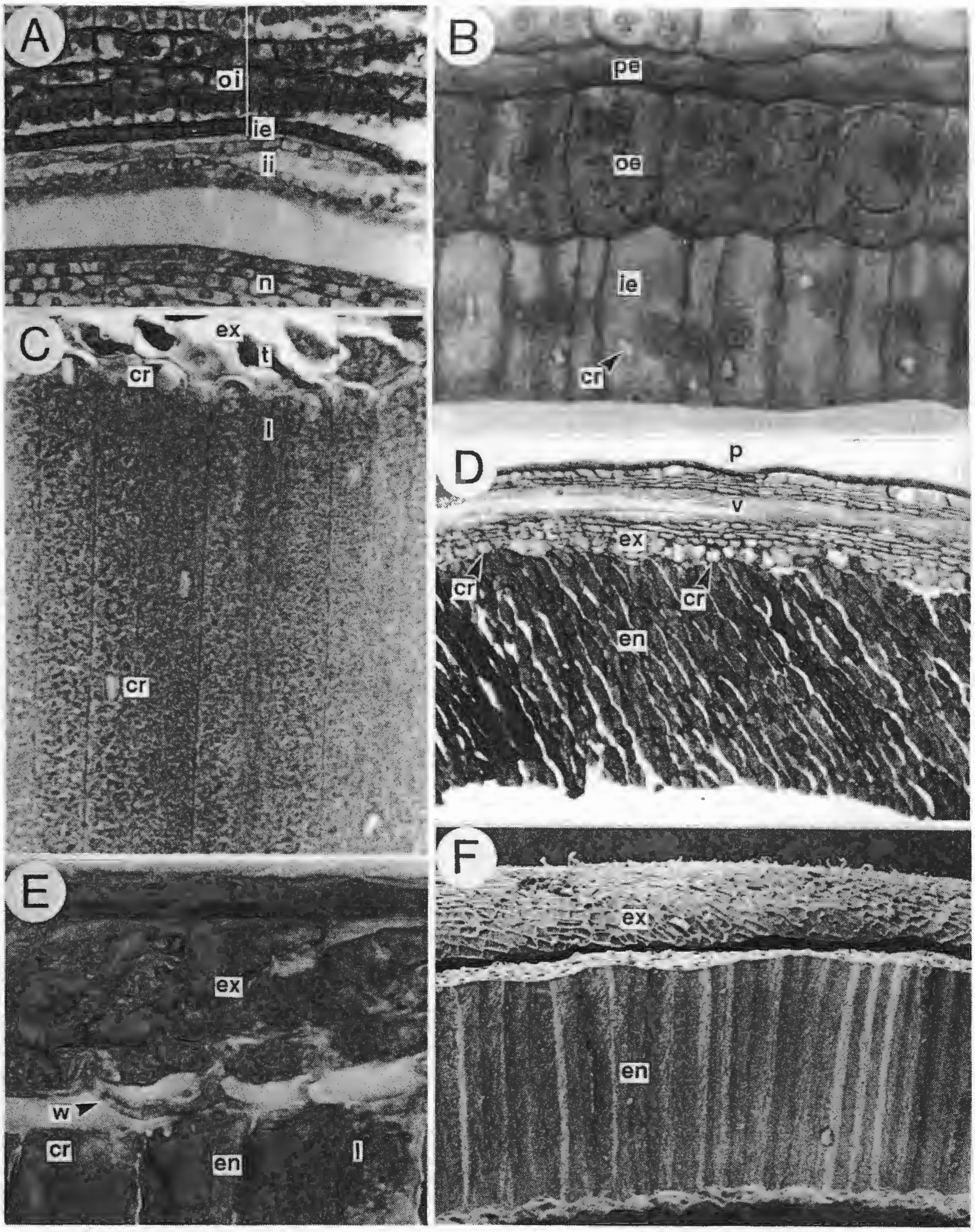
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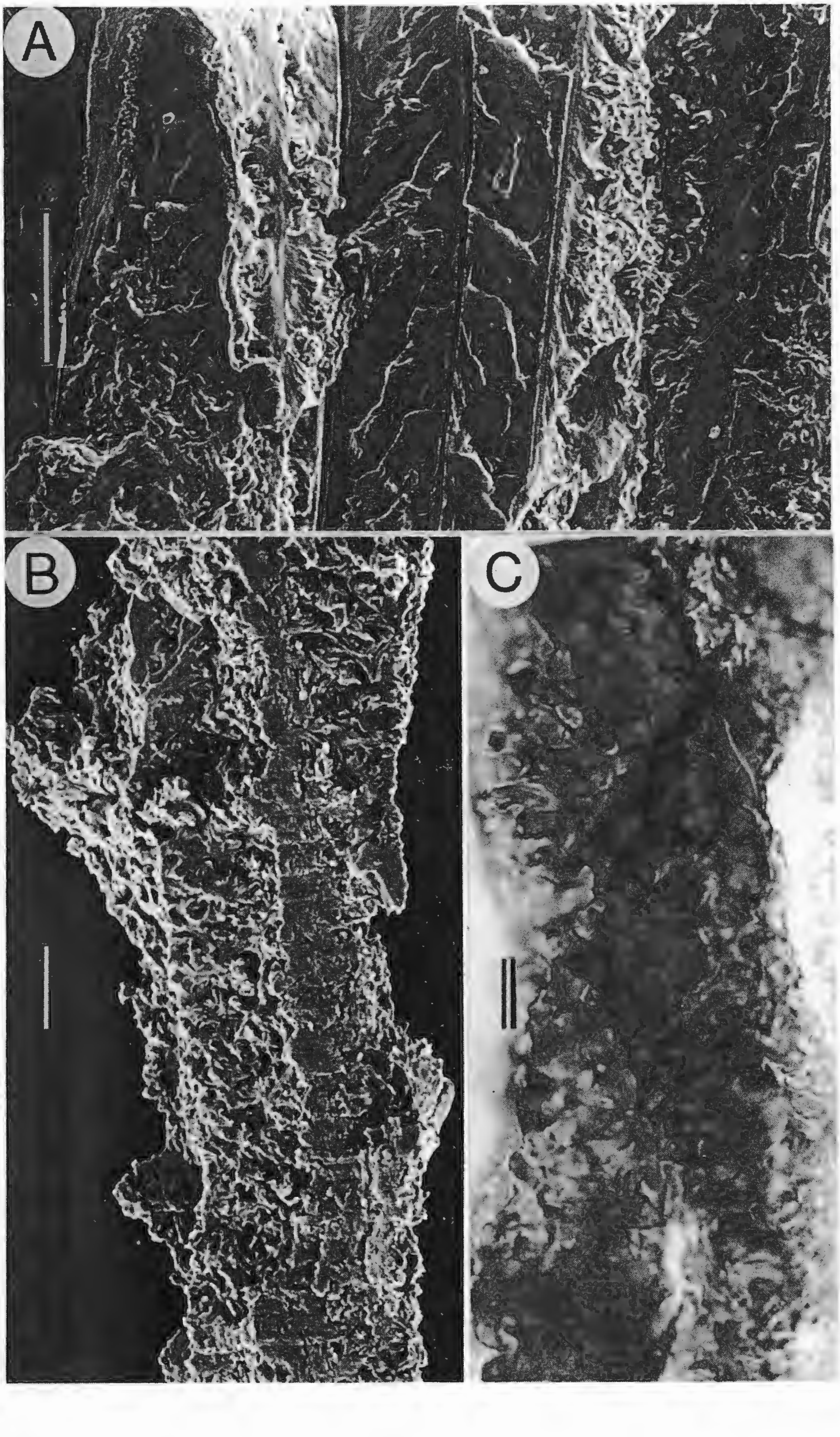
Figure 6. Detail of the woody palisade: A: Cell wall facets showing the compact alignment of palisadal cells; B: Detail of individual cell showing rough sculpturing of faceted cell walls; C: Light micrograph of palisadal cells in chalazal region showing twisted appearance and rough wall sculpturing. Scale bars = 10 µm.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The outer epidermis. The most prominent cell layer in the exotesta, overlying 0 - 7 layers of smaller cells. Initially brick-shaped and radially orientated, the cells become obliquely compressed during maturation (Figure 3). The outer cell wall is greatly thickened. The radial walls of the brick-shaped epidermal cells are undulated forming a strongly interlocking pattern (Figure 3).

The main feature of mature exotestal cells is their dense tannin content. Tannin deposition is evident from an early stage and enlarging aggregates of tannin granules in the cytoplasm later completely occlude the cell lumen (Figure 3).

