# SEED DORMANCY AND

# GERMINATION IN PROTEA COMPACTA R.Br.

# AND LEUCADENDRON DAPHNOIDES Meisn.

by

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CONTENTS

## ABSTRACT

The germination responses of seed to a wide variety of treatments showed that both the pericarp and the embryo contribute to the dormant condition in Protea compacta. Germination can be improved to a greater or lesser extent by scarification, high oxygen tensions, stratification and applied hormones. In Leucadendron daphnoides dormancy is mainly imposed by the pericarp which apparently acts as a barrier to oxygen diffusion to the embryo. A considerable increase in germination is brought about by scarification and high oxygen tensions applied to intact seeds. Stratification and applied hormones improve germination to a lesser extent. Leaching and light treatments fail to improve germination in both species.

Attempts to characterize the major inhibitor present in seed leachates indicated that it was coumarin-like in its properties. No evidence could be found that inhibitors leached from seed were actually involved in the regulation of germination.

Poor germination in both species is apparently due to the lack of germination promoters rather than to the presence of inhibitors. Germination of seed of Leucadendron daphnoides was increased 50% by chilling at 5°C and 500% by incubation in oxygen. Both these

treatments brought about a four-fold increase in the level of butanol-soluble cytokinins. The latter apparently play the primary role in promoting germination. The greater effectiveness of high oxygen tensions in improving germination appears to be due to the additional effect oxygen has in stimulating the production of acidic gibberellin-like substances, to a level 30 times higher than with chilling. The acidic gibberellin-like substances do not appear to have a primary role in promoting germination but their effect is additive in the presence of a threshold level of butanol-soluble cytokinins. Maximum germination does not appear to depend on phasic changes in promoter levels, but on whether an increase in the level of gibberellin-like substances coincides with an increase in the levels of butanol-soluble cytokinins.

In Protea compacta the effectiveness of chilling and oxygen incubation in improving germination by approximately 50%, is apparently mediated through their effect in increasing the levels of butanol-soluble cytokinins. The latter appear to play the primary role in promoting maximum germination, which apparently does not depend on a phasic change in promoter levels.

# INTRODUCTION

The family Proteaceae comprises 60 genera and approximately 1300 species, all of which are evergreen trees or shrubs or perennial herbs. It is particularly well represented in South Africa and Australia but is also fairly widely distributed throughout the southern hemisphere in South America, Malaya, New Zealand, the Pacific islands, and in tropical Africa and Madagascar. The South African Proteaceae consist of 13 genera and well over 300 species. None of these is found in any other continent and, except for certain species in the genera Protea and Faurea, which are found as far north as Central Africa and in Madagascar, the majority are found growing wild only in the south western Cape (Levyns, 1958; Hutchinson, 1967).

Many species of Protea, Leucospermum, Serruria and Leucadendron are outstandingly effective both as garden shrubs and cut flowers. The trees or shrubs flower for long periods and the flowers, which carry and last well, are in considerable demand on the cut-flower markets both in South Africa and overseas.

One of the major obstacles to large scale cultivation of members of this family is that many are difficult to propagate. Although vegetative propagation by means of cuttings has proved successful and has a

number of advantages (Salinger, 1964; Rousseau, 1965; 1967; Topper, 1966) most plants are still raised from seed, as the latter method requires relatively unspecialized facilities. Propagation from seed, however, is complicated by the fact that germination of many species either occurs sporadically over a period of time or is poor due to a large proportion of dormant seed in seed samples (Horn, 1962; Van Staden, 1966). Much of the early literature on the propagation of species of Proteaceae by seed is of a popular nature and is not very specific as to the treatments that have been used in attempts to improve germination (Thorns, 1943; Werner, 1951; Vogts, 1960). Poor germination has been attributed to ineffective seed screening methods (Horn, 1962). However, in Horn's investigations and those of Van Staden (1966) more effective seed screening methods were used but these also gave samples with a low percentage germination. Although Horn (1962) and Watson and Parvin (1970), did not regard endogenous inhibitors as being of importance in preventing germination, Vogts (1960) maintained that poor germination was due to the presence of such inhibitors which could be leached out of the seed.

Against the background of the increasing economic importance of the protea flower industry in South Africa and the necessity to resolve the conflicting reports and

opinions regarding poor germination, it was decided to undertake an investigation, along the lines of current research into seed dormancy, on seed of Protea and Leucadendron. It was hoped that a study of a single species from each of the two largest genera might give a lead to improving germination in the family as a whole.

# CHAPTER 1

## EXPERIMENTAL PROCEDURE

### 1.1 Plant material

Seed of *Protea compacta* R.Br. and *Leucadendron daphnoides* Meisn. was obtained from the Department of Forestry, Pretoria and from Mr. M.J. Middelmann, Heuningklip Nurseries, Newlands, Cape. A relatively large proportion of the seed of these two species was known to be dormant. In addition the seeds were large in size and easily sorted. This helped to eliminate the problems of seed sorting which are encountered particularly in species with small seeds. The genus *Leucospermum* was avoided as seed sorting is particularly difficult in this genus. Atkinson (1961) showed that samples of apparently viable *Leucospermum* seed often contained a large proportion of seed with shrivelled, non-viable embryos.

The seed used was carefully hand sorted and only plump undamaged seed was used in experiments. Seed was stored in sealed containers at 5°C until used.

### 1.2 Germination procedure

Seed was germinated in petri dishes on acid-washed sand (40 - 100 mesh) which was kept at field

capacity by the addition of distilled water. In a preliminary investigation (Brown and Van Staden, 1971) it was found that temperatures above 20°C had a detrimental effect on germination. Unless otherwise stated seed was germinated in a germinator with controlled temperature and light conditions, at alternating temperatures of 10°C for 16 hours followed by 20°C for 8 hours. Light (ca.1,0 lumen/$m^2$) was supplied by cool white fluorescent tubes to coincide with the higher temperature period.

The protrusion of the radicle through the covering structures was taken as the criterion for germination. In experiments where the covering structures were removed, the geotropic curvature of the radicle and greening of the cotyledons was taken as the criterion for germination. In a preliminary trial seed was treated with 5% Kaptan fungicide to cut down fungal infection. This, however, was found to be detrimental to germination and was not repeated in later experiments.

# CHAPTER 2

## THE MODE OF GERMINATION IN PROTEA COMPACTA AND LEUCADENDRON DAPHNOIDES

In both Protea compacta and Leucadendron daphnoides the fruit is an achene. In Protea compacta it is elongated (ca. 15mm long and 7mm wide) and is covered with a dense layer of fine brown hairs (Fig. 2.1). The first visible sign of germination is the protrusion of the radicle through the hard, woody pericarp. As the cotyledons expand and become green in colour so the pericarp is split progressively and eventually shed.

In Leucadendron daphnoides the fruit is flattened with smooth sides (ca. 9mm long and 10mm wide). From the side, it appears to be almost circular in outline (Fig. 2.2). The first visible sign of germination is the splitting of the hard, woody pericarp. This splitting occurs in a very characteristic manner and always starts at the "radicle end" and extends along the edge of the flattened sides of the seed. The radicle protrudes only once the pericarp has split. This mode of germination suggests that radicle elongation is preceded by cotyledon expansion and that without the latter the radicle would not be able to emerge from within the seed coat.

In the text, for convenience, the fruits of the two species are referred to as "seeds" and the covering structures (pericarp and testa) are referred to as the "seed coat".

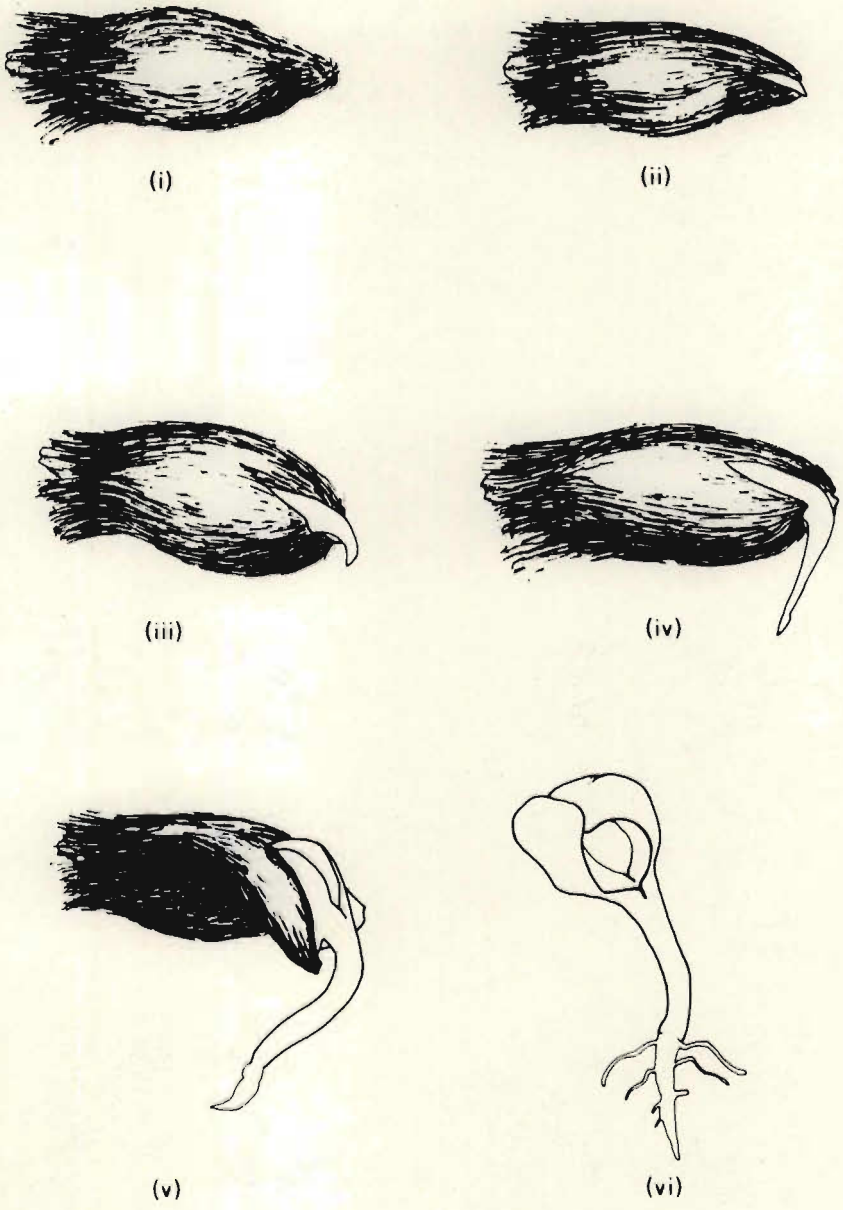



Fig. 2.1

Stages in the germination of fruits ("seeds") of Protea compacta: (i) dry seed; (ii) radicle protruding through the pericarp ("seed coat"); (iii) further growth of the radicle and splitting of coat; (iv) young seedling further splitting the coat; (v) young seedling shedding the coat; (vi) young seedling. (Magnification: x 2).

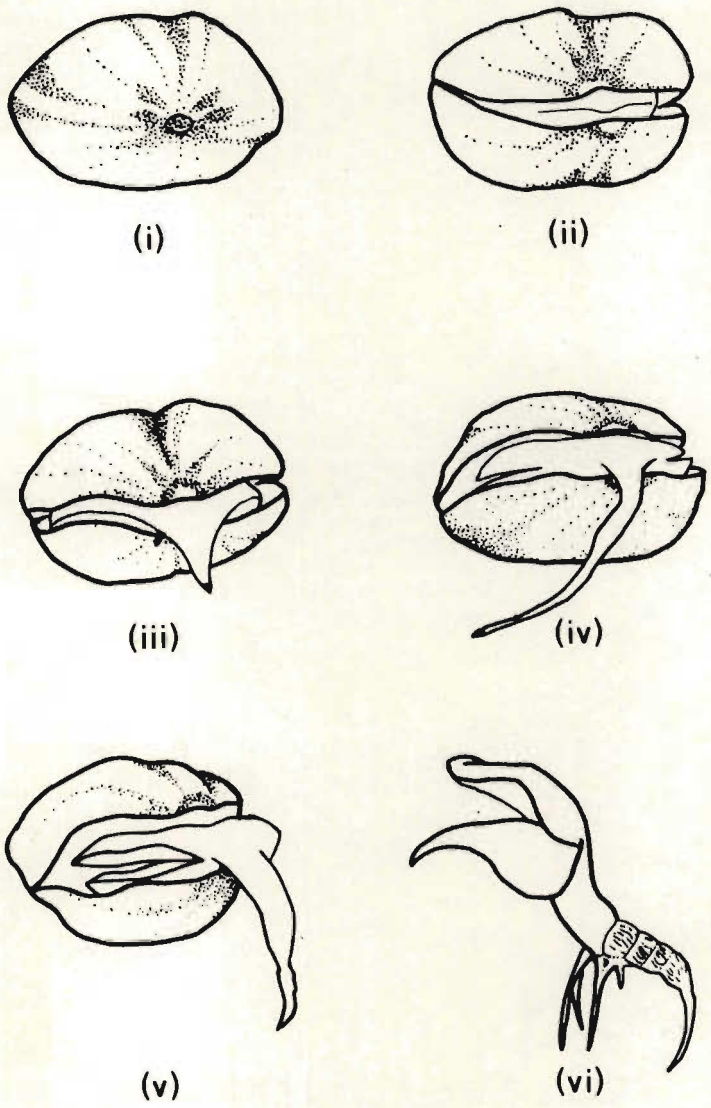



Fig. 2.2

Stages in the germination of fruits ("seeds") of Leucadendron daphnoides: (i) dry seed; (ii) the splitting of the pericarp ("seed coat") apparently as a result of cotyledon expansion; (iii) elongation and protrusion of the radicle; (iv) further growth of the radicle; (v) young seedling shedding seed coat; (vi) young seedling. (Magnification: x 3).