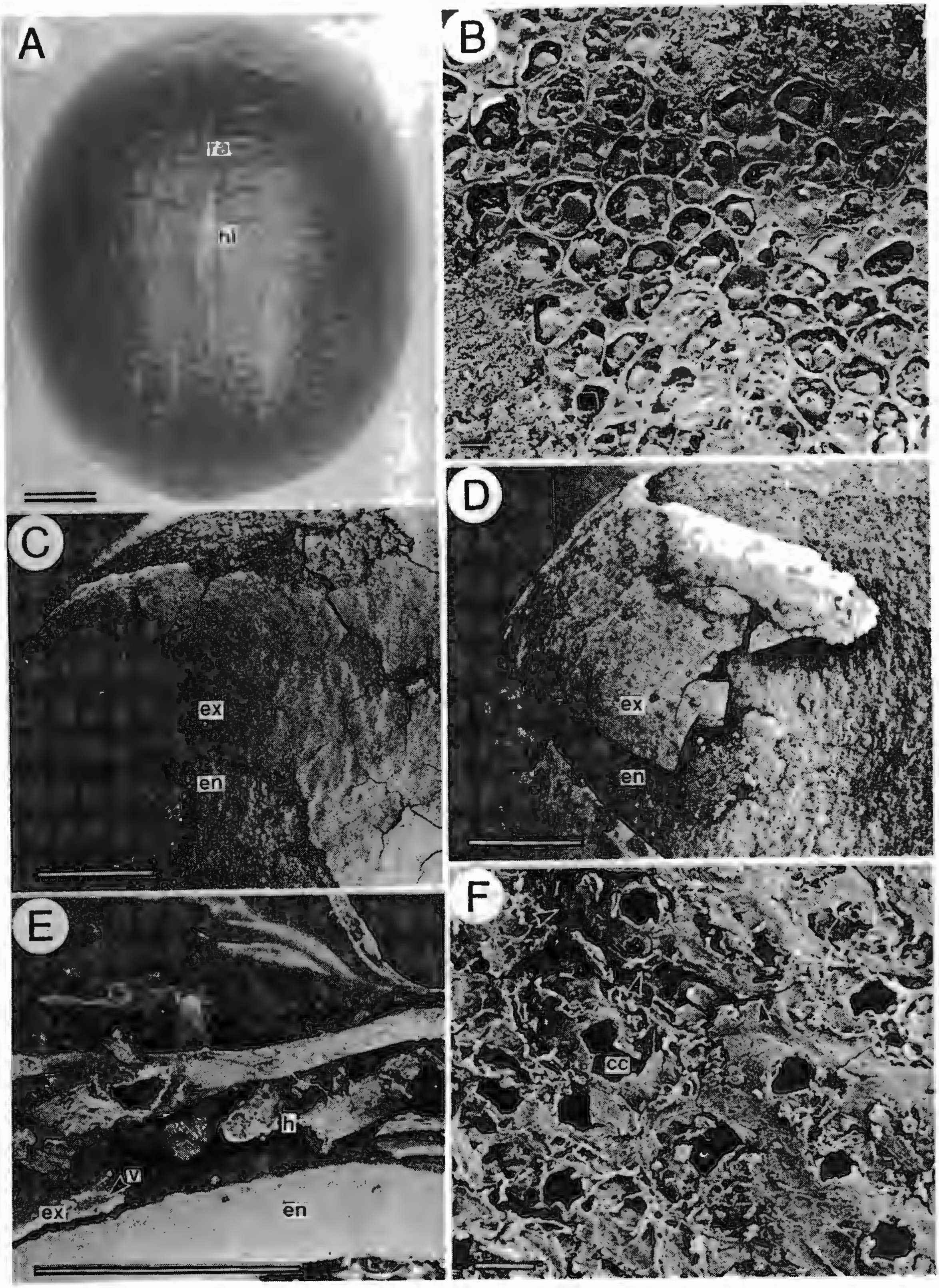
The cells of the inner layer of the mature exotesta are loosely attached to the top of the crystalliferous palisade, or endotesta cells. This attachment is readily broken under mechanical stress (Figure 3D - F; Figure 5E).

Endotesta

The endotesta forms a stony shell at maturity (Figures 1C, 5F) consisting of a single-cell layer of columnar palisade cells (at the chalazal pole the outer epidermis of the inner integument also develops into a palisade, the cells of which lack crystals (Figure 2B)). The inner epidermis cells of the outer integument (future endotesta) are isodiametric at pollination (Figure 5A) but rapidly elongate after fertilization (Figure 5B - C) until they attain a mature length of 250 - 350 µm at w 5 (Figures 2C, 5C). Adjacent endotestal cell walls become irregular, laterally interlocking and sometimes twisting (Figures 5D, 6). Following elongation the cell lumen occludes with granular lignin, becoming completely lignified at maturity (Figure 5). Enlarging rod-shaped, centrifugally migrating crystals are seen from w 2 onwards. At maturity the crystals are large (5 - 8 µm) and prismatic, lodging at the top end of each cell immediately internal to the outer tangential cell wall (w 7 to 11 - Figure 5). These form an outer crystal field when the exotesta pulls away from the stony palisade post-dispersal (w 13 - Figure 7B).

The endotesta has a single opening at the chalazal pole through which the raphal vascular bundle enters the seed (Figure 2B). At the micropylar end the inner epidermis of the outer integument (future endotesta) overgrows the inner integument shortly after fertilization (Figure 2A), forming a seam in the palisade at w 5 (Figure 2C). The inner integument atrophies after fertilization (Figure 2A) forming part of a thin layer, the seed pellicle, at maturity (Figure 1B).

Figure 7. Fates of the embryo covering layers during experimental soil storage. A: Intact exotesta after hand removal of pericarp. B: Intact endotesta surface, after hand removal of exotesta, showing crystal field atop palisade cells. C: Testa after 1 year of soil storage showing lack of pericarp and broken exotesta still attached to the endotesta. D: Testa after 2 years of soil storage showing loosened, extensively scarified exotesta. E: Testa after 4 years of soil storage showing remnants of the exotesta, overgrown with hair roots. F: Endotestal surface after 4 years of soil storage showing hairline crack (arrows) and dissolved-crystal cavities. Scale bars: A, C - E: = 1mm; B and F: = 10 µm. cc, crystal cavities; en, endotesta; ex, exotesta; h, hair roots; hi, hilum; ra, raphe; v, vascular bundle.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Functions of the embryo covering layers

The covering layers of the embryo have three main functions: to aid in dispersal (pericarp); in recently dispersed seeds, to exclude oxygen from the embryo during otherwise favourable conditions for germination (exotesta); and to mechanically protect the embryo during its soil storage phase between the occurrence of fires (endotesta). These functions relate to developmental patterns of the covering layers.

1. Pericarp as elaiosome

The pericarp functions in its entirety as an elaiosome, or edible attractant for ants (Figure 1A). Unpalatable tannins, which occur abundantly in the underlying testa, are absent in the pericarp (Figure 2B). *Leucospermum* diaspores, shed during midsummer afternoons, are located by ants through smell (Slingsby and Bond, 1985). During shedding the elaiosomal tissue remains fully turgid, or nearly so (Brits, unpublished). The thick cuticle could contribute to maintaining turgidity of the elaiosome following shedding. The nutritional and attractant substances of the elaiosome are largely unknown (Midgley and Bond, 1995).

2. Seed coat: mechanical defences / resistances

2.1 Exotesta

When the elaiosome is removed, the remaining, germination unit, is the seed proper with the exotesta now functioning as the outer covering (Figure 7A). There is no evidence for a mechanical role for the exotesta, other than providing a durable oxygen impermeable envelope during the soil storage phase.

2.2 Endotesta

The main function of the endotesta (crystalliferous palisade) is mechanical protection against pathogens and seed eaters. Only the endotesta (and disintegrating exotesta) cover the embryo during perennial underground storage. Endotestal sclereids form a solid layer at maturity (Figure 5F) and the unscarified endotesta resists strong inward pressure (Chapter 6.2). The thickened endotesta outer tangential cell walls are loosely connected to both its underlying cell contents (including crystals) and the exotestal layer above (Figure 5E). Thus upon shrinking through the process of desiccation-scarification the exotesta tears away (Figure 5F) partly exposing the palisadal crystals (Figure 7B). With progressive disintegration of the exotesta only the endotesta remains (Figure 1D). The field of large crystals (Figure 5D) in young dispersed seeds probably contributes to protection of the embryo. The presence of crystals only on the outer palisade layer in the double palisadal wall at the chalazal pole is consistent with this proposal. The double palisade could function to strengthen the seed coat where the vascular system enters the seed.

The intact woody endotestal envelope constrains the emergence of weaker embryos (Brits, 1990; Chapter 6.3). Relatively small seed banks were estimated for soil-stored Proteaceae (Le Maitre, 1988) and this may include a proportion of weak embryos, including ageing embryos of previous flowering seasons. Within each flowering season a marked proportion of embryos is incompletely developed (Van Staden and Brown, 1973). Weakening of the tough endotesta through scarification results in a higher seed germination rate (Chapter 4.3; Chapter 6.4) and emergence percentage (Chapter 4.3). The endotestal seam at the micropyle end (Figure 2C) forms a fracture line where breakage occurs during germination (Figures 1C; 10).

3. Seed coat: permeability to water and oxygen

3.1 Exotesta

The exotesta forms a continuous film of c. 15 - 30 µm thick, brittle and relatively non-elastic, around the *Leucospermum* seed (Figures 3F, 7A). Of the testal sublayers the relatively thin exotesta is the main oxygen excluding organ (Chapter 6.3). It is therefore imperative that the exotesta functions as an airtight (unbroken) capsule around the embryo (Brown and Van Staden, 1973). Both encapsulation, and saturation of the exotesta with water, appear to contribute to embryo anoxia. The exotesta cellular layers and the cuticle are strongly hydrophilic, water droplets being rapidly absorbed from any part of its suface (Brits, personal observation).

The persistent pericarp lower cell wall layer resembles a film covering the underlying structure of cuticular ridges (Figure 4D). The functional sublayers of the mature exotesta are thus: the lower epidermal cell wall layer of the sloughed pericarp; the cuticular ridges; and the cellular layer proper, with extreme thickening of the epidermal tangential cell walls, plus tanniniferous cell contents.

The specialization and structural integrity of these sublayers indicate a specialized role in *Leucospermum* germination ecology. The data suggest that the cuticular ridges and pericarp cell wall remnants may in combination contribute to exclusion of gaseous oxygen, as follows. The ridge microsculpturing may form a hydrophilic matrix which supports and stabilizes a c. 5 µm film of free water under wet conditions. Thus in saturated seeds, water entering between ridges is trapped and immobilised within the between-ridge canals, these being capped by the roof-like structure of spreading ridge-tops and pericarp cell walls. A static water film thus results, impeding the diffusion of gaseous oxygen through the exotesta. The saturated subcuticular tanniniferous cell layer(s) will likewise restrict the diffusion of oxygen. Captured water films in a mucous matrix are a strong barrier to oxygen penetration in many seed species through poor diffusion of oxygen in water (Come and Tissaoui, 1973) coupled with the prevention of convective water flow in the film (Gutterman, 1993). Saturated tanniniferous layers rich in phenolic compounds function as a biochemical barrier to oxygen diffusion in seeds (Come and Tissaoui, 1973; Werker, 1980/81).

Oxygen impermeability of the exotesta apparently develops late during morphogenesis. This is suggested by the fact that prematurely harvested seeds are markedly less dormant than fully matured seeds (floater vs. sinker seeds - Van Staden and Brown, 1973; Brits and Van Niekerk, 1986; Chapter 4.3). Figure 3 shows that tannin occlusion of exotestal cells is incomplete at w 7 and only near-complete at w 9.

3.2 Endotesta

The opening at the chalazal end which allows entrance of the vascular system into the seed (Figure 2B) remains an entrance to water in the dispersed seed. However the entire woody palisade is highly permeable to water (Brits, personal observation) resulting in rapid imbibition of free water by the whole seed. When soaked the seed is fully saturated in c. 48 h (Brown *et al.*, 1986). Desiccation occurs at the same rate, stabilizing in c. 48 h (Table 1).

The intact endotesta may partially reduce oxygen diffusion to the embryo (Chapter 6.3). Thus scarification of the endotesta by fracturing increases seedling emergence also through improved embryo oxygenation.

Scarification and fates of the embryo covering layers

1. Elaiosome / Pericarp

Little is known about the short-term fate of the pericarp (elaiosome) after harvesting of freshly released achenes by ants. The elaiosome is apparently removed (Bond and Slingsby, 1983). Remaining tissue is however soon lost in the soil (Figure 7C). The pericarp is particularly prone to microbial decomposition when incubated under moist conditions in germination experiments (Brits, 1986). It can therefore be assumed that pericarp tissue not consumed by ants would rapidly decompose during soil storage. However, in recently dispersed seeds the presence of a non-decomposed pericarp may be important, since pericarp removal was shown to improve oxygenation of seeds (Kelly *et al.*, 1992).

Table 1. *Leucospermum cordifolium* seed weight (mg) changes during drying. Fresh, mature, pericarpless seeds were soaked for 48 h and dried under ambient early winter conditions (22 °C, 60% RH average). Weights were calculated from means of two replicates of 48 seeds.

Day of weighing	Dry (control)	Soaked
0*	270,0	286,1
1 (24 h)	269,9	272,3
2	269,9	270,6
3	269,6	269,8
4	269,7	269,8
5	269,9	270,0

Table 2. Mean linear dimensions (mm) of *Leucospermum* seeds before and after soaking in water for 24 h. Three seeds were measured, three micrometer readings per seed per variable.

	Length	Width	Thickness
Before	7,059	5,605	4,500
After	7,157	5,712	4,625
Difference	0,098*	0,107	0,125*

*Significant at P = 0,05

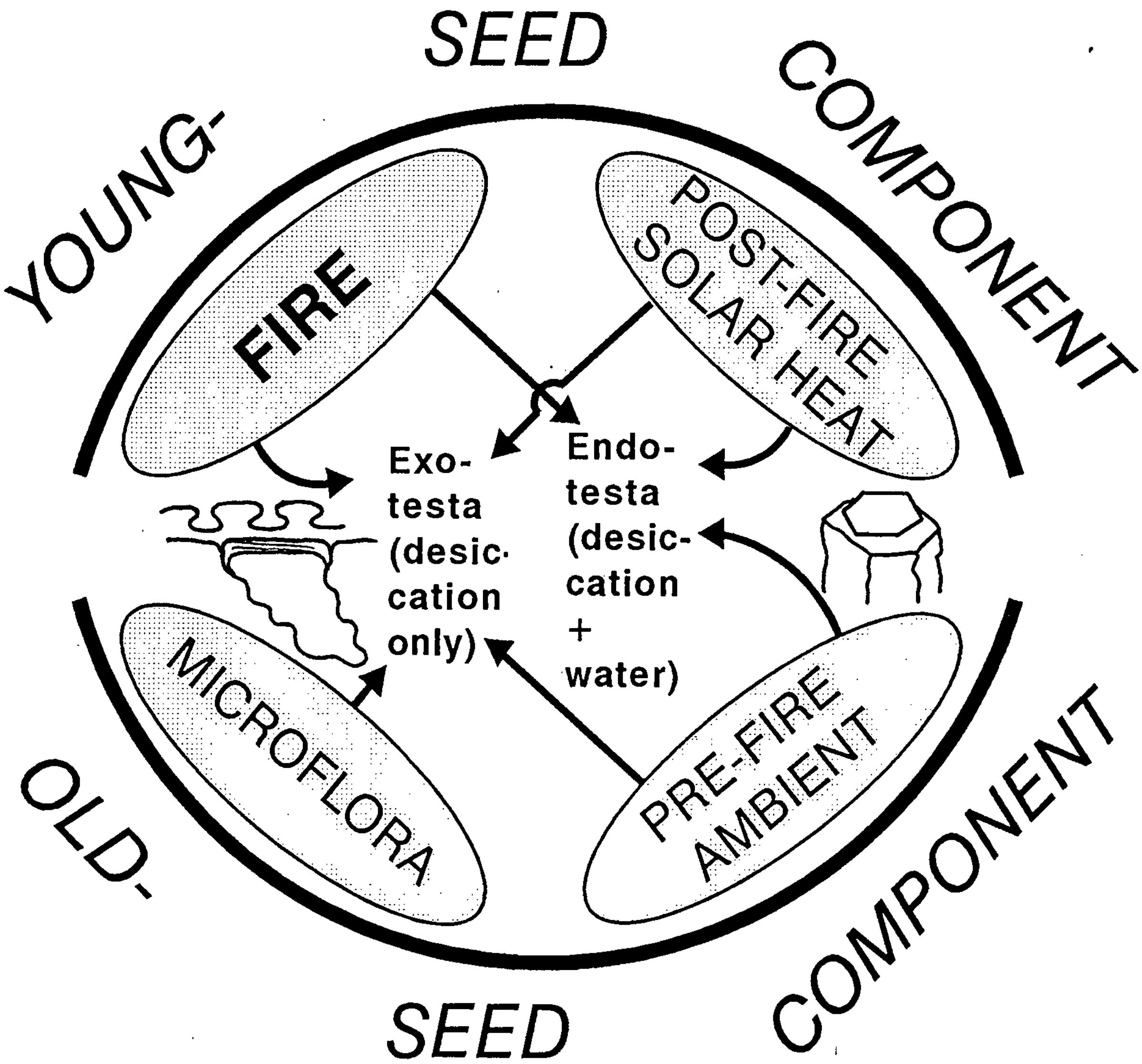


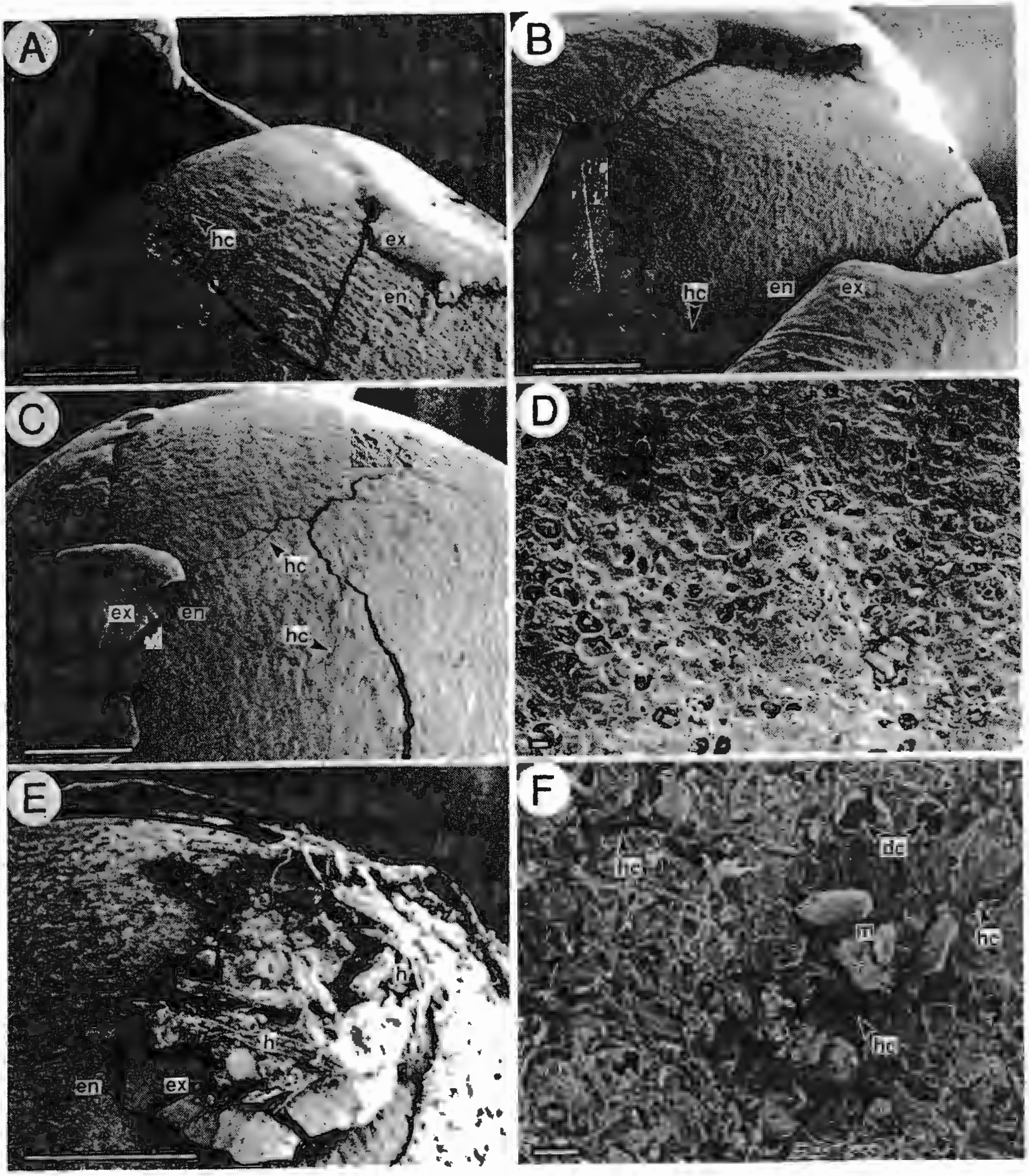
Figure 8. Diagram of scarification by desiccative, hydrative and microbial factors interacting on the testal layers in young and old *Leucospermum* seed bank components during soil storage.