# CHAPTER 3

## ATTEMPTS TO IMPROVE GERMINATION

Other than a report by Horn (1962) that light and low temperature do not improve seed germination in species of Proteaceae, there are no reports in the literature of the effects of scarification, leaching, light, stratification, high oxygen tensions and applied hormones on germination. The effects of these treatments, commonly applied in germination studies to overcome dormancy, were investigated.

### 3.1 Scarification treatments

When dormancy is imposed by the covering structures of the seed their complete or partial removal often results in increased germination (Barton, 1965; Ballard, 1973). The coat may offer a mechanical restriction to radicle elongation or embryo enlargement (Barton, 1965; Esashi and Leopold, 1968; Ballard, 1973). It may exert its influence by acting as a barrier to the diffusion of oxygen and/or water (Barton, 1965; Edwards, 1968; Ballard, 1973). The covering structure may also prevent the leaching of inhibitors (Black, 1959; Webb and Wareing, 1972b). The presence of hard woody coats in the species under investigation

suggested that dormancy could be coat-imposed.

Seed was mechanically scarified by filing away small portions of the coat. The treatments were as follows:

(i) coat scarified at the "radicle end";

(ii) coat scarified at the "style end";

(iii) coat scarified midway on the side of the seed, and germinated with the scarified surface facing upwards; and

(iv) the seed coat completely removed (embryo excised).

Table 3.1

THE EFFECT OF SCARIFICATION OF THE SEED COAT ON GERMINATION OF PROTEA COMPACTA AND LEUCADENDRON DAPHNOIDES

Figures are mean percentages of 10 replications of 10 seeds over 40 days.

| SCARIFICATION TREATMENTS | PROTEA COMPACTA | LEUCADENDRON DAPHNOIDES |
|---|---|---|
| Intact seed | 50 ± 5 | 30 ± 5 |
| Scarified at "style end" | 78 ± 5 | 90 ± 5 |
| Scarified at "radicle end" | 80 ± 3 | 75 ± 6 |
| Scarified on side between "style" and "radicle ends" | 45 ± 3 | 88 ± 2 |
| Excised embryos | 30 ± 8 | 30 ± 0 |

The results in Table 3.1 show that while scarification on the side of the seed had no effect on the germination of Protea compacta, germination was significantly increased when portions of the coat at either end of the seed were removed. The latter treatments either weaken or remove the mechanical effect of the seed coat. When the coat was weakened by scarifying at the "style end", "abnormal" germination, where the extending radicle pushed the cotyledons out of the covering structures, frequently occurred. Scarification on the side of the seed did not improve germination, possibly because the mechanical forces exerted by the coat were not altered sufficiently to allow the embryo to develop. These results suggest that the dormant condition of Protea compacta is, at least in part, brought about by the mechanical restriction of the embryo by the seed coat. Where germination does not occur it is possible that the radicle is unable to develop sufficiently to rupture the coat. If this were the case it might be expected that complete removal of the seed coat would result in maximal germination. However, few excised embryos germinated, as exposure to the atmosphere made them particularly subject to microbial attack. The finding that few excised embryos of Protea compacta germinate under sterile conditions in vitro suggests that embryo dormancy also contributes

to the dormant condition (Van Staden, Brown and Button, 1972a).

In contrast to Protea compacta, all scarification treatments resulted in significant increases in the germination of Leucadendron daphnoides (Table 3.1). In the latter species the mode of germination did not deviate from that characteristic for intact seed, irrespective of the site of scarification. Furthermore scarification at the "radicle end" of the seed did not appear to enhance radicle emergence from within the coat. This suggests that the events leading to germination follow a set pattern. The first visible sign of germination is the splitting of the seed coat, apparently as a result of cotyledon expansion and this is followed by radicle elongation (Fig. 2.2).

To obtain information about the mechanism by which the seed coat imposes dormancy on the embryo of Leucadendron daphnoides, scarified seed was germinated in two positions. When the scarified surface was placed on the sand, only 30% germination was recorded. However, 80% germination was obtained with the scarified surface uppermost. These results suggest that exposure to the atmosphere is beneficial in increasing germination. This was tested by covering the exposed surface with lanolin, a procedure which resulted in a reduction of germination to 50%. These results suggest that the seed

coat of Leucadendron daphnoides acts as a barrier to oxygen uptake. In addition it is significant that when the scarified surface was placed downwards on the sand, a treatment that would favour leaching of compounds from the embryo, germination was reduced to a level equal to that of the controls.

Seed of Protea compacta was also scarified on the side and then germinated in a variety of positions. Seed placed with the scarified surface on the sand gave germination results identical to those of the unscarified control. Similar germination results were obtained where the scarified surface faced upwards and was covered with lanolin. In a similar treatment, but without the lanolin covering, germination was depressed to below that of the control, apparently as a result of severe microbial attack. This suggests that the coat also has a protective function and prevents microbial attack of the embryo. In the same experiment seeds scarified at the radicle end gave germination 30% higher than the untreated control, again suggesting that the coat plays a predominantly mechanical role in preventing the protrusion of the radicle.

Microbial attack is probably responsible for the low levels of germination observed in excised embryos. This is suggested by the fact that embryos that germinated did so within the first twelve days of

each experiment, during which time microbial activity was not very marked.

The results of the scarification experiments lead to the conclusion that dormancy of Protea compacta is apparently partly a result of the mechanical restriction of radicle elongation by the seed coat and partly due to embryo dormancy. In Leucadendron daphnoides however, dormancy appears to be mainly coat-imposed. Here the coat apparently exerts its influence by restricting gaseous diffusion to the embryo. Further experiments were carried out to verify these findings (3.4).

## 3.2 Leaching treatments

Coat-imposed dormancy may be due to the presence of inhibitors in the coat (Bradbeer, 1968; Irving, 1968) or alternatively their outward diffusion from the embryo may be impeded (Black, 1959; Webb and Wareing, 1972a). Although Vogts (1960) postulated that inhibitors are involved in the regulation of germination of proteaceous seed, their actual involvement in imposing dormancy has not been shown.

The possible removal of inhibitors by leaching and its subsequent effect on germination (if any) was investigated by the following procedures: Seed was shaken in 100ml distilled water for 4, 8 and 12 days,

respectively. During this time the water was renewed every two days. The bulked leachates were concentrated to 100ml, their pH adjusted to 2,0 and then extracted with ether. The ether extracts were strip-loaded onto Whatman No. 1 chromatography paper and separated by descending chromatography in iso-propanol:ammonia: water (10:1:1 v/v). The chromatograms were dried, cut into ten equal strips and assayed for inhibitors using the lettuce seed germination bioassay (See 4.1.5 for details).

Inhibiting substances were removed from intact seeds by leaching (Fig. 3.1). The inhibition produced by leachates increased up to eight days, whereafter it remained more or less constant. Most of the inhibitor activity occurred between $R_f$ 0,8 and 1,0. In spite of this evidence which indicated that inhibitors are removed by leaching, the treatment did not increase germination in either species (Table 3.2). Although this suggests that the inhibitors may not be active in regulating germination, the low germinability may be the result of other factors related to the treatment, such as oxygen stress.

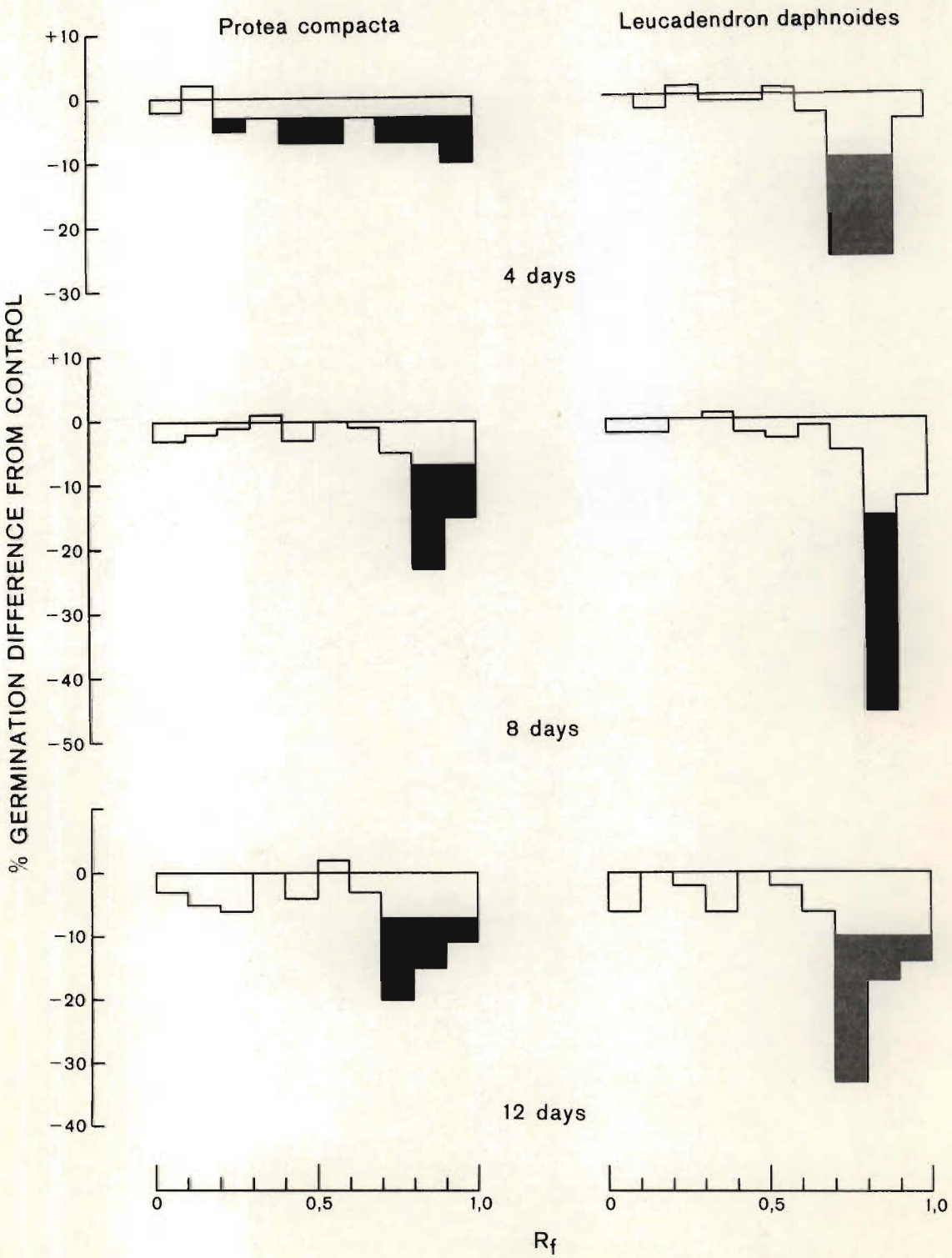



Fig. 3.1

Inhibitors from seed leachates of Protea compacta and Leucadendron daphnoides. Leachates were chromatographed on paper in iso-propanol:ammonia:water (10:1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level.

Table 3.2

THE EFFECT ON GERMINATION OF *PROTEA COMPACTA* AND *LEUCADENDRON DAPHNOIDES* OF LEACHING SEEDS BY SOAKING IN DISTILLED WATER

Figures are mean percentages of 8 replications of 25 seeds over 63 days.

| TIME (DAYS) | *PROTEA COMPACTA* | *LEUCADENDRON DAPHNOIDES* |
|---|---|---|
| 0 | 49 ± 11 | 27 ± 1 |
| 4 | 6 ± 1 | 11 ± 1 |
| 8 | 2 ± 1 | 4 ± 1 |
| 12 | 0 | 7 ± 1 |

In order to eliminate inadequate aeration, seed was leached intermittently (12 hours each day) in running water for 4, 8 and 12 days respectively. Care was taken that they never dried out. Under these conditions it was considered that seeds would receive sufficient aeration for germination. However, subsequent germination showed the same pattern as seed that had been submerged in water (Table 3.3).

Table 3.3

THE EFFECT ON GERMINATION OF PROTEA COMPACTA AND LEUCADENDRON DAPHNOIDES OF INTERMITTENT LEACHING OF SEED IN RUNNING WATER

Figures are mean percentages of 8 replications of 25 seeds over 63 days.

| TIME (DAYS) | PROTEA COMPACTA | LEUCADENDRON DAPHNOIDES |
|---|---|---|
| 0 | 45 ± 8 | 26 ± 4 |
| 4 | 20 ± 4 | 9 ± 2 |
| 8 | 17 ± 4 | 6 ± 1 |
| 12 | 9 ± 2 | 9 ± 2 |

If the dormant state of the seed is the result of high inhibitor concentrations in the embryos and if the inhibitors cannot be leached out through the seed coat, then scarification and placement of the seed in a position to favour leaching would be expected to increase germination. Such a treatment applied to seed of both species gave germination results no different to unscarified controls. According to the evidence obtained it would appear as if the seed coats did not exert an influence on germination by impeding the leaching of inhibitors. If inhibitors play a role in the control of germination it is apparently not an over-riding effect.

The fact that the germination of both species is progressively inhibited with increasingly high levels of hydration (Tables 3.2 and 3.3) could be due to the phenomenon of "water-sensitivity". Here a state of dormancy is induced by an over-abundant supply of moisture (Hay, 1962). According to Hay (1962) and Negbi, Rushkin and Koller (1966) this inhibition of germination is due to the resistance of the water layers surrounding the embryo to diffusion of oxygen into the embryo. Such an argument would be consistent with the reports by Vogts (1960) that for maximum germination, seeds should be watered in such a way that the water washes through the rooting medium and the seeds are not submerged in water for any length of time.

## 3.3 Stratification treatments

In Protea compacta germination is apparently only partly regulated by the seed coat, as Van Staden, et al. (1972a) have shown that a large proportion of excised embryos failed to germinate when cultivated under sterile conditions in vitro. In Leucadendron daphnoides dormancy appears to be wholly coat-imposed. It is well known that both embryo dormancy and coat-imposed dormancy may be overcome by a period of storage under moist conditions at low temperature (Wareing and Phillips, 1970; Wareing and Saunders, 1971). The

effect of stratification on the germination of both species was investigated by imbibing seed on moist filter paper for 24 hours and then incubating them at 5°C and 25°C for 0, 30, 60 and 90 days respectively. After incubation the seed was germinated under standard conditions (1.2).

Table 3.4

THE EFFECT ON GERMINATION OF PROTEA COMPACTA AND LEUCADENDRON DAPHNOIDES OF INCUBATION OF SEED UNDER MOIST CONDITIONS AT 5°C AND 25°C

Figures are percentages of 250 seeds

| TEMPERATURE | TIME (DAYS) | PROTEA COMPACTA | LEUCADENDRON DAPHNOIDES |
|---|---|---|---|
| | 0 | 46 | 20 |
| 5°C | 30 | 59 | 30 |
| | 60 | 59 | 23 |
| | 90 | 65 | 18 |
| 25°C | 30 | 3 | 5 |
| | 60 | 0 | 3 |
| | 90 | 0 | 0 |

Incubation of seed at 25°C depressed germination very markedly (Table 3.4). Stratification of seed of both species at 5°C for 30 days increased germination. However, longer periods of stratification

did not improve germination further in Protea compacta and had a depressing effect on germination in Leucadendron daphnoides. A considerable proportion of Protea compacta seed germinated at 5°C during the 60 day and 90 day chilling periods. Stratification apparently results in certain changes occurring in the seeds which result in an increased ability of the cotyledons to split the coat in Leucadendron daphnoides and the radicle to pierce the coat in Protea compacta.

## 3.4 Oxygen treatments

The effect of the seed coat on the free passage of oxygen was investigated by germinating seeds of both species in air, oxygen and nitrogen. The seed was placed on moist sand in 500ml flasks. The flasks were flushed with the appropriate gas, care being taken to ensure that atmospheric pressure was not exceeded in the flasks. At two day intervals the gas was renewed and the germinated seed removed.

Table 3.5

THE EFFECT ON GERMINATION OF PROTEA COMPACTA AND LEUCADENDRON DAPHNOIDES OF INCUBATING SEED IN AIR, OXYGEN AND NITROGEN

Figures are mean percentages of 5 replications of 20 seeds.

| TREATMENT | PROTEA COMPACTA | LEUCADENDRON DAPHNOIDES |
|---|---|---|
| Air | 47 ± 17 | 12 ± 7 |
| Oxygen | 72 ± 7 | 85 ± 14 |
| Nitrogen | 0 | 0 |

Oxygen increased germination (Table 3.5), the effect being more marked in Leucadendron daphnoides than in Protea compacta. No germination was recorded in nitrogen and when after 44 days, the nitrogen was replaced by oxygen, 55% of the seeds of Leucadendron daphnoides germinated, indicating that they had not suffered adversely in the absence of oxygen. Similar treatment of Protea compacta seed, however, did not result in any germination. This difference in response to oxygen, after nitrogen treatment, may be related to fundamental differences in the metabolism of seed of the two species. Differences have been reported in the response of excised embryos of Betula and Fraxinus to incubation in nitrogen. Whereas embryos of Betula

germinated in nitrogen (Black and Wareing, 1959), those of Fraxinus excelsior did not (Smith and Villiers, 1973). After 25 days in nitrogen embryos of Fraxinus excelsior were no longer viable if returned to air to germinate. This was thought to be due in part to a loss of membrane integrity resulting in the confluence of lipid droplets and the lysis of the cytoplasm by hydrolytic enzymes released on the rupture of the vacuolar membranes.

The results in Table 3.5 clearly suggest that in Protea compacta and Leucadendron daphnoides the seed coat may act as a barrier to gaseous diffusion and hence impose dormancy. It also seems most likely that the deleterious effect on germination of prolonged soaking (Tables 3.2 and 3.3) is due to the resistance of the water layers surrounding the embryo to the diffusion of oxygen.

## 3.5 Applied hormones

Growth promoters such as kinetin and gibberellic acid can substitute for the chilling requirement of certain seeds (Webb and Dumbroff, 1969; Baskin and Baskin, 1970). In order to determine whether they can bring about a similar effect in the seed of Protea compacta and Leucadendron daphnoides, seed was soaked for 24 hours in each of a range of solutions of benzyladenine (BA) and kinetin (1,10,50 mg/l), and gibberellic acid ($GA_3$) (1,10,100 mg/l).

Table 3.6

THE EFFECT ON GERMINATION OF *PROTEA COMPACTA* AND *LEUCADENDRON DAPHNOIDES* OF SOAKING SEED IN KINETIN, BENZYLADENINE AND GIBBERELLIC ACID

Figures are mean percentages of 5 replications of 10 seeds after 72 days.

| CHEMICAL | CONCENTRATION | *PROTEA COMPACTA* | *LEUCADENDRON DAPHNOIDES* |
|---|---|---|---|
| | Control | 57 ± 3 | 20 ± 2 |
| Kinetin | 1 mg/l | 67 ± 5 | 13 ± 1 |
| | 10 mg/l | 78 ± 1 | 33 ± 4 |
| | 50 mg/l | 58 ± 3 | 30 ± 2 |
| Benzyladenine | 1 mg/l | 72 ± 3 | 35 ± 3 |
| | 10 mg/l | 54 ± 5 | 20 ± 2 |
| | 50 mg/l | 76 ± 2 | 10 ± 1 |
| Gibberellic acid | 1 mg/l | 62 ± 3 | 22 ± 2 |
| | 10 mg/l | 68 ± 1 | 30 ± 2 |
| | 100 mg/l | 66 ± 1 | 23 ± 3 |

Although germination in both species can be increased by treatment with the three growth promoters, the species differ in their responses (Table 3.6). In *Protea compacta* the largest response was obtained in kinetin (10 mg/l), BA (50 mg/l) and $GA_3$ (10 mg/l), whereas in *Leucadendron daphnoides* kinetin (10 mg/l), BA (1 mg/l) and $GA_3$ (10 mg/l) gave the largest responses. These results agree with those obtained by Van Staden

*et al.* (1972a) with excised embryos cultured under sterile conditions *in vitro*.

The effect of the applied growth promoters may be to promote radicle extension in *Protea compacta*, as has been shown to occur in lettuce by Haber and Luippold (1960) and in *Acer pseudoplatanus* by Pinfield and Stobart (1972). In *Leucadendron daphnoides* the effect may be to promote cotyledon expansion as found by Ikuma and Thimann (1963) in lettuce; Kursanov, Kulaeva and Mikulovich (1969) in pumpkin, Esashi and Leopold (1969a) in *Xanthium* and Kumar and Sastry (1973) in cucumber.

## 3.6 Light treatments

Seeds of both species, which had been imbibed in the dark for 24 hours, were subjected to red and far-red light treatments. The results (Table 3.7) supported the contention of Horn (1962) that light does not influence the germination of proteaceous seed.

Table 3.7

GERMINATION OF SEED OF *PROTEA COMPACTA* AND *LEUCADENDRON DAPHNOIDES* AFTER TREATMENT WITH RED AND FAR-RED LIGHT

Figures are mean percentages of 10 replications of 10 seeds over 48 days.

| LIGHT TREATMENT | *PROTEA COMPACTA* | *LEUCADENDRON DAPHNOIDES* |
|---|---|---|
| Red light (2 hours) | 56 ± 3 | 15 ± 2 |
| Far-red light (2 hours) | 59 ± 5 | 13 ± 1 |
| Red light (2 hours) and far-red light (2 hours) | 56 ± 1 | 13 ± 2 |
| Dark | 60 ± 2 | 15 ± 2 |

## 3.7 Discussion

The germination responses of seed to a wide variety of treatments show that both the coats and the embryos apparently contribute to the dormant condition in *Protea compacta*, while in *Leucadendron daphnoides* dormancy is apparently coat-imposed.

Although inhibitors are leached from intact seed, no evidence could be found that they are actually involved in the regulation of germination, or that their leaching is a pre-requisite for germination. In addition the covering structures do not appear to restrict the leaching of such compounds from the embryo.

In Protea compacta the seed coat appears to mechanically restrict radicle elongation, and to a lesser extent, to retard oxygen uptake. These effects are overcome to a greater or lesser degree by scarification, high oxygen tensions and stratification. The effect of stratification may be due to physical changes in the covering structures, enabling the radicle to emerge more readily, or to chemical changes in the embryo enabling the radicle to develop sufficient thrust to pierce the coat. Wareing and Saunders (1971) considered it unlikely that the loss of dormancy after stratification could be due to any direct changes in the properties of seed coats. Boucaud and Ungar (1973), however, maintained that cold treatment promoted germination in Suaeda spp., chiefly by bringing about a change in the mechanical resistance offered by the testa to the development of the embryo.

In Leucadendron daphnoides the seed coat imposes dormancy by acting as a barrier to oxygen diffusion to the embryo. Scarification treatments which broke that barrier, as well as high oxygen tensions applied to intact seeds resulted in dramatic increases in germination.

The poor germination obtained in both species after prolonged soaking and leaching may be due to an increased barrier to oxygen diffusion to the embryo brought about by an over-abundance of water. The effect

of stratification in improving germination may be an indirect one brought about by the influence of low temperature on oxygen diffusion to the embryo. Wareing (1969) has stressed that for stratification to be successful, seeds must be fully imbibed and have adequate aeration. Côme and Tissaoui (1973) have pointed out that at low temperatures oxygen is more soluble in water and more reaches the embryo, which also has a lower oxygen requirement for germination. The combination of these two factors apparently leads to increased metabolic activity resulting in changes in hormone levels that promote radicle elongation in Protea compacta and cotyledon expansion in Leucadendron daphnoides. Both these growth phenomena have previously been shown to be affected by the application of exogenous growth promoters (Ikuma and Thimann, 1963; Pinfield and Stobart, 1972) while in the present study exogenous gibberellic acid, kinetin and benzyladenine have been shown to increase germination.

In order to follow up the results of the preliminary seed treatments it was decided to look more closely at the nature of the inhibitors removed by leaching. It was also decided to investigate whether the increase in germination brought about as a result of stratification and high oxygen tensions could be mediated by changes in endogenous hormone levels.

# CHAPTER 4

## CHARACTERIZATION OF GERMINATION INHIBITORS

Water leachates of seed of both species have been shown to inhibit germination in the lettuce seed germination bioassay (Fig. 3.1). It has also been shown (Brown and Van Staden, 1971) that the inhibition produced by leachates was more than could be accounted for on the basis of their osmotic pressure alone. Using relatively simple techniques an attempt was made to characterize the inhibiting substances. The inhibiting effects of seed extracts were compared with inhibition produced by two known germination inhibitors viz., coumarin and abscisic acid (ABA). Coumarin has been reported by Evenari (1949, 1961), Wareing (1965), Ketring (1973) and Valio (1973) to be of wide occurrence in plant tissue and to be one of the most potent of the naturally occurring germination inhibitors. Since the discovery of ABA by Okhuma, Smith, Lyon and Addicott (1963), it has been postulated that it plays a role in the dormancy of seed of a number of species e.g. peach (Lipe and Crane, 1966); rice (Dey and Sircar, 1968) and apple (Rudnicki, 1969).

## 4.1 Extraction and bioassay of germination inhibitors

### 4.1.1 Aqueous extracts

Fifteen grammes of seed was divided into seed coats and embryos and each component (unground) was shaken in 100 ml distilled water for 12 hours at room temperature. The leachate was concentrated to dryness in a rotary flash evaporator under reduced pressure at 40°C. The residue was taken up in one ml distilled water and 500 µl of this was immediately strip-loaded onto Whatman No. 1 chromatography paper.