2. Exotesta

Desiccation scarification

The *Leucospermum* seed is killed by temperatures exceeding c. 80 °C (Brits, unpublished) - in contrast to many taxa in mediterranean-type climates which require heat-shock to physically break testa-imposed dormancy (Keeley, 1991; Bell *et al.*, 1993). The *Leucospermum* exotesta however ruptures when desiccated following removal of the protective pericarp (Chapter 6.2). Exotesta rupturing is gradual in unburnt fynbos

Figure 9. Scarification mechanisms of the testa. A: Intact seed desiccated at 40 °C, cooled and immersed in water. B: Seed desiccated over silica gel and immersed in water. C: Seed treated with (c) H_2SO_4 for 7 min and washed in water. D: Endotestal surface following (c) H_2SO_4 treatment showing partly dissolved crystals and absence of carbonized residue. E: Testa after 4 years of soil storage showing strong scarification of the exotesta. F: Hairline cracks in endotesta of soil-stored seed (4 years) showing dissolved-crystal cavities and soil microflora. Scale bars: A - C, E: = 1mm; D and F: = 10 µm. dc, dissolved crystals; en, endotesta; ex, exotesta; h, hair roots; hc, hairline cracks; m, soil microflora.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during perennial soil storage (Figure 7) but can predictably be much accellerated by the direct drying heat of fire or, in fire-exposed soil, through heating-desiccation by the sun during the summer season following a burn (Chapter 6.2). Cycles of wetting-drying improved germination in *Clematis microphylla,* compared with continuous saturation (Lush *et al,* 1984). This is thought to be caused by seed coat cracking during drying (Bell *et al,* 1993). However the strong response in the *Leucospermum* exotesta to desiccation alone, (and in the endotesta to wetting following desiccation) without the need for wetting-drying cycles, suggests a different, presently unique, scarification mechanism in *Leucospermum.*

Microbial digestion

The exotesta is more subject to microbial decomposition than the endotesta (although much less so than the pericarp). Thus microbial decomposition combined with repeated hydration and desiccation gradually cause the breakage and disintegration of the exotesta during soil storage (Figure 7).

A model for fire-dependent scarification

Natural scarification of the exotesta is thus achieved by one or more of a number of processes: rapid removal of the protective pericarp/elaiosome by ants plus, alternatively, its microbial decomposition; in unburnt fynbos, by slow microbial decomposition of the exotesta; plus slow seasonal differential stretching and shrinkage of the exotestal layers following repeated hydration and dessication; in burning fynbos by strong direct desiccation pulse; indirectly, subsequent post-fire desiccation by high summer / autumn temperatures in the superficial fire-exposed soil layer (Figure 8).

3. Endotesta

A parallel set of fire and climatic variables determine the scarification history of the endotesta, as with the exotesta (Figure 8). The exceptions are the apparent resistance to microbial decomposition (Figure 9E, F) and the requirement for free water after desiccation to effect fracturing (Chapter 6.2). In old, scarified endotestas the crystals are lost (Figure 7F).

Endotesta scarification through desiccation-wetting

The hard, nut-like endotestal shell fractures when desiccated and re-wetted in free water, but gradual hydration under high RH has no destructive effect (Chapter 6.2). The physical principle(s) of endotesta fracturing is not known. The pattern of desiccation-cum-water effects are similar in all artificial and natural modes of endotesta scarification (Figure 9).

4. Scarification mechanisms

Exotesta breakage resulting from desiccation was first observed in routine drying of wetted seeds from which the pericarp was removed. This occurred even at mildly warm temperature (40 °C - Chapter 6.2) and included non-patterned tearing as well as pulling away of the exotesta from the adnate endotesta (Figure 9A).

Exotesta: differential tension of seed coat layers

There is very little increase in seed size on imbibition (c. 1 - 3%, Table 1) suggesting that moisture-induced changes in the volume of the endotesta are negligible. Rupturing of the exotesta is therefore unlikely to be a result of differential shrinking or expansion between the endo- and exotestal layers. Moreover desiccation is known to cause rupturing of the exotesta also in seeds containing no free water (Chapter 6.2).

A more likely explanation is therefore differential shrinking of only the exotestal sublayers when airdry (in the absence of free water) during progressive desiccation. It appears that desiccation alone could provide sufficient tension for the exotesta to rupture and simultaneously pull away from the endotesta. Thus upon continued drying the tanniniferous lower exotestal stratum shrinks more than the surface layer(s) (thickened epidermal outer cell walls and cuticle of unknown composition). Consequently the drying exotesta curls strongly towards the inside (adaxially).

Incurling is controlled in the non-dispersed achene by the fully hydrated condition of the exotesta, which in addition is overlain by the turgid pericarp. When present in the dried, dispersed achene the adnate, now parchment-like pericarp continues to hold the exotesta in place mechanically (Brits, personal observation). However after the pericarp is removed by ants or microbially, both slow and pulsed desiccation mediated by fire will cause the exotesta to shrink, curl inwards and tear away from the palisade to which it is only weakly joined.

(c) H_2SO_4 scarification

Sulphuric acid is commonly used to scarify hard-coated seeds (Campbell *et al.*, 1988). Its typical action is to digest the impermeable outer testa layer(s) and relatively long treatment periods of up to 4 h may be required. During treatment the acid carbonizes the testa, leaving a charred residue which in some cases has to be removed as well, e.g. in the treatment of *Rubus cuneifolius* seeds, before germination can take place (Campbell *et al.*, 1988). The digestive action of (c) H_2SO_4 in most organic compounds is based on its extreme affinity for water. Thus the acid extracts water even from organic molecules, leaving only a charred residue.

Sulphuric acid treatment for short periods of 7 - 10 min is extremely effective in stimulating *Leucospermum* germination, although the mechanism involved is unknown. It seems likely that sulphuric acid effectively scarifies both of the testa layers through desiccation, within 7 - 10 min. of treatment, paralleling the process of natural desiccation-scarification. Thus the exotesta ruptures primarily through strong drying, although not excluding possible carbonizing digestion. Little if any carbon residue however appears to remain on the seed (Figure 9C, D) contrasting with the effects of prolonged acid treatment in other seeds. The same applies to the endotesta which, when acid treated, appears almost intact. Apparently desiccation by the concentrated acid causes breakage of the hard palisade only during subsequent washing (Figure 9C; Brits and Van Niekerk, 1976). This is analogous to other artificial desiccative and natural scarification processes in the soil (Figures 1D, 9). Scarification by (c) H_2SO_4 based on desiccation, rather than chemical dehydrative digestion, appears to be unique in seed science technology.

Acid treated seed can often be heard to break during washing (Brits, personal observation). The tough endotesta is rendered brittle and easily breakable by finger pressure by this procedure. This equals the effects of natural scarification after 2 - 4 years of soil storage (Chapter 6.4).

Embryo physiology

The physiological component of seed dormancy in *Leucospermum* involves embryo requirements for daily alternating temperature (Brits, 1986; Chapter 4.4). Temperature requirements restrict germination to the post-fire environment during winter. This has three potential advantages: avoidance of canopy competition, optimalization in interspecific competition through synchronous germination of a large accumulated seed bank, and avoidance of moisture stress. These oxygen-dependent processes occur only after embryo oxygenation has been effected by testa scarification (Chapter 6.3).

Optimum low and high incubation temperatures in *Leucospermum* seed species are correlated with average early winter daily minimum and maximum air temperatures in the natural habitats of species (Chapter 4.4). Thermodormancy in species is thus narrowly adapted to the natural environment, as is the case with many plants surviving in stressfull habitats (Abdulfatih, 1983; Keeley, 1991).

Germination and establishments

During germination cotyledon expansion at the micropylar end causes the endotesta to crack at the micropylar seam. The endotestal shell is thinner on its lateral edges than on the raphal and antiraphal faces (Figure 1C) causing the seam crack to extend along these edges. This results in a typical breakage pattern which allows radicle protrusion and cotyledon emergence (Figure 10). Germination is epigeal (Figure 10) and considerable force is needed to lift the large cotyledons from c. 3 - 5 cm below the soil surface (Brits, 1987) into emergence. Large-seededness (Figure 7A) and largely oily seed reserves (Mitchell, 1983) could be adaptations that provide such energy.

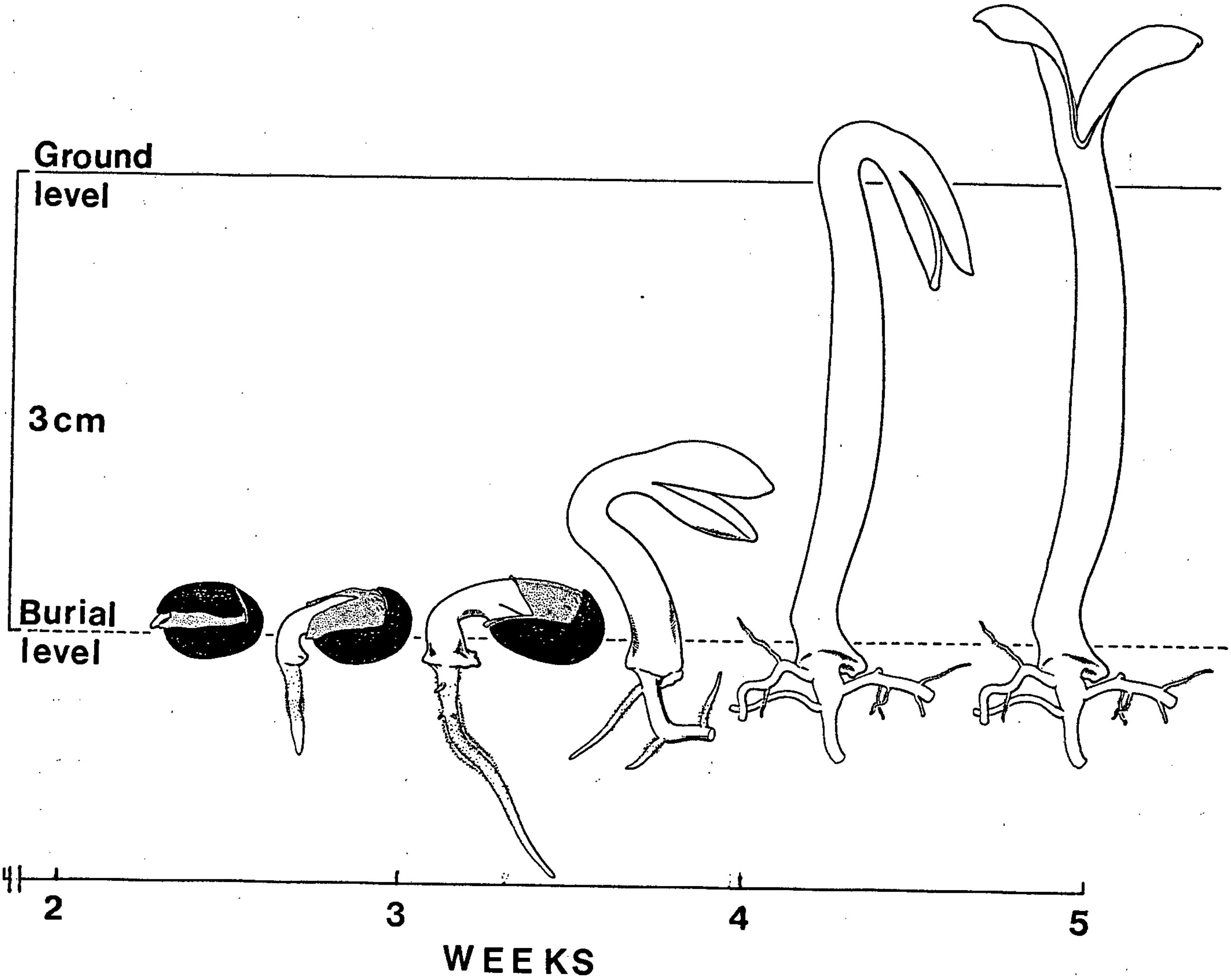

Figure 10. Germination and epigeal emergence in *Leucospermum*. The endotesta splits at the micropylar end and breaks in a characteristic pattern. The hypocotyl foot enlarges prior to elongation.

A prominent flat structure develops at the lower end of the hypocotyl (Figure 10). The "hypocotyl foot" in fynbos Proteaceae is found only in *Leucospermum* and other myrmecochores which emerge from depth (Brits, 1987). It provides a base in the sandy fynbos substrate from which upward thrust is applied during emergence.

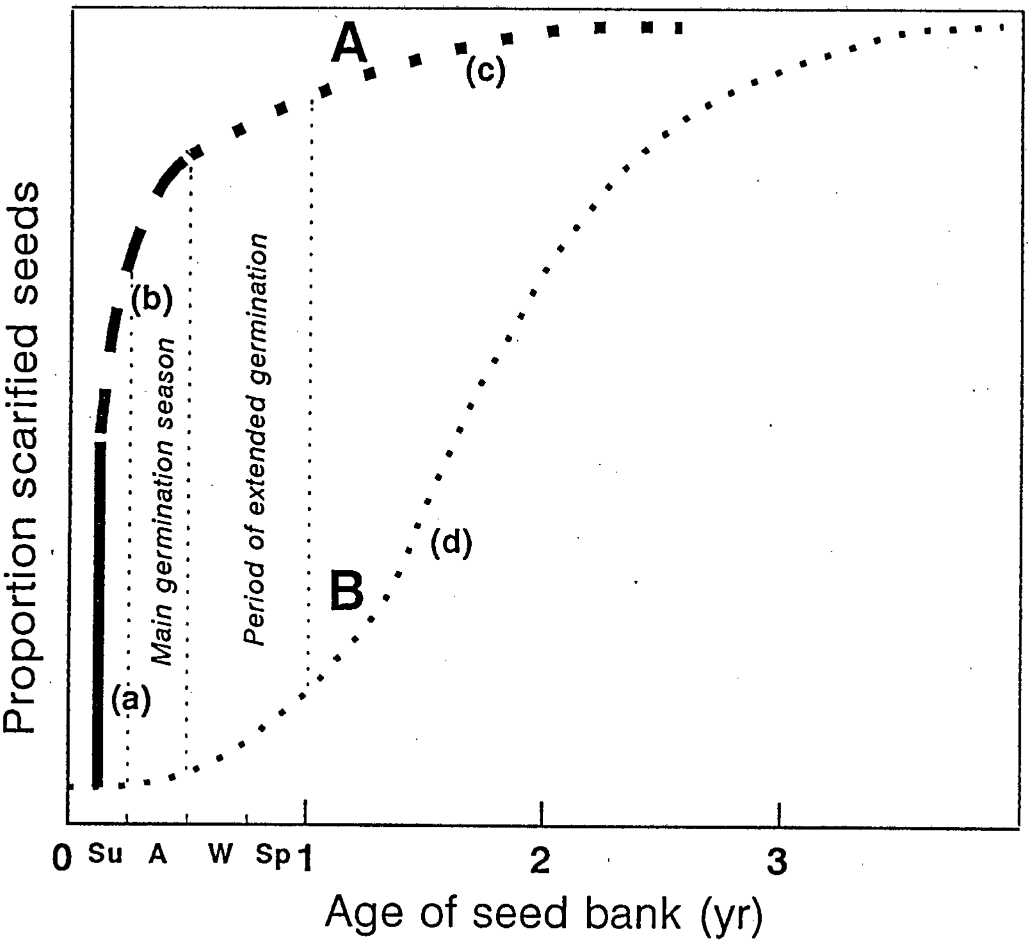

Figure 11. Proposed model for scarification regimes acting on youngest-season *Leucospermum* seeds in fynbos post- (A) and pre-fire (B). The desiccative effect of fire in mid-summer (a) represents the potentially strongest single (pulse) effect; desiccation effects by the sun in fire-exposed soil during summer (b) represents the next strongest effect; while scarification of remaining intact seeds beyond the first autumn post-fire (c) progresses essentially at the same slow rate as for seeds in unburnt fynbos (d, latter part of curve) and extending beyond the first germination season. Seedling recruitment efficiency is assumed directly related to the proportion of seeds scarified; thus after fire effectively all seeds in A and B combined will germinate synchronously, with only the portion (c) participating in extended germination.

Figure 12. *L. cordifolium* seedlings in fynbos emerging from under a stone four months after fire, presumably from an abandoned ant nest.

The rate of *Leucospermum* germination is relatively slow: typically 14 - 21 days to radicle protrusion (for fresh, intact seeds), plus another 7 - 10 d to emergence, even under optimal conditions of oxygenation and temperature (Chapter 5). The lag time between sowing and emergence from 1 cm depth in seed beds is typically 4 - 6 w (Brits and Van Niekerk, 1986). At dispersal the embryonic axis in *Leucospermum* is undifferentiated consisting of only meristems where the plumule and radicle will in time develop (Figure 1B).

Once imbibed, dormant *Leucospermum* embryos endure long periods of saturation and an indefinite number of cycles of desiccation / wetting, without loss of viability (Brits, personal observation). *L. cordifolium* seeds stored artificially in the soil for 2 - 4 years germinated significantly faster than fresh, intact seeds (Chapter 6.4). Germination rate in the Australian heathland species *Clematis microphylla* increased markedly when seeds were repeatedly desiccated and rewetted before germination (Lush *et al.*, 1984).

Seed bank dynamics

Seed longevity

Mature *Leucospermum* plants produce seeds consistently over many seasons, adding to the seed bank at a rate of 25 - 250 seeds per plant p.a. (Brits, personal estimate). Seed longevity in the soil is inversely related to age (Roberts, 1972), however the rate of decline is not known in *Leucospermum*. One seed sample buried in the present simulated dispersal study germinated 60% after 4 years (Chapter 6.4). It is therefore likely that the soil-stored seed bank is larger than the seed crop of the latest flowering season. Most of these seeds would be scarified (Figure 7C - F). Le Maitre (1988) in a study of *Leucadendron pubescens* R.Br., a Proteaceae species with large soil-stored seeds, calculated an annual seed crop per plant of 181 seeds and estimated the seed bank size in the soil at 1,5 times the annual crop.

Rate of scarification

The results above suggest that the rate of testa scarification in the seed bank may differ greatly under different combinations of climate and fire regime.

1. *Old-seed component*. This is the portion of the seed bank which accumulates in the soil during the pre-fire phase (lasting naturally 6 - 20+ y - Bond and Slingsby, 1983). The soil surface is effectively shaded by the vegetation canopy (Brits, 1987). During autumn ambient diurnal temperature alternates relatively strongly and condensation (dew) typically occurs during the night on exposed soil. This suggests a regime of weak daily desiccation-hydration cycles which could continue over many years. The resulting endotestal fractures would be small and accumulate gradually. Such a process could explain the brittle, strongly scarified condition of seeds buried for 4 years in unburnt fynbos (Figure 1D). The pattern of gradual scarification (Figure 7) suggests a cumulative normal scarification rate curve (Murdoch and Ellis, 1992) for the combined testa layers in the seed bank, approaching complete scarification of seeds after 2 - 3 years of soil storage (Figure 11B; Chapter 6.4).
2. *The young-seed component desiccated by fire* (present flowering season seeds). Plant canopy destruction and heating of soil by fire will result in the strongest degree of desiccation (pulse) of these seeds, comparable to heating in an oven (cf. Chapters 6.2, 6.3). Soil is heated to c. 50 °C at 2 cm depth in low-intensity fynbos fires (De Lange and Boucher, 1993a). Fire followed by rain will predictably result in the strongest degree of both exotesta rupturing and endotesta fracturing. Fire intensity in fynbos varies (Bond *et al.*, 1990) and differential seedling recruitment responses in *Leucospermum* are therefore predicted according to the above model (Chapter 6.2).

3. *The young-seed component remaining unscarified in fire-exposed soil after fire during the first summer/autumn season.* Daily surface soil temperatures are high achieving an average maximum of 55 °C in January (Brits, 1986). Superficial soil temperature in exposed soil may exceed 60 °C on individual days (J.H. de Lange, personal communication). This and strongly desiccating winds (De Lange and Boucher, 1993a) could accellerate exotesta rupturing in young seeds. Rainy spells during this phase of strong desiccation would increase the degree and rate of endotesta fracturing. Such spells do occur at low frequency in fynbos, especially under "bergwind" conditions when exceptionally high ambient, desiccating temperature is often rapidly succeeded by rain (Chapter 6.2). Since freshly dispersed seeds constitute a major part of the myrmecochore seed bank (Le Maitre, 1988) this process could increase the rate of scarification of the seed bank as a whole after fire. This could partly explain the strong and rapid recruitment response in *Leucospermum* following the first autumn rains after fire. Published (Brits *et al.*, 1993) and present results (Figure 9A) suggest scarification rates for different young components of the seed bank (Figure 11A). In California chaparral, older soil-stored seeds of *Arctostaphylos canescens* were found to be more easily stimulated to germinate than fresh seeds, indicating variable responsiveness of different seed bank components to favourable germination conditions (Parker, 1987).