### 4.1.2 Ether extracts

The method of extraction was based on that of Eagles and Wareing (1964) and Irving and Lanphear (1968). Fifteen grammes of seed was separated into coats and embryos; each was homogenized in a blendor at room temperature in 80% aqueous methanol. The fractions were kept overnight in a refrigerator at 4°C and then filtered through Whatman No. 42 filter paper. The fractions were concentrated at 30°C under reduced pressures in order to remove the methanol. The aqueous fraction was acidified to pH 2,0 with 5% $H_2SO_4$ and then extracted six times with 50 ml ether. The ether fraction was reduced to dryness under reduced pressure at 40°C. The residue was redissolved in six ml dry ether and

immediately strip-loaded onto Whatman No. 1 chromatography paper.

4.1.3 Ethyl acetate extracts

The extraction technique was similar to that used by Davis, Heinz and Addicott (1968) for the extraction of ABA. Fifteen grammes seed was separated into coats and embryos. Each was homogenized in 100 ml 80% acetone, kept in a refrigerator at 4°C overnight and then filtered through Whatman No. 42 filter paper. The extracts were then evaporated to an aqueous residue, acidified to pH 2,0 with HCl and extracted twice with equal amounts of 5% $NaHCO_3$. The pH of the combined sodium bicarbonate fractions was adjusted to 2,0 and it was extracted twice with equal volumes of ethyl acetate. After being reduced to dryness the residue was taken up in four ml ethyl acetate and immediately strip-loaded onto Whatman No. 1 chromatography paper.

4.1.4 Chromatographic separation of extracts

All extracts were separated at room temperature using descending paper chromatography. The concentrated extracts and the standards were strip-loaded onto Whatman No. 1 paper. The chromatograms were equilibrated for four hours prior to development. After the solvent front had travelled about 30 cm, the chromatograms were

dried and cut into ten equal transverse strips. The biological activity of each strip was determined in the lettuce seed germination bioassay.

### 4.1.5 Lettuce seed germination bioassay

The techniques used in this bioassay were similar to those described by Sankhla and Sankhla (1968). Each chromatogram strip, representing a separate $R_f$ value, was cut up and placed in a petri dish and three ml distilled water was added. One hundred seeds of lettuce (var. Grand Rapids) were then scattered on the paper in each petri dish and these were placed in the dark for two hours. The lettuce seeds were subsequently exposed to cool white fluorescent light, with an intensity of 3,0 x 10 lumen/$m^2$, for 30 minutes. After illumination, the seeds were incubated in the dark in a growth cabinet maintained at 26°C. The percentage germination was recorded after 48 hours.

Each extraction and bioassay was carried out at least twice.

## 4.2 Results and Discussion

### 4.2.1 Chromatographic separation of aqueous extracts

Figure 4.1 shows the lettuce seed bioassay results after the separation of aqueous extracts in

iso-propanol:ammonia:water (10:1:1 v/v). The seed coat and embryo extracts showed a band of inhibition corresponding to $R_f$ 0,9 - 1,0. The coumarin standard alone gave a band of inhibition corresponding to $R_f$ 0,9 - 1,0 and the ABA standard alone gave inhibition at $R_f$ 0,8 - 0,9. [(RS) - abscisic acid by courtesy of R.J. Reynolds Tobacco Co., North Carolina, U.S.A.] A mixture of the two standards gave inhibition at $R_f$ 0,8 - 0,9. Thus in this solvent system the inhibitory effects of ABA and coumarin could not be distinguished and the band of inhibition showed by seed extracts corresponded to both.

Aqueous extracts were subsequently separated in n-butanol:ammonia:water (200:6:36 v/v). In this solvent system ABA separated out at $R_f$ 0,6 (Davison, 1965; Bowen and Hoad, 1968) and coumarin at $R_f$ 0,9 (Swain, 1953). The results (Fig. 4.2) show that in addition to the inhibition obtained at $R_f$ 0,9 - 1,0, the seed coat extract of Protea compacta gave a second band of inhibition corresponding to $R_f$ 0,6. These results suggested that the major inhibitor of germination in the bioassays was a compound or compounds with a $R_f$ similar to that of coumarin. The second band of inhibition obtained from the seed coat of Protea compacta had an $R_f$ value corresponding to that of ABA.

In a third experiment aqueous extracts were separated in ethyl acetate:2N ammonia (1:1 v/v). In

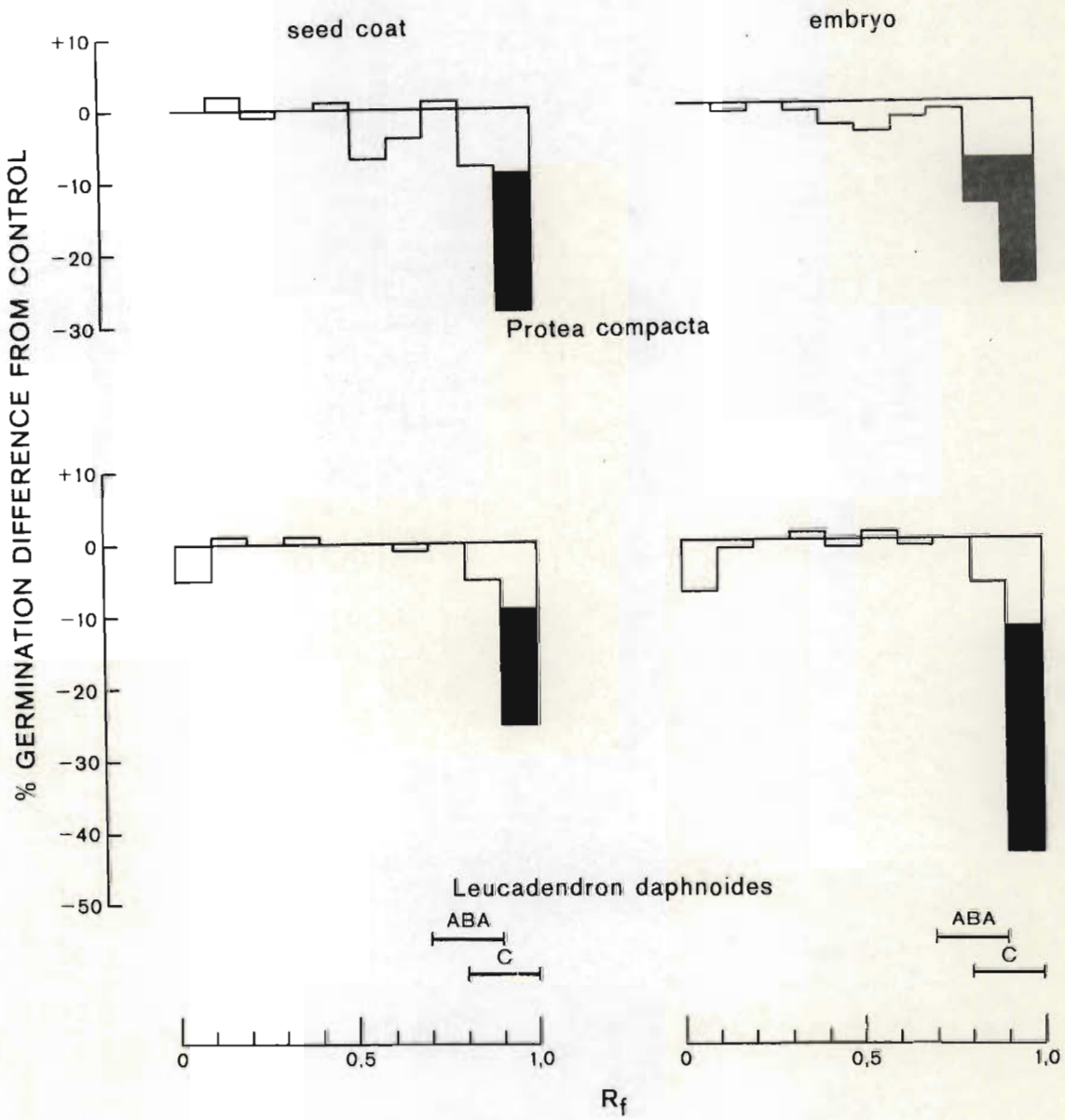



Fig. 4.1

Inhibitors from aqueous extracts of seed coats and embryos of Protea compacta and Leucadendron daphnoides. Extracts were chromatographed on paper in iso-propanol:ammonia:water (10:1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level. ABA = Abscisic acid. C = Coumarin.

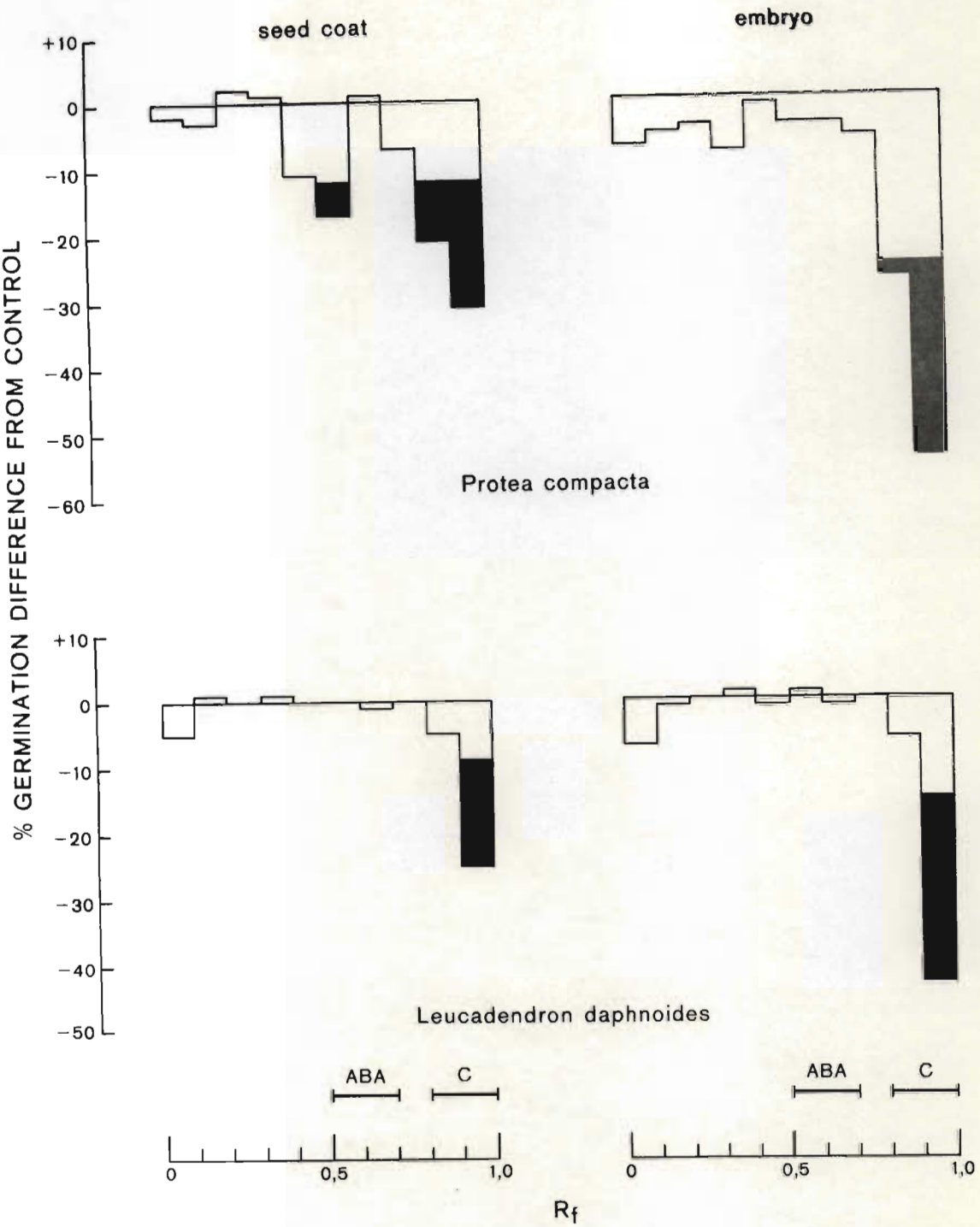



Fig. 4.2

Inhibitors from aqueous extracts of seed coats and embryos of Protea compacta and Leucadendron daphnoides. Extracts were chromatographed on paper in n-butanol:ammonia:water (200:6:36 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level. ABA = Abscisic acid. C = Coumarin.

this solvent system ABA separated out only very slowly and gave a band of inhibition at $R_f$ 0,1. The coumarin standard on the other hand, inhibited germination at $R_f$ 1,0. All the seed extracts gave a band of inhibition at $R_f$ 1,0 (Fig. 4.3). These results indicated that the major inhibitor present in the seed extracts behaved physically in a manner similar to coumarin.