Syndrome of extended seed bank germination

Plant species subject to severe environmental stress commonly extend their seedling recruitment phase over the greater part of the natural germination season (Gutterman, 1993). The spreading of germination over time results in a staggered ("erratic") emergence pattern and this "polymorphic germination time" or extended germination, has been widely researched (Cavers and Harper, 1966). Extended germination of intact seeds has been observed in at least six *Leucospermum* species (as slow germination rate - Chapter 4.3) and the character is therefore defined as a syndrome (Stebbins, 1974; Angevine and Chabot, 1979). Its prevalence as a natural characteristic of nut-fruited Proteaceae is reflected in horticulture in the vexation experienced - and documented - with respect to (coat-imposed) seed dormancy. In *Leucadendron argenteum* (L.) R.Br., with seeds structurally resembling *Leucospermum* (Jordaan, 1945), disappointingly poor germination extending over several seasons is described in the earliest document on the cultivation of Cape Proteaceae (Knight, 1809). In *Leucospermum* this problem has been characterized as the "most challenging" of Proteaceae germination problems (Brown *et al.*, 1986).

Extended germination is a primary survival strategy adapting seedling populations to unpredictable and severe stress / disturbance events that occur during the otherwise favourable germination season at low frequencies, e.g. drought, frost, insect predation, rodent herbivory, disease, etc. (Cavers, 1983; Gutterman, 1993). Such events, especially drought, are potentially cataclysmic because the gene pool, i.e. the entire local species population, could be extinguished should all seeds in the seed bank have germinated early and simultaneously. Specht (1981) demonstrated that seedling survival of especially slow-germinating species in Australian heathlands regenerating after fire depends directly on the length of the moist period of the first germination season. The sporadic occurrence of drought during winter, observed in fynbos (Brits, 1987) could clearly limit the period of moisture availability, and thus regenerative success in *Leucospermum*, during the critical first post-fire season. Although the probability for a disturbance event in a given germination season is often low, its recurrent nature determines a high probability in the evolutionary history of species.

The old-seed component of the seed bank is arguably fully oxygenated (scarified) in the ground when moistened in the pre-fire phase during successive winters. Old seeds nevertheless remain ungerminated because dormancy is enforced physiologically by unsuitable temperatures (Brits, 1986). However when the appropriate temperature cues and moisture conditions co-occur, the older component of the seed bank germinates without delay. This results in the synchronous emergence of the majority of seedlings, often characterised as *en masse* germination in post-fire fynbos and resulting in even-aged seedling stands (Rourke, 1972; Brits, 1987). The characteristic of extended germination is therefore confined to the young component of the seed bank, in which seeds have an undamaged exotesta. Only a small portion of the young-seed component will participate in extended germination (Brits, 1987; Figure 11).

The most important environmental and genetic variables contributing to differential disintegration of the seed coat in individual young seeds (seed-to-seed variation) are discussed by Murdoch and Ellis (1992) and shown for *Leucospermum* in Figure 8. Other variables affecting seed coat / physiological dormancy may include: temperature effects dependent on differences in burial depth of seeds (Brits, 1987); maternal effect, manifesting as maturation differences in individual seeds influenced by position within the ageing infructescence (Vogts, 1982; Gutterman 1985); and genetic differences in physiological dormancy (Murdoch and Ellis, 1992) and seed coat permeability (Brits, unpublished).

Gradual testa scarification in nature conceivably determines differential oxygenation of individual seeds in the soil-stored seed bank. Thus only embryos in which the individual oxygenation threshold is exceeded will germinate (Chapter 5). In a young (present flowering season, unscarified) seed batch this will manifest as the erratic emergence pattern often observed in seed beds (Brits and Van Niekerk, 1986).

Apparently none of the above factors will cause the exotesta in all freshly dispersed seeds to break during the main seedling recruitment phase. This is suggested by a similar tendency observed in desiccation-scarification experiments. Thus a small but significant proportion of fresh seeds will remain non-oxygenated - therefore dormant - in the now depleted seedbank (Figure 11). Natural germination may therefore extend over several years (Brits, personal observation). This phenomenon was observed in

Australian heathland species regenerating after fire (Specht, 1981) and germination may even extend over successive fire cycles. The latter is suggested by the germination pattern in seed beds extending over more than 4 years (Knight, 1809; Chapter 6.4). An inadequate diurnal fluctuation in germination temperature regime soon establishes after fire in fynbos through rapid growth of the vegetation canopy. It is therefore likely that some *Leucospermum* seeds provide a genetic continuum at least until the regenerated population releases its first new seed crop (in c. 3-4 years - Rourke, 1972).

Conclusions

Adaptive equilibria in reproductive strategies

An equilibrium in the responses to opposing ecophysiological forces is evident in the dynamics of *Leucospermum* soil-stored seed banks. Such equilibria are a strategy for responding to potentially conflicting selective pressures in the environment (Willson, 1992). The following discussion summarises current understanding of germination dynamics in *Leucospermum*. In many instances the germination characteristics are a compromise between two or more comflicting requirements in seed behaviour.

Seed coat scarification vs. embryo protection

The scarification of the endotesta, for example, may relieve mechanical constraint of weaker (or deeply buried) embryos, which in turn could assist in their emergence. However the embryo needs protection from soil-borne pathogens and predators during extended inter-fire periods. Progressive scarification implies increased exposure of the long-lived embryo to seed destroyers through the fissures present in the now weakened testa (Figure 1D) and this may lead to reduced viability (Lush *et al.*, 1984; Gutterman, 1993).

Extended germination vs. pre-emption of space through early germination

Old seeds form the seed bank component that germinates fully, synchronously and early within the habitat after fire and the function of extending germination is restricted to a young-seed component which is not, or only partly, scarified (Figure 11). Clearly *Leucospermum* species risk efficiency in pre-empting available establishment space (Fenner, 1985) by allocating a significant proportion of young seeds to the strategy of extended germination. An adaptive equilibrium is therefore suggested between the conflicting advantages of immediate and delayed germination.

Anoxia vs. oxygenation of the embryo

Dormancy in *Leucospermum* imposed through oxygen deprivation appears to have the single function of extending emergence of young seeds over the entire germinative season. The principle of regulated oxygen deprivation is by definition a risk for a seed bank dependent for recruitment on the respiring embryo. The poor emergence found after low-intensity fires (Bond *et al.*, 1990) suggests that substantial proportions of the seed bank remained ungerminated with a consequent loss in recruitment potential for the species. Plants using other germination cues from fire, e.g. smoke, could therefore in many situations predictably outperform *Leucospermum* recruitment relying on desiccation-scarification alone. Clearly a lesser degree of seed coat dormancy could potentially compensate for the negative effect of low fire intensity - at the expense of the capacity of the population to extend germination of young seeds.

Slow germination rate vs. rapid establishment

Rapid germination and establishment is a common strategy in species subject to occasional rain in dry environments (Gutterman, 1972; Jurado and Westoby, 1992). The spectacular displays of Asteraceae ephemerals in semi-deserts after rainy spells (e.g. Bell *et al.*, 1993) exemplify this strategy. Why does *Leucospermum* in an arid summer environment opt for the apparent opposite, an unusually slow rate of germination? The long incubation period in *Leucospermum* (Chapter 5) is similar to that of some species in moisture-stressed habitats which germinate only after c. 16 d of wetting (Gutterman, 1993).

High soil moisture levels prevail for extended periods during the winter rainy season in Cape fynbos, but occur also in short, unpredictable spells during the unfavourable dry Cape summer (Deacon *et al.*, 1992). The latter characteristically last for only a few days. Rapidly germinating seed will predictably not survive the ensuing moisture stress (cf. Keeley, 1991). Thus seeds which require a long germinative period would be buffered against short spells of high moisture availability (spurious recruitment opportunities) during summer. When the dry season resumes, non-germinated seeds desiccate without damage, postponing germination to the main germinative period of winter. This "stop-go" germination mode is adaptive and has been observed in plants growing in habitats that receive occasional rain in summer (Lush *et al.*, 1984). In *Leucospermum* it is complemented by the additional requirement of seeds for incubation temperatures markedly cooler than the ambient temperature in wet fynbos during summer rainy spells (Brits, 1986). Certain perennials in habitats receiving mainly winter rains exhibit the latter strategy (Gutterman, 1993).

Slow germination in *Leucospermum* during the favourable early-winter season however puts the genus at a potential disadvantage with sympatric competitors. Competition is stringent for developmental space by other rapidly germinating species (cf. fast-germinating Australian heathland species - Specht, 1981) and resprouters in the post-fire habitat (Yeaton and Bond, 1991; Bond *et al.*, 1992). The latter occupy available growing space quickly (Kruger, 1983) using large amounts of lignotuber reserves to effect bursts of growth that could exclude seedlings (Specht, 1981; Keeley, 1986). A critical balance of selective pressures is therefore implied in the evolution of slow germination rate in *Leucospermum*.

Myrmecochory: a price for fire- and predator escape

The main predators of *Leucospermum* propagules are rodents (Le Maitre and Midgley, 1992). The relatively large, plump achenes are released during early and midsummer afternoons and there is no telechoric mechanism to aid in dispersal away from potential predators, as is the case in other taxa (Van Staden *et al.*, 1989). Achenes fall directly to the ground, ants rapidly collect them, during daytime, and bury them in shallow nests (Bond and Slingsby, 1983). Achenes not removed on the same day are apparently quickly consumed by species of mice (Le Maitre, 1988).

The early and midsummer period of achene release in *Leucospermum* immediately precedes the peak of the fire season in the fire-prone fynbos during late-summer and early autumn (Van Wilgen *et al.*, 1992). Both dispersal phenology and soil burial thus provide effective escape mechanisms also from the intense, albeit short-lived, heat peaks of fire. The mutualism between ants and plants in escaping destruction by both granivores and fire therefore appears highly profitable (Slingsby and Bond, 1985; Bennett and Krebs, 1987).

The system is however less than perfect for *Leucospermum* survival. Many achenes are, for instance, buried too deep for emergence. Deep emergence depth is an important seedling mortality factor (Fenner, 1985) and de Lange and Boucher (1993b) found an inverse relation between the rate of *Audouinia capitata* (Bruniaceae) seedling emergence in fynbos and the burial depth of seeds. *Leucospermum* seedlings have been found to emerge from up to 11 cm deep (Brits, 1987). Reduced ability to emerge would apply especially to ageing seed in the seed bank (Chapter 6.4) in which vigour has diminished sufficiently so that emergence from 3 - 5 cm (average) depth is no longer possible. In addition a marked proportion of seeds are buried in nests under stones (Figure 12) which further impedes emergence, compared with emergence from nests in open ground. Thus a large proportion of seeds may perish through the myrmecochorous strategy.

Another costly aspect of myrmecochory is the concentration of large numbers of seeds in small emergence sites (nests). Up to 40 *L. cordifolium* seedlings have been found congesting in one site (Brits, 1987). Of these seedlings only one will mature (Brits, personal observation) and this implies considerable waste of the nutrient-costly propagules.