### 4.2.2 Chromatographic separation of ether extracts

A problem encountered in chromatographing the aqueous extracts was that the large number of water-soluble substances present interfered with the efficient separation of the constituents of the extracts. In some cases poor separation was partially overcome by loading smaller amounts of extract on a number of sheets of chromatography paper and then combining corresponding $R_f$ strips in the bioassay. A more satisfactory solution to the problem was to use different techniques for extracting the seeds. Aqueous extracts were acidified and partitioned against ether in a technique based on those of Eagles and Wareing (1964) and Irving and Lanphear (1968) and described earlier (4.1.2). The ether extracts were separated in n-butanol:ammonia:water (Fig. 4.4). The seed extracts and the coumarin standard inhibited germination at $R_f$ 0,9 - 1,0.

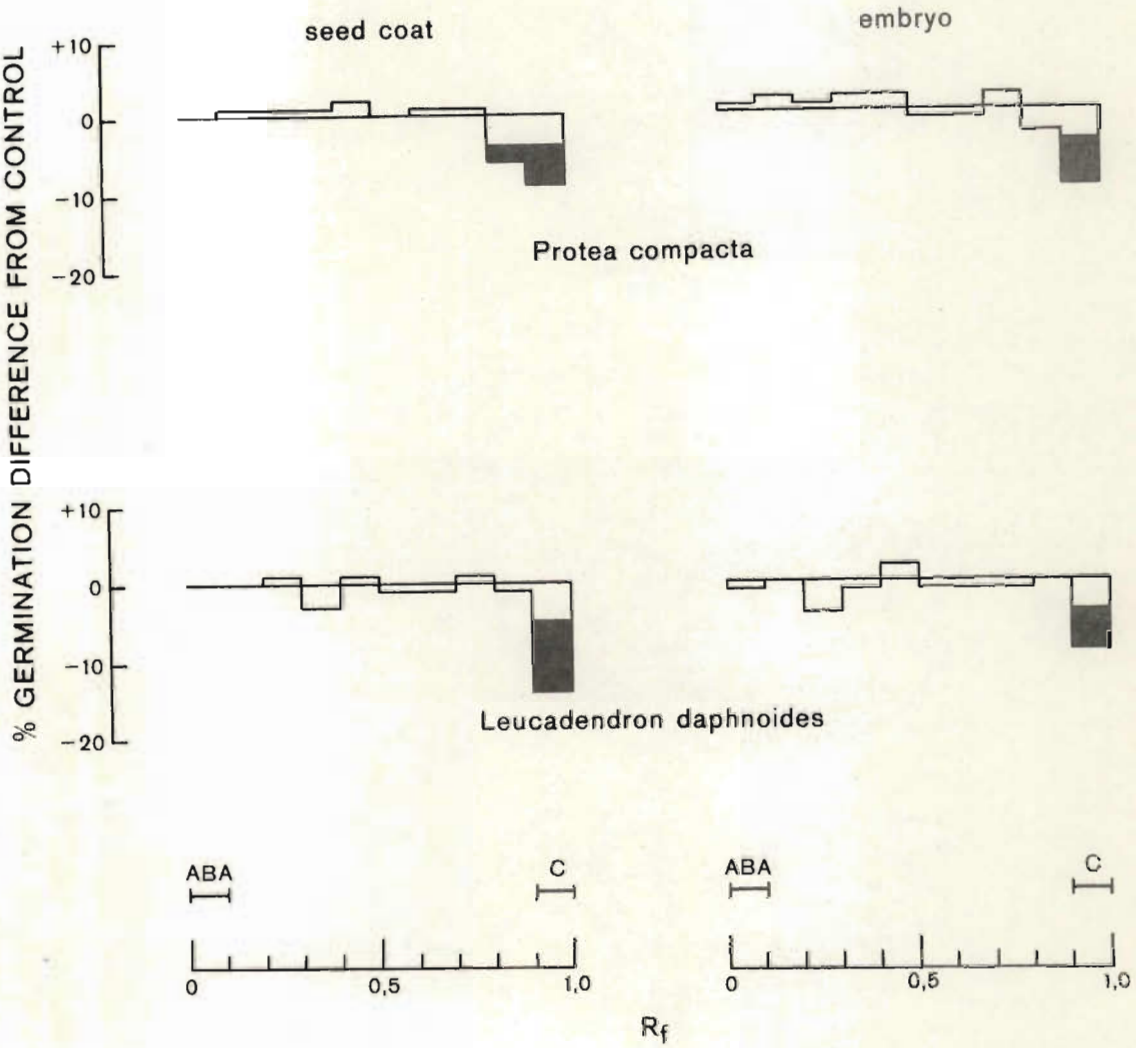



Fig. 4.3

Inhibitors from aqueous extracts of seed coats and embryos of Protea compacta and Leucadendron daphnoides. Extracts were chromatographed on paper in the organic phase of ethyl acetate:2N ammonia (1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level. ABA = Abscisic acid. C = Coumarin.

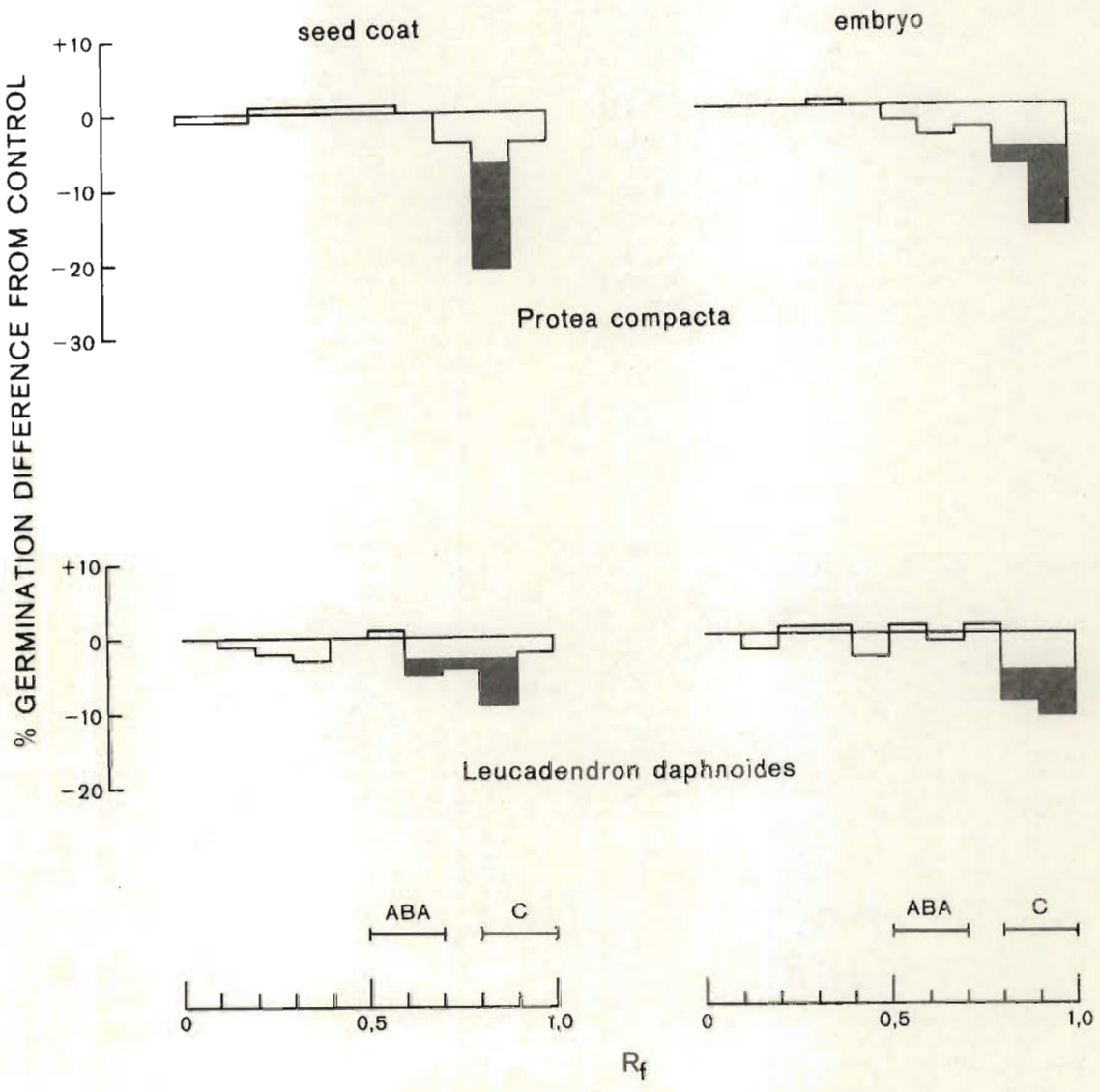



Fig. 4.4

Inhibitors from acidic ether extracts of seed coats and embryos of Protea compacta and Leucadendron daphnoides. Extracts were chromatographed on paper in n-butanol:ammonia:water (200:6:36 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level. ABA = Abscisic acid. C = Coumarin.

4.2.3 Chromatographic separation of ethyl acetate extracts

Aqueous extracts of the seed coat of Protea compacta gave inhibition in the bioassay in the region of $R_f$ 0,5, a position which corresponded with that of the ABA standard (Fig. 4.2). In an attempt to determine whether ABA was in fact present, an extraction technique used by Davis et al. (1968) specifically for ABA, was used. Seeds were extracted using ethyl acetate (4.1.3) and the extracts separated in n-butanol:ammonia:water. The bioassay results (Fig. 4.5A) show that the seed extracts of both species gave significant inhibition at $R_f$ 0,9 or 1,0. The coumarin standard gave a band of inhibition at $R_f$ 0,9 - 1,0. It was interesting to note that although this extraction technique was not the most suitable for extracting coumarin (as the latter is not very soluble in acetone or in ethyl acetate) a compound(s) with chromatographic properties similar to coumarin was extracted in sufficient quantity to show significant inhibition in the bioassay.

The seed coat extracts of Leucadendron daphnoides were the only extracts to show significant inhibition (1% level) in the region $R_f$ 0,5 - 0,6 which broadly corresponds with the ABA control. These results indicate that if ABA is present at all, it is present only in very small quantities.

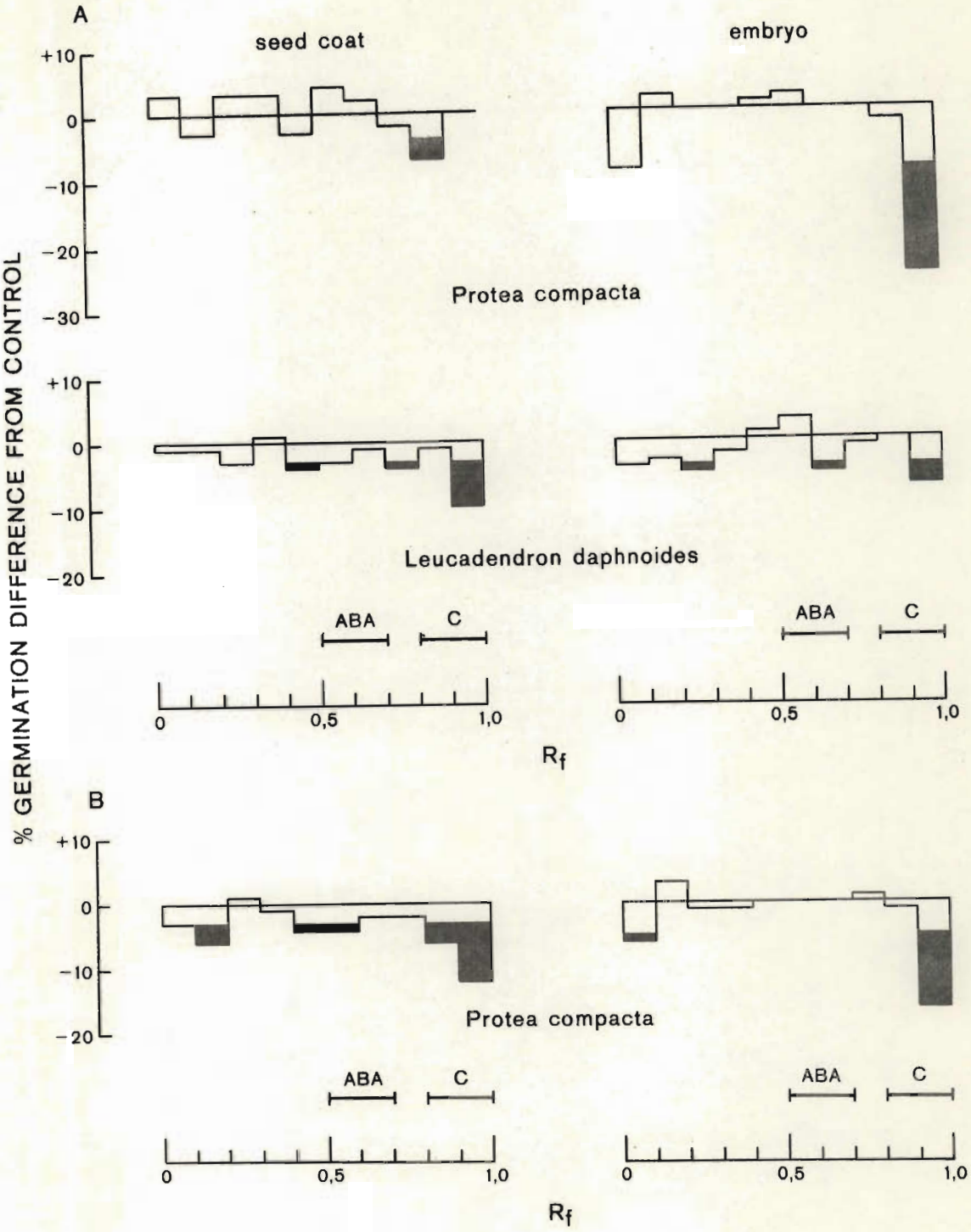



Fig. 4.5

Inhibitors from acidic ethyl acetate extracts of seed coats and embryos of Protea compacta and Leucadendron daphnoides. Extracts were chromatographed on paper in n-butanol:ammonia: water (200:6:36 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. A: extracts from 15 g seed. B: extracts from 90 g seed. Shaded areas represent differences significant from water controls at the 1% level. ABA = Abscisic acid. C = Coumarin.

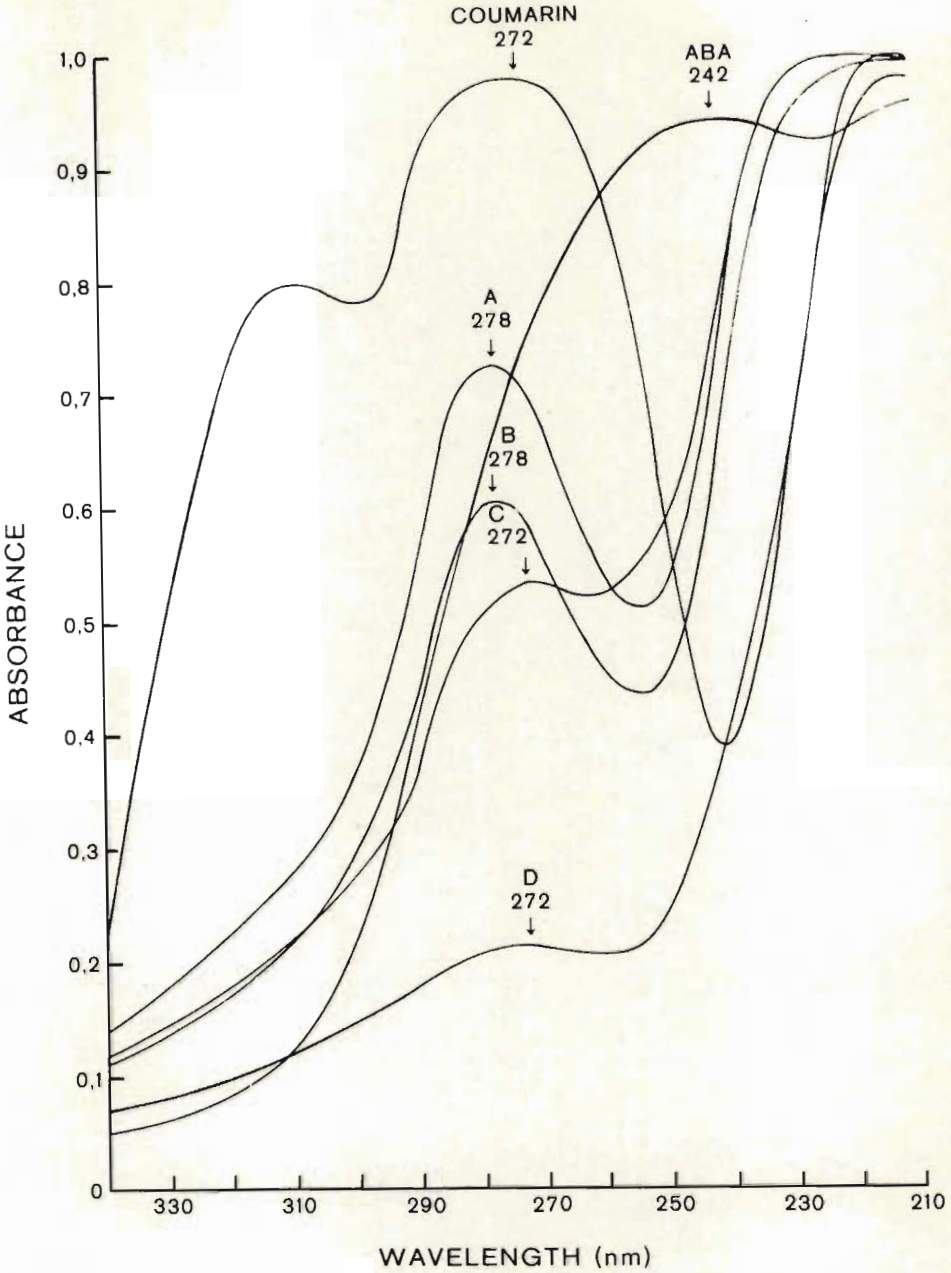



Fig. 4.6

A comparison of UV absorption spectra of Abscisic acid (ABA) and Coumarin with those of inhibitors from acidic ethyl acetate extracts of Protea compacta. Extracts were separated on paper in n-butanol:ammonia:water (200:6:36 v/v).

A = coat ($R_f$ 0,5) B = coat ($R_f$ 0,6)

C = coat ($R_f$ 1,0) D = embryo ($R_f$ 1,0)

In an attempt to eliminate the concentration factor, 90 grammes seed (i.e., six times the weight of seed used in earlier extractions) was extracted using ethyl acetate (4.1.3). As seed supplies were limited only Protea compacta was investigated. The ethyl acetate extracts were separated in n-butanol:ammonia: water, the chromatogram cut into ten equal strips and each strip was eluted in 80% methanol for 24 hours. The pattern of absorption of each fraction of the seed coat and embryo extracts under UV light was then recorded in a Zeiss DMR 21 Spectrophotometer. The results (Fig. 4.6) show the absorption spectra of coumarin and ABA standards together with the chromatographed fractions of seed coat and embryo extracts. Abscisic acid shows peak absorption at 242nm and coumarin at 272nm. The inhibitor in the seed coat and embryo extracts at $R_f$ 1,0 (Fig. 4.5B) shows a peak similar to coumarin at 272nm. The inhibitor from the seed coat at $R_f$ 0,5 - 0,6 (Fig. 4.5B) has peak absorption at 278nm. This inhibitor thus appears not to be ABA.

### 4.2.4 Spray reagents specific for coumarin

In order to obtain more information about the inhibiting compound with properties similar to coumarin, acidic ether extracts of seed of both species were chromatographed in three different solvent systems and

the chromatograms were then treated with a number of spray reagents. Each reagent gave a characteristic colour reaction in the presence of coumarin (Swain, 1953). The mean $R_f$ values for the inhibiting compound and the coumarin standard in the three solvent systems are given in Table 4.1.

Table 4.1

A COMPARISON OF THE $R_f$ VALUES OF COUMARIN AND THE MAJOR INHIBITING COMPOUND IN ACIDIC ETHER EXTRACTS OF PROTEA COMPACTA (P) AND LEUCADENDRON DAPHNOIDES (L), WHEN SEPARATED IN THREE SOLVENT SYSTEMS

Figures are the means of the combined values for the coat and embryo of each species and the means of two replicates of the coumarin standard.

| CHROMATOGRAPHIC SOLVENT SYSTEM | INHIBITING COMPOUND ($R_f$) | COUMARIN ($R_f$) |
|---|---|---|
| iso-propanol:ammonia: water (10:1:1 v/v) | P 0,91<br>L 0,91 | 0,92 |
| n-butanol:ammonia: water (200:6:36 v/v) | P 0,94<br>L 0,94 | 0,92 |
| ethyl acetate:2N ammonia (1:1 v/v) | P 0,95<br>L 0,93 | 0,94 |

The inhibiting compound showed up with a yellow/green fluorescence when irradiated with UV light after spraying with 2N NaOH. No fluorescence was obtained without

pretreatment with NaOH. The active area also reacted with 1% aqueous potassium permanganate giving a yellow colour. These reactions suggest that the compound is coumarin-like, as it behaves physically and chemically in the manner described for coumarin by Swain (1953).

Although the data presented are not conclusive chemical proof that the inhibitor is coumarin, the bulk of the evidence nevertheless seems to indicate that it is similar, if not identical, to coumarin. Although there were a number of other indications of inhibitory activity in certain extracts, no evidence for the presence of ABA was found.

# CHAPTER 5

## CHANGES IN ENDOGENOUS HORMONE LEVELS DURING STRATIFICATION

The major factor responsible for poor germination of Protea compacta seed is apparently the dormant condition of the embryo. Germination of isolated embryos is poor both when incubated on sand (Table 3.1) or under sterile conditions in vitro (Van Staden et al., 1972a).

In Leucadendron daphnoides dormancy is apparently coat-imposed (3.1). Although the germination pattern of the two species differs (Figs. 2.1 and 2.2), germination of both species can be improved by stratification (Table 3.4). In addition both species can be induced to germinate by the exogenous application of cytokinins and gibberellic acid (Table 3.6). Both of these growth promoters are known to be able to substitute for chilling requirements (Webb and Dumbroff, 1969; Baskin and Baskin, 1971; Pinfield and Stobart, 1972). The involvement of cytokinins in radicle elongation (Haber and Luippold, 1960; Pinfield and Stobart, 1972) and cotyledon expansion (Ikuma and Thimann, 1963; Kursanov et al., 1969; Sveshnikova and Khokhlova, 1969 and Rijven, 1972) is well established. In addition their ability to promote

expansion of excised cotyledons forms the basis of at least three bioassays (Esashi and Leopold, 1969a; Letham, 1971; Kumar and Sastry, 1973).

Webb, Van Staden and Wareing (1973a) showed that stratification of dormant embryos of Acer saccharum led to considerable changes in the levels of endogenous cytokinins, gibberellin-like substances and germination inhibitors. In order to follow up the results in Table 3.4 and to determine whether stratification of proteaceous seeds had similar effects to those obtained in Acer saccharum, a study was made of changes in endogenous hormone levels during stratification.

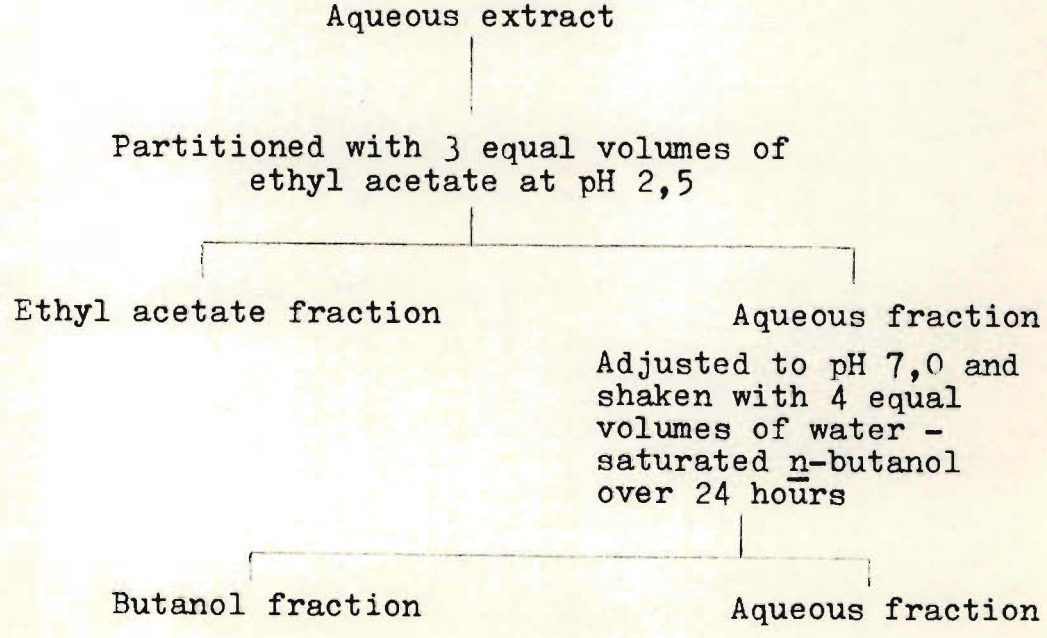
## 5.1 Materials and Methods

Seeds of Protea compacta and Leucadendron daphnoides were soaked in distilled water for 24 hours and then incubated on moist filter paper at 5°C and 25°C for 0, 30, 60 and 90 days respectively. At the end of the treatment period the woody seed coats were removed and samples of embryos were extracted for cytokinins, gibberellin-like substances and germination inhibitors. Where samples of seed rotted when incubated at 25°C, these were not extracted. Whenever sufficient seeds germinated during incubation, they were extracted separately. At the end of each stratification treatment, a sample of seed was germinated under standard conditions

(1.2). Results were based on at least two replicates of each experiment done at different times.