Myrmecochory moreover results in the strongly localized emergence of seedlings around parent plants, the large majority within a radius of 1, or at most 2 m, from the main stem (Slingsby and Bond, 1985; Brits, 1987). The advantage of this is replacement of the parent plant after fire in a site which is evidently suitable for survival. However seed dispersal is the single most important factor promoting gene flow in plants, next to pollen transport (Van Staden *et al.*, 1989). Strongly topochoric (short-distance) dispersal therefore suggests extremely limited geneflow ("genetic neighbourhood" effect - Levin, 1981) in *Leucospermum* by means of the seed phase (Bond and Slingsby, 1983). In addition the long-distance dispersal potential in evolutionary time is poor (Willson, 1992). Small barriers for ants, e.g. streamlets, can apparently create a major barrier to the territorial expansion of myrmecochores. Ant dispersal could have contributed to the high degree of endemism found in *Leucospermum* (Cowling *et al.*, 1992).

The above constraints imposed by myrmecochory suggest compromising adaptation in *Leucospermum*. Despite its prevalence in fynbos, Willson (1992) questioned whether this dispersal mode is especially advantageous.

Narrow vs. wide response to temperature cues

Leucospermum seeds are physiologically dormant at high temperature. At constant temperature above 20 °C less than 3% of seeds will typically germinate (Brown *et al.*, 1986; Chapter 4.4). The pattern of occasional summer rain in fynbos might stimulate germination of non-dormant seed populations in the absence of this temperature constraint, leading to subsequent seedling loss through drought. This strategy may however be costly. Warm weather spells occur also during the main germinative season of autumn when soil moisture levels have already risen and remain high (Brits, personal observation) and this has been shown for Australian heathlands (Specht, 1981). Such recruitment opportunities could be lost with strong enforcement of high temperature dormancy.

The temperature levels enforcing or relieving physiological dormancy moreover lie closely together, relative to the overall pattern of shifting temperatures in fynbos soil (Brits, 1986). The strong alternation required for maximal germination, for example, rapidly weakens with increasing soil storage depth of seeds (Brits, 1987). Thus a substantial part of the seed bank may be subjected to weak or even confounding temperature cues.

Some seeds in the seed bank however germinate at all temperatures, with the percentage response characteristically increasing as average seasonal temperature falls (Brits and Van Niekerk, 1986; Brits, 1986). The response of seeds to atypical temperature cues could be a worthwhile investment of the species in utilizing unusual, yet favourable, weather patterns.

It therefore appears that the genetic systems regulating dormancy and germination characters are effectively balanced to secure an optimal recruitment response for the *Leucospermum* population. Thus the genetic constitution of the seed population regulates recruitment in terms of multiple equilibria of complex and often conflicting ecological forces / principles. Study of the occurrence and magnitude of selection pressures on reproductive traits is clearly needed (Wilson, 1992).

***Leucospermum* in the Cape a young genus?**

Based on phytogeography and edaphic data, Rourke (1972) proposed a recent and rapid radiation for *Leucospermum* species in their areas of greatest concentration. The above-mentioned common seed characters in *Leucospermum* leads to a similar conclusion for at least the group of fynbos species considered here. *Leucospermum* seed character syndromes are foremostly adaptive responses, directly and indirectly, in avoiding a complex of moisture-stress phenomena (Chapters 4 - 6). Even fire-disturbance is considered a companion phenomenon to prolonged summer moisture stress in mediterranean vegetation types of the world (Gill, 1975). Significantly the main fire season in fynbos coincides with the months of peak moisture stress (Le Maitre and Midgley, 1992). Extended germination as well, operates primarily to counter the effects of episodic-drought patterns on recruiting populations (Brits, 1987). The complex of moisture-stress phenomena in fynbos thus manifests primarily at three levels in the seedling recruitment of *Leucospermum* species: suppressive influence of summer drought on germination and establishment potential; disturbing (canopy competition-removing) effect of fire, in addition fire is potentially destructive to exposed seeds; during the cool winter germination season, the occasional occurrence of dry spells which in evolutionary time could lead to local extinction (Brits, 1987). Thus the suite of *Leucospermum* seed dormancy characters discussed above are all strongly related to drought-avoidance and xerophily in fynbos.

The complex nature of this germination strategy suggests a unique evolutionary origin within the fynbos members of the family (possibly including other nut-fruited Proteaceae genera). Uniqueness and the syndrome nature (spread over a group of species) of the characters suggest that species radiation in *Leucospermum* took place recently. This could have been after the change of a wetter to the present drier mediterranean-type climate, dated to the Mio-Pliocene (Deacon *et al.*, 1992). Had radiation occurred earlier, during wetter periods, then some of the numerous other drought-avoiding / fire-cued strategies found in Mediterranean-type climate plants (Keeley, 1991; Bell *et al.*, 1993) could have evolved independently in different species of *Leucospermum*. Smoke and vegetation charate, for example, function as germination cues in a large number of non-proteaceous fynbos and chaparral taxa respectively (Keeley, 1987; Brown, 1993) indicating convergent evolution. In *Protea* serotiny evolved in response to fire disturbance. Among the fynbos members of *Leucospermum* no significant divergence from these features has yet occurred. However adaptations appear to have been modified by differential selection pressures in new habitats (Chapter 4.4).

Adaptation is essential for plant survival in harsh and unpredictable environments (Gutterman, 1993) and the results suggest that eco-dormancy characters in *Leucospermum* are of primary fitness value to species. Not only the severity of dormancy experienced in horticulture, but also the extent of the range and the complexity of interaction of characters, support this view. Evolution in stressful and unpredictable environments is a continuously driven process (Harper, 1977), in contrast with "old" species in relatively stable environments, in which characters show little genetic variation (Stebbins, 1950). Thus *Leucospermum* species appear to remain in an adaptive phase, constantly equilibrating their reproductive gene pool amongst the conflicting pressures of stresses and disturbances in the fynbos environment.

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CHAPTER 8

CONCLUDING REMARKS

This thesis aimed to characterise the seed biology of *Leucospermum* species in fynbos as fully as possible. The objective was to construct a working model of functional relationships from seed anatomy, physiology and environmental cues. In summary this study revealed a surprising degree of complexity of the *Leucospermum* seed's internal and external environments and their interrelationships in fynbos. The following remarks integrate key aspects of the genus' seed biology from foregoing chapters, at the same time listing some contributions of the work to, and areas for future research in, fynbos seed science.

Current status of understanding

Seed ontogeny: The confusion in the literature of *Leucospermum* seed structure terminology was corrected and published. This served as the basis for correlating seed structure and function in fynbos.

Artificial measurement and control of dormancy

This thesis has shown the critical importance, in laboratory investigations, of distinguishing between non-viable and viable-but-dormant fractions of seed populations. The ability to germinate seeds fully is the most important tool to this end.

Maximizing treatments. Germination of intact seeds can presently be maximized under laboratory conditions in a number of *Leucospermum* species. A combination of treatments are required, each with a diffferent role:

1) (c) H_2SO_4 scarification: increases oxygen permeation during incubation and softens the hard coat, facilitating emergence of the radicle;

2) incubation in oxygen: enhances germination also in acid scarified seeds. Scarification and natural aeration can apparently not substitute fully for the effect of incubation in oxygen, in realizing maximal response;

3) alternating temperatures: the average required levels for six species were 9,9 (low) and 21,2 °C (high) respectively. However, for maximal response the correct temperatures for individual species must be used;

4) soaking in a mixture of gibberellin and (synthetic) cytokinin ("Promalin") further enhances germination. Thus any blocks interfering with the embryo's own synthesis / interconversion of hormones (e.g. non-optimal temperature regime) can be partly overcome by exogenous supplementing of hormones;
5) fungicide treatment, which significantly suppresses strong fungal growth during the long incubation period of 3 to 6 weeks; some of the fungi are presumed to be seed-borne diseases.

Comparison with viability estimates indicated that all viable and even sub-viable seeds can be germinated with these combined procedures. It can therefore be concluded that all requirements for dormancy breaking and germination are met in such seeds. Untreated seed of the semi-domesticated *L. cordifolium* showed markedly less dormancy than those of other species and this indicates the importance of working with wild (or near-wild) material in fynbos seed studies.

Tetrazolium testing for viability. Large seed size and ease of application renders tetrazolium testing of viability extremely practical in *Leucospermum* seed studies. The results with tetrazolium were however variable. Large discrepancies occurred occasionally between viability scores and actual germination / emergence. The best results with this method can be obtained with freshly harvested, fully matured seeds.

Characterisation of dormancy and germination mechanisms

Germination syndromes in Leucospermum. Similar germination percentage and rate response patterns to a variety of treatments, in contrasting species (Chapter 4.3; 6.3) suggest that the physiological and physical processes involved in seed germination are common in the fynbos *Leucospermum* group of species. The common requirement for two daily incubation temperatures during germination, in a range of species, also supports this notion. The complex of seed features spread over many species are defined as character syndromes (Chapter 4.4).

Type of dormancy. The *Leucospermum* seed employs two types of dormancy, primary and enforced. Freshly dispersed seeds are fully dormant by means of the oxygen-excluding testa, thus showing primary dormancy. However the embryo is not primarily dormant, quiescence only is enforced physiologically by the lack of two incubation temperature levels in unburnt fynbos and in burnt fynbos during summer.

"Complex" dormancy. The three conditions defined as comprising complex dormancy, either alone or in combination (Chapter 1), are all met in the *Leucospermum* seed.
1) Several types of dormancy operate in the same seed, as was shown above.

2) Several environmental conditions (cues) can act singly or in combination to relieve one dormancy type. In *Leucospermum* the testa is desiccation-scarified by a combination of three heat phenomena in fynbos (Chapter 6.3). Furthermore two temperature levels in the incubation environment are required.

3) Several functional seed characters are receptive to the same dormancy breaking cue. The functionally different exo- and endotestas in *Leucospermum*, both scarified by heat pulse, are an example.

The *Leucospermum* seed is therefore a "multifunctional organism" (Manning, 1987) employing multiple dormancy mechanisms and ecological cues.