5.1.1 Extraction and bioassay of cytokinins

The method used was based on that of Van Staden, Webb and Wareing (1972b). Ten grammes of non-germinated embryos were homogenized in a blendor with 300 ml 80% ethanol. The homogenate was extracted for 24 hours at 5°C and then filtered. The residue was re-extracted with 200 ml ethanol and then discarded. The combined filtrates were concentrated to the aqueous phase under vacuum at 40°C, brought to 100 ml and the pH adjusted to 2,5. The aqueous extracts were then extracted as follows:



In initial experiments, once the ethanol extracts had been concentrated to the aqueous phase, they were first adjusted to pH 9,0 and extracted with petroleum ether, before being acidified and extracted with ethyl acetate. However, it was found here and also reported by Van Staden (1973), that considerable amounts of cytokinins were removed by the petroleum ether. Although the petroleum ether was useful in removing excess lipids which otherwise might interfere later with chromatography, this latter advantage was negated by the loss of cytokinins. In all experiments reported here, this step was omitted from the extraction procedure.

The butanol and aqueous fractions were taken to dryness under vacuum at 40°C, redissolved in 35% ethanol, strip-loaded onto Whatman No. 1 chromatography paper and separated with iso-propanol:ammonia:water (10:1:1 v/v). The dried chromatograms were divided into ten equal strips and assayed for cytokinin activity with the soybean callus bioassay (Miller, 1965). All cultures were grown for 28 days at 26 ± 2°C under continuous illumination. The ethyl acetate partitioning was included to remove lipids and acidic growth inhibitors Van Staden (1973) reported that considerable quantities of cytokinins could be lost in the acidic ethyl acetate fractions and thus these were routinely assayed for cytokinin activity.

5.1.2 Extraction and bioassay of gibberellins

The method of extraction was based on that of Phillips (1972) but omitting the agar-diffusion technique. Ten grammes of embryo material was homogenized in a blendor with 200 ml 80% methanol. The homogenate was extracted for 24 hours at 5°C and then discarded. The combined filtrates were concentrated to the aqueous phase under vacuum at 35°C, brought to 50 ml, the pH adjusted to 2,5 with 5% HCl, and then partitioned with three equal volumes of redistilled ethyl acetate. The pooled ethyl acetate fraction, containing acidic and possibly neutral gibberellins was reduced to dryness under vacuum at 35°C and taken up in six ml redistilled ethyl acetate. The pH of the aqueous fraction was adjusted to 7,0, whereupon it was reduced to dryness and the residue taken up in six ml 35% ethanol. This fraction was assumed to contain the basic and most of the neutral gibberellins. Both fractions were strip-loaded onto Whatman No. 1 chromatography paper and separated with iso-propanol:ammonia:water (10:1:1 v/v).

The dried chromatograms were divided into ten equal strips and assayed for gibberellin activity. The lettuce hypocotyl assay (Frankland and Wareing 1960) was used initially. However, it was found that the variability in the results could not be reduced significantly and thus the assay was abandoned. The Rumex leaf senescence

retardation bioassay developed by Whyte and Luckwill (1966) was then used. The chromatogram strips were cut up and placed in 30 mm diameter petri dishes with lids. Three ml distilled water was added to the paper strips in each dish. Leaves were detached from Rumex plants and pre-aged in the dark at 25°C for 24 hours. Ten millimetre leaf discs were cut from the leaves and four discs (each from a different leaf) were placed abaxial surface downwards on the moistened paper strips in each petri dish. In order to reduce variability and to obtain consistent results in the assay, it was found essential to use only four leaves at a time. A disc from each leaf was allocated systematically to each petri dish and to the controls. In the latter, blank chromatography paper was moistened with distilled water or a range of $GA_3$ standards ($10^{-2}$ to $10^2$ µg/l). A separate set of controls was assayed with every four leaves used.

The petri dishes containing the discs were placed on large enamel trays which had been lined with moist filter paper. The trays were covered with aluminium foil and then incubated at 25°C in darkness. When the chlorophyll had almost disappeared from the discs on the blank paper controls (normally after three to five days), the four leaf discs from each treatment were placed overnight in six ml methanol to extract the chlorophyll. The optical density of the solution at 665 nm

(chlorophyll a) was measured on a spectrophotometer (Spectronic 20). The chlorophyll content of each treatment was expressed as a percentage of that in the relevant blank paper controls.

5.1.3 Extraction and bioassay of germination inhibitors

Ten grammes of embryo material was extracted as for gibberellins (5.1.2), except that the acidified aqueous extract was partitioned with three equal volumes of redistilled ether. The pooled ether fraction, containing acidic and possibly neutral inhibitors, was reduced to dryness under vacuum at 35°C and taken up in six ml pure dry ether. The pH of the aqueous fraction was adjusted to 7,0, reduced to dryness under vacuum and the residue taken up in six ml 35% ethanol. The aqueous fraction was assumed to contain the basic and most of the neutral inhibitors. Both fractions were strip-loaded onto Whatman No. 1 chromatography paper and separated with iso-propanol:ammonia:water (10:1:1 v/v). The dried chromatograms were divided into ten equal strips and assayed for inhibitors using the lettuce seed germination bioassay (4.1.5).

## 5.2 Results

### 5.2.1 Germination

Incubation of the seed at 25°C had an extremely unfavourable effect on germination (Fig. 5.1). In contrast seed that had been stratified for 30 days showed an increase in germination of approximately 50%. Longer periods of stratification did not further improve the germination of Protea compacta significantly and they led to a drop in germination in Leucadendron daphnoides.

### 5.2.2 Cytokinin activity

No significant cytokinin activity could be detected in the aqueous extracts of unchilled and chilled embryos of Protea compacta (Fig. 5.2). The butanol extracts of the unchilled embryos did, however, yield significant activity. The $R_f$ values of this activity corresponded with the $R_f$ values (0,6 - 0,7) of authentic zeatin and zeatinriboside. Stratifying the seed for 30 days, a treatment which greatly improved germination, resulted in an 84% increase in butanol-soluble cytokinins (calculated as kinetin equivalents). A further increase in the chilling period resulted in a decrease in cytokinin activity. Incubation of the seed at 25°C had a detrimental effect on germination and led to a decrease in their cytokinin content to a level below that of the untreated

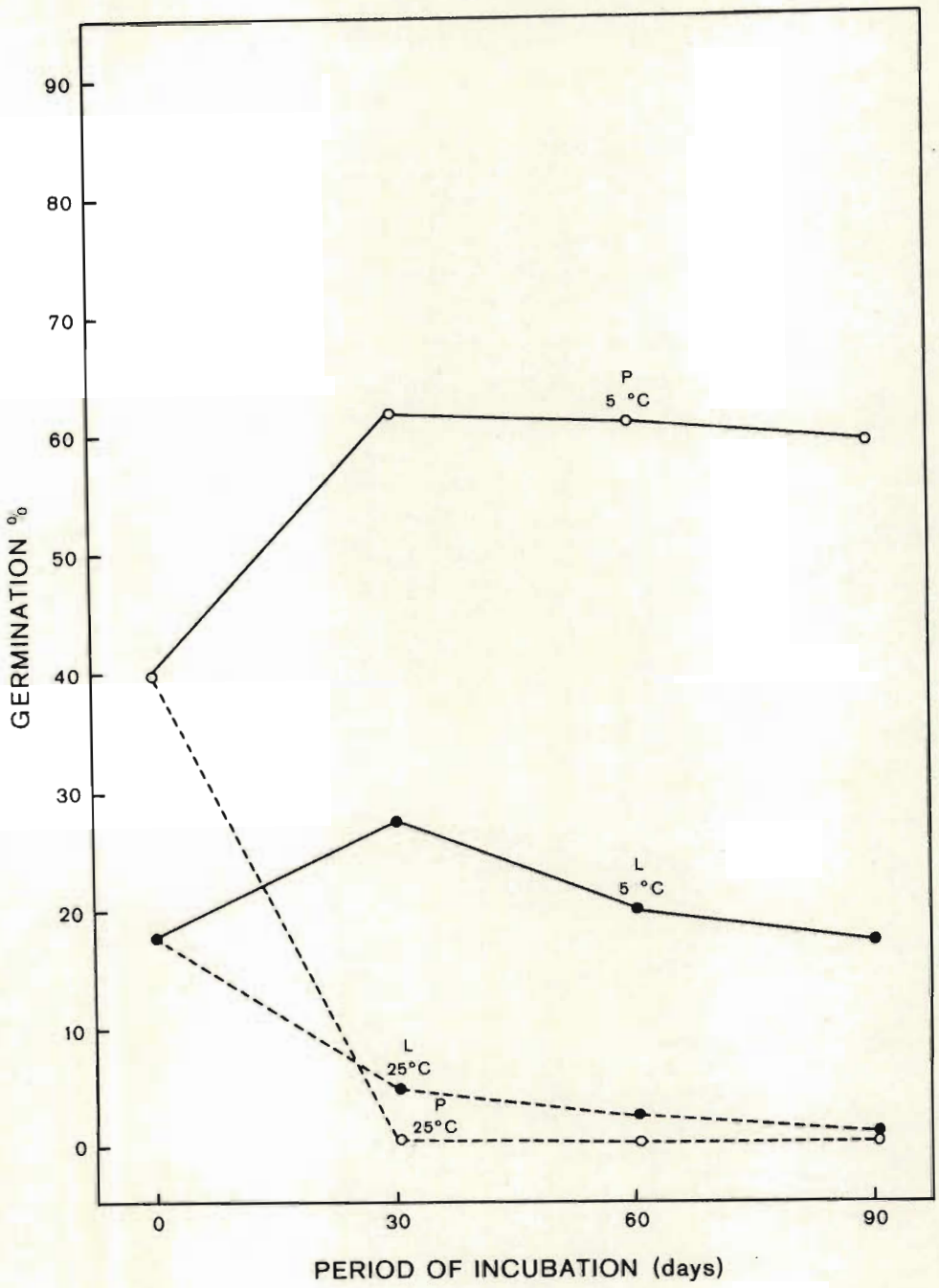



Fig. 5.1

Germination of seed of Protea compacta (P) and Leucadendron daphnoides (L) following incubation at 5°C and 25°C. Figures are means of 250 seeds.

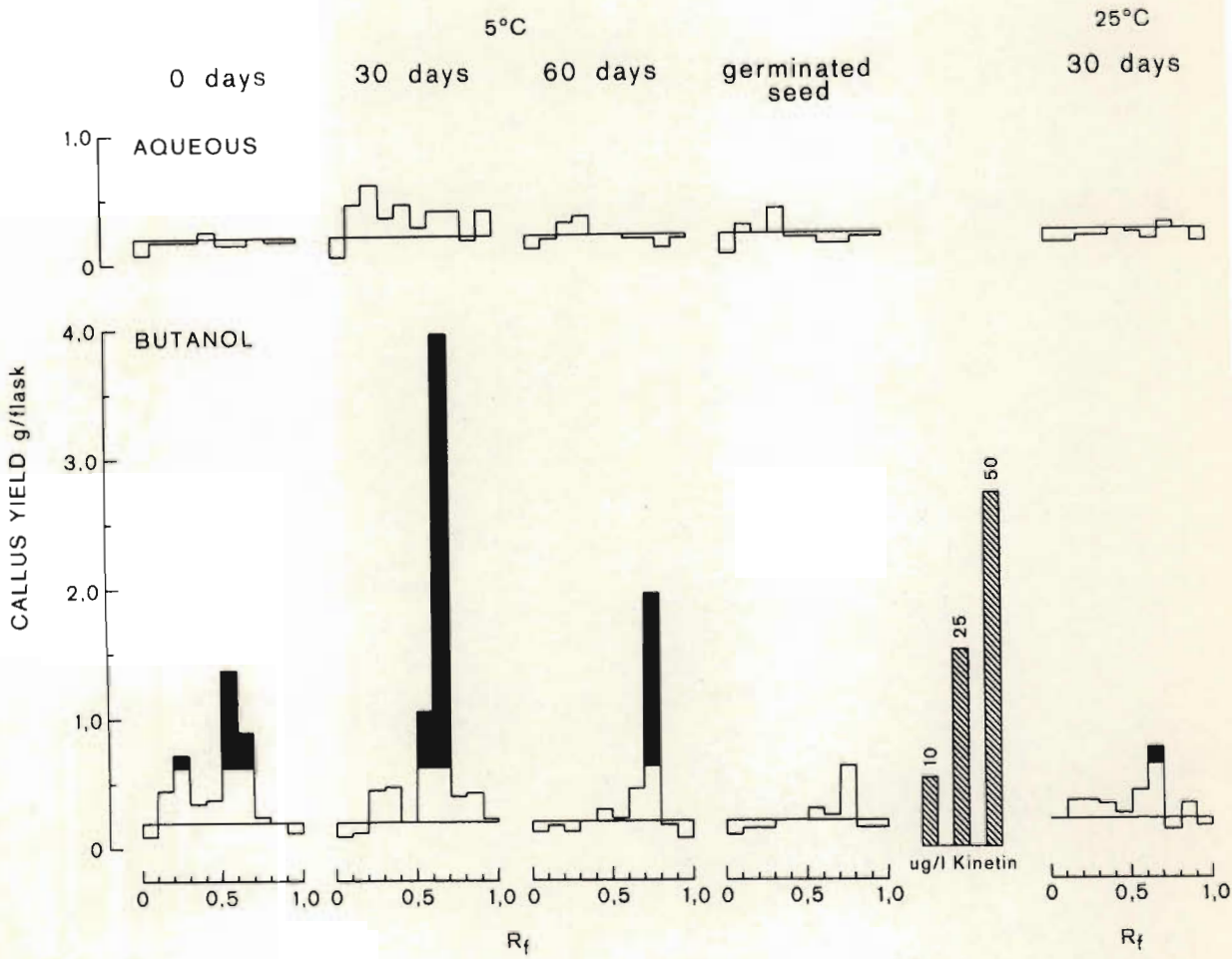



Fig. 5.2

Soybean callus assays of paper chromatograms loaded with aqueous and butanol extracts of Protea compacta seed, incubated at 5°C and 25°C respectively. The equivalent of 10 grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and bioassays. Blackened areas represent regions significantly different from the controls at the 1% level.

controls.