Phenology of dormancy breaking and germination. Contributing to complexity is a sophisticated phenology of successive seed processes and events leading to successful species recruitment in fynbos. This seasonal pattern is as follows:

Process / event	**Peak season**
Seed ripening	spring to early summer
seed dispersal	early to midsummer
removal of pericarp	following dispersal
fire season	late summer
testa desiccation-scarification	during / shortly after fire
onset of rainy (high moisture) season	mid- to late autumn
embryo oxygenation	following testa scarification and rain
incubation temperature requirements met	post-fire in autumn to early winter
germination season	late autumn and early winter.
season favourable for seedling establishment	winter to early spring

Alternating temperature requirements. This study showed that *Leucospermum* species are narrowly adapted to their natural environments, with respect to temperature requirements. It thus confirms for fynbos what has been found in other mediterranean-type vegetations (Chapter 7). The advantages of narrow adaptedness to a particular niche could be increased sensitivity of a seed population to a unique environment. This could lead to improved seed responsiveness, avoidance of seed loss to spurious germination cues and the pre-emption of growing space by early germination and establishment. Temperature requirements were shown to be adaptive, independent and genetically stable characters in *Leucospermum*.

Temperature amplitude was proposed not to be a seed ecological parameter in *Leucospermum*, as in the case of many weed seeds (Chapter 4.4). Rather, the two temperature requirements function independently. It was shown before that serotinous Proteaceae seeds require only low temperature for germination (Brits, 1986) and this further supports the notion of independence of temperature requirements, especially since a specific function for high temperature requirement was identified in *Leucospermum*. The requirements in fynbos *Leucospermum* species should therefore aptly be characterized as a diurnal bi-temperature requirement.

Testa desiccation-scarification. High temperature *per se* has been ruled out as a factor in the breakage/scarification of the testa layers in *Leucospermum* and also as a physiological factor. Exotesta as well as endotesta scarification is therefore caused only by desiccation effects (excepting microbial scarification) although in nature desiccation is positively correlated with temperature. The primary function of the desiccation-scarification mechanism is to differentially increase oxygenation in seeds and thus extend the emergence pattern of the recruiting population. In effecting this the exotesta is the main instrument, and to a lesser degree the endotesta. The results underline the high degree of adaptedness in *Leucospermum* seeds to fynbos stress and disturbance factors. This type of scarification and the underlying desiccation mechanisms as proposed in this thesis, is presently unknown in seed science.

Hormonal model. An analysis of the seed hormone literature on nut-fruited Cape Proteaceae (Chapter 1) indicated that the endogenous levels of the known promoter hormone groups in *Leucospermum*, gibberellins (GAs) and cytokinins (CKs) depend primarily on the provision of low incubation temperature. For CK synthesis low temperature provision only was deduced but for GA synthesis a daily period of high temperature was indicated, or alternatively high in combination with low temperature (Chapter 1, Table 1). These deductions were tested successfully in Chapter 5. In optimally germinating *L. glabrum* seeds the endogenous levels of GAs and CKs were found to change phasically under a single alternating temperature regime. It was found that a daily pulse of high temperature, in addition to the primary provision of low temperature, control the rise of GA levels in the embryo. Only low temperature appeared to be required for CK synthesis/interconversion. Early increases in GAs were proposed to control the induction of germination *sensu stricto*. Subsequent sequential peaks of CKs, appearing in cotyledons and the embryonic axis, are thought to indicate CK control of at least three major processes during germination: 1) early mobilization of protein and lipid reserves in the axis, and later in cotyledons, 2) cotyledon expansion which causes the endotesta to split, permitting radicle protrusion and 3) later, radicle growth. The above view contrasts with that of Brown and Van Staden (reviewed in Chapter 1) who proposed that CK is the primary plant growth substance to break dormancy in certain nut-fruited Proteaceae. This study suggests that *L. glabrum* could serve as a model for *Leucospermum* in fynbos on account of the highly homogeneous seed biology of this group of species.

Scenario for regeneration from young seeds after fire. Seeds of the present flowering season are the primary source for seedling recruitment in fynbos. The arguments above suggest that the majority of such young seeds in *Leucospermum* would be scarified during and shortly after fire. Fire could influence the degree of scarification and therefore also of subsequent oxygenation in such seeds, i.e. the recruitment success of the species correlates with fire intensity. The removal of the vegetation canopy by fire leads, in the ensuing autumn, to warm soil temperatures during daytime and this, together with low autumn nighttime temperature, induce the formation of the primary germination hormone (group), gibberellin(s), after the occurrence of rain. Low nighttime temperature alone regulates the increase in cytokinin levels. Thus the two germination promoter hormone groups will regulate the now well aerated embryo to commence utilization of food reserves, split the endotesta at the radicle end and resume radicle growth. Unscarified young seeds will germinate erratically through at least the first germination season.

Older, long-living seeds in the seed bank, mostly scarified, may germinate faster than young seeds following the occurrence of high day temperature in autumn.

Unresolved problems and future research

Smoke and other unknown stimulants. Although a role for smoke as a germination stimulant could not so far be confirmed for *Leucospermum*, this possibility is not ruled out completely. Considering the complexity of dormancy control in nut-fruited Proteaceae, it remains a distinct possibility that other stimulative factors may yet be found to be involved (Chapter 4.4). The discovery of new cues in some *Leucospermum* species would indicate unsuspected divergence in this assumed homogeneous genus in fynbos.

Physical mechanisms of testa scarification and impermeability. The breakage mechanisms of the exo- and especially the endotesta need more clarification. This is necessary to test the proposition that desiccation-scarification is a new seed biological phenomenon, as opposed to other kinds of wetting-drying-scarification (Lush *et al.*, 1984; Bell *et al.*, 1993). Oxygen impermeability of the exotesta has also not been demonstrated directly in *Leucospermum* and has received scant attention in seed science generally (Werker, 1980/81).

Incubation temperature requirements. A comparative study of temperature requirements in weed and *Leucospermum* seeds would be required to adequately test the proposed, unconventional, concept of diurnal bi-temperature requirement in *Leucospermum* (Chapter 4.4), as opposed to the established concept of temperature amplitude *per se* (Murdoch *et al*, 1989) as an ecophysiological parameter. Such study should include a comparison of physiological control of dormancy in these seeds types.

Partial completion of germination in unburnt fynbos leading to fast germination rate. Soil-stored seeds germinate surprisingly fast (Chapter 6.4). Seeds may have completed and accumulated the initial stages of germination during repeated wet periods in nature, shortening the final period of continuously favourable conditions required for germination (Lush *et al.*, 1984). This aspect of *Leucospermum* seed ecology deserves more study.

Cellular repair mechanisms during intermittent wetting in the soil-storage phase. A much higher longevity was found in seeds stored for 4 years in the soil than in dry-stored seeds (Chapter 6.4). The possibility exists that periodic hydration of seeds in the soil may increase longevity by means of cellular repair. This principle may also account for the apparently much higher longevity of naturally, as opposed to artificially stored seeds in serotinous Proteaceae. In nature these seeds are stored in a turgid condition in the mother plant canopy. This aspect of seed longevity, and the implied potentially improved seed storage technology for Proteaceae, deserves further study.

Leucospermum *seed bank dynamics,* especially the rate of seed attrition during soil storage and the hypothesis of seed bank partitioning, represent gray areas in present knowledge. These aspects could be investigated using hypothetical prediction and field experiments. For example the hypothesis that extended germination is primarily an adaptive response to rare dry spells during the moist germination season, could be tested as follows. Germination and emergence patterns of species occurring in permanently moist environments (e.g. *L. glabrum, L. formosum, L. reflexum*) could be compared with those in drier environments (*L. cordifolium, L. erubescens*). The hypothesis predicts that germination in the former should tend to be more synchronous and in the latter more staggered (assuming that drought is the only factor that depresses recruitment during winter).

Effects of heat intensity of fire on subsoil temperature and soil moisture content (desiccative potential) in fynbos could be investigated to test the hypothesis of testa desiccation-scarification under field conditions. The proposed disintegration of the exotesta by the heat of fire has not been verified in the field. In addition the prediction that rainy spells following fire and dry summer weather will increase endotesta scarification, needs field testing.

Germination biology of other fynbos taxa. The models developed in this thesis suggest that equally complex, but different, dormancy mechanisms may occur in other fynbos taxa. Dormant seed in several myrmecochorous *Restio* genera, for example, could not yet be germinated successfully under controlled conditions. The complexity of cues associated with myrmecochory in *Leucospermum* may thus be parallelled in other genera.

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ACKNOWLEDGEMENTS

I am grateful to a large circle of leaders, supervisors, colleagues, friends and interested persons who contributed to this thesis.

The invaluable help and encouragement of my supervisors, William Bond, Owen Lewis and Jill Farrant (University of Cape Town) and Neville Brown and John Manning (National Botanical Institute) are sincerely appreciated. Their spirited and competent comment contributed much to the work and for this, too, the inputs of Jonty Cutting and Hannes van Staden (University of Natal), Hannes de Lange (National Botanical Institute) and Gerhard Malan (Agricultural Research Council) are much appreciated.

The Agricultural Research Council showed unfailing support and patience with this project, bordering on the magnanimous. I thank the Council also for financial support. My thanks go to the National Botanical Institute for the opportunity to work for two years in the Kirstenbosch Research Centre, Division of Ecology and Conservation, and especially to Neville Brown for thoughtful guidance during this period.

Statistical analysis and interpretation was generously provided by Frikkie Calitz (Agrimetrics Research Institute, Stellenbosch) and his colleagues Dennis Capatos, Jeanne Bezuidenhout, M. Terhaar and Marieta van der Rijst. John Randall (Stellenbosch University) assisted with the analysis of seed coat breakage data (Chapter 6). Statistical input was sometimes expanded to the drawing of technically demanding figures, at which Frikkie also excells, assisted by E.L. McArdell.

Technical assistance with the execution of experiments contributed much to the project, and this was always provided in a supportive spirit, by Gerrit and Brigitte van den Berg (Agricultural Research Council) and Teri Dennison (University of Natal). I thank the Electron Microscope Units of the University of Cape Town and INFRUITEC, Stellenbosch, especially Messrs. Dane Gericke and Rassie Van Zyl, for technical assistance and Mr J. Nel, photographer at INFRUITEC, for preparation of photographs (Chapter 7).

Several nurseries donated seed for experiments and these gifts are gratefully acknowledged (Chapter 4.4, Table 1). The Weather Bureau and the Institute for Soil, Climate and Water provided climatical data (Chapters 4, 6).

I dedicate this thesis to my mother and my wife, Cecilia.