The unchilled embryos of Leucadendron daphnoides contained low levels of butanol-soluble cytokinins and, in contrast to Protea compacta, high levels of water-soluble cytokinins (Fig. 5.3). The activity of the aqueous extracts occurred between $R_f$ 0,2 and 0,3, suggesting that it is due to cytokinin ribotides. Chilling the seed at 5°C progressively decreased the activity in the aqueous fractions over the 30, 60 and 90 day treatments. During the same period the cytokinin activity of the butanol fractions increased approximately four-fold, reaching a peak between 30 and 60 days of stratification. Essentially the same tendency was observed when the seed was incubated at 25°C. The important difference between the temperature treatments was that the cytokinin levels of stratified seeds were always about 20% higher than those of seed maintained at the higher temperature (Fig. 5.4). In both species no activity was found in germinated seed (Figs. 5.2 and 5.3). To obtain some information about the nature of the activity in the butanol and aqueous extracts of seed stratified at 5°C for 30 days the extracts were separated on a LH-20 Sephadex column and eluted with 35% ethanol (Armstrong, Burrows, Evans and Skoog, 1969). In Leucadendron daphnoides the aqueous extract containing cytokinin ribotides from four grammes embryo material

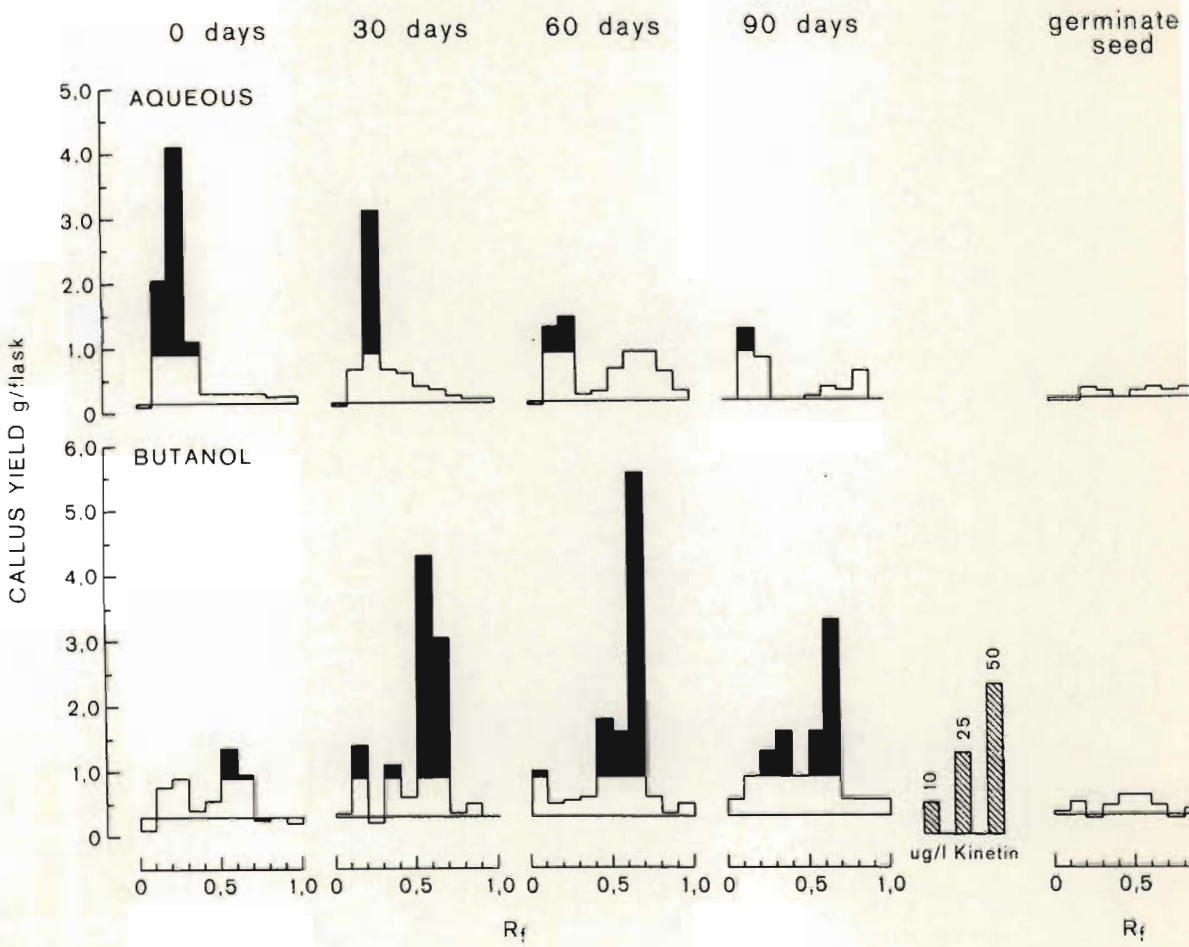



Fig. 5.3

Soybean callus assays of paper chromatograms loaded with aqueous and butanol extracts of Leucadendron daphnoides seed, stratified at 5°C. The equivalent of 10 grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia: water (10:1:1 v/v). The values presented are the means of two separate extractions and bioassays. Blackened areas represent regions significantly different from the controls at the 1% level.

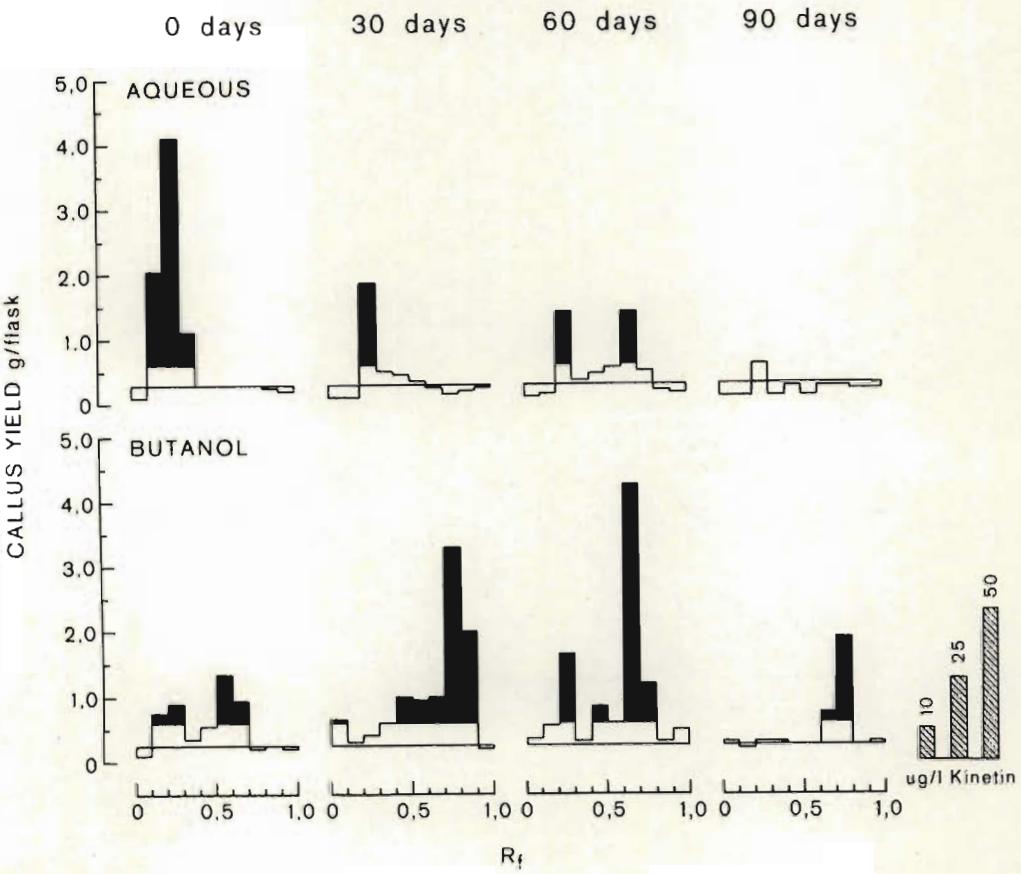



Fig. 5.4

Soybean callus assays of paper chromatograms loaded with aqueous and butanol extracts of Leucadendron daphnoides seed, incubated at 25°C. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia: water (10:1:1 v/v). The values presented are the means of two separate extractions and bioassays. Blackened areas represent regions significantly different from the controls at the 1% level.

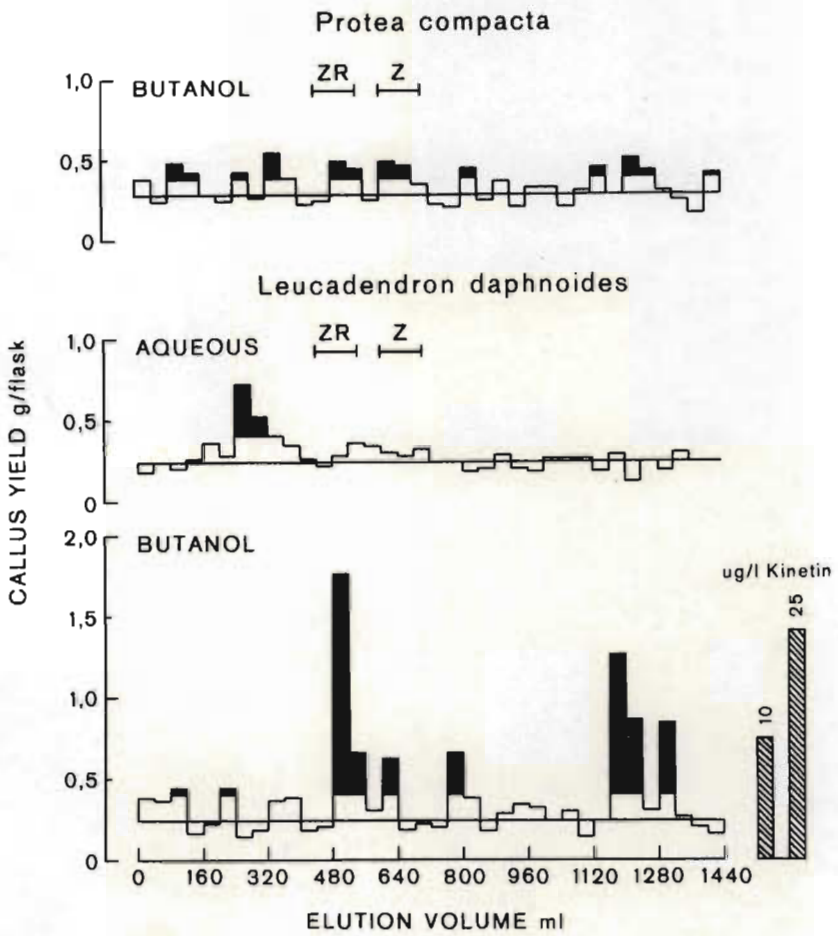



Fig. 5.5

The distribution of cytokinin activity of a Sephadex LH-20 fractionation of extracts obtained from non-germinated embryos of Leucadendron daphnoides (four grammes) and Protea compacta (nine grammes), stratified at 5°C for 30 days. Blackened areas represent regions significantly different from the controls at the 0,1% level. Z = Zeatin, ZR = Zeatinriboside.

yielded one significant peak of activity which eluted between 240 and 320 ml (Fig. 5.5). The butanol extract, which would normally contain free bases and ribosides, yielded a number of significant peaks of activity. In Protea compacta the aqueous extracts of nine grammes embryo material yielded no significant activity and the results are not presented. The butanol extracts, however, showed a number of significant peaks of activity (Fig. 5.5). Significant peaks of activity in the butanol extracts of both species, which eluted between 480 and 520 ml and between 600 and 640 ml, corresponded with the elution patterns of zeatinriboside and zeatin, respectively.

### 5.2.3 Gibberellin-like activity

After being imbibed for 24 hours, seed of both species showed a relatively low level of gibberellin-like activity in the acidic ethyl acetate fraction and a high level in the aqueous fraction. In Leucadendron daphnoides (Fig. 5.6) the level of activity in the ethyl acetate fraction was increased slightly by incubation, irrespective of the incubation temperature and peaked after 30 days. The level of activity in the aqueous fraction was due mainly to the presence of slow moving promoters ($R_f$ 0,1 - 0,2). The level of activity of these promoters dropped with time at both temperatures. At 5°C, however, there was a concomitant increase in the level of fast moving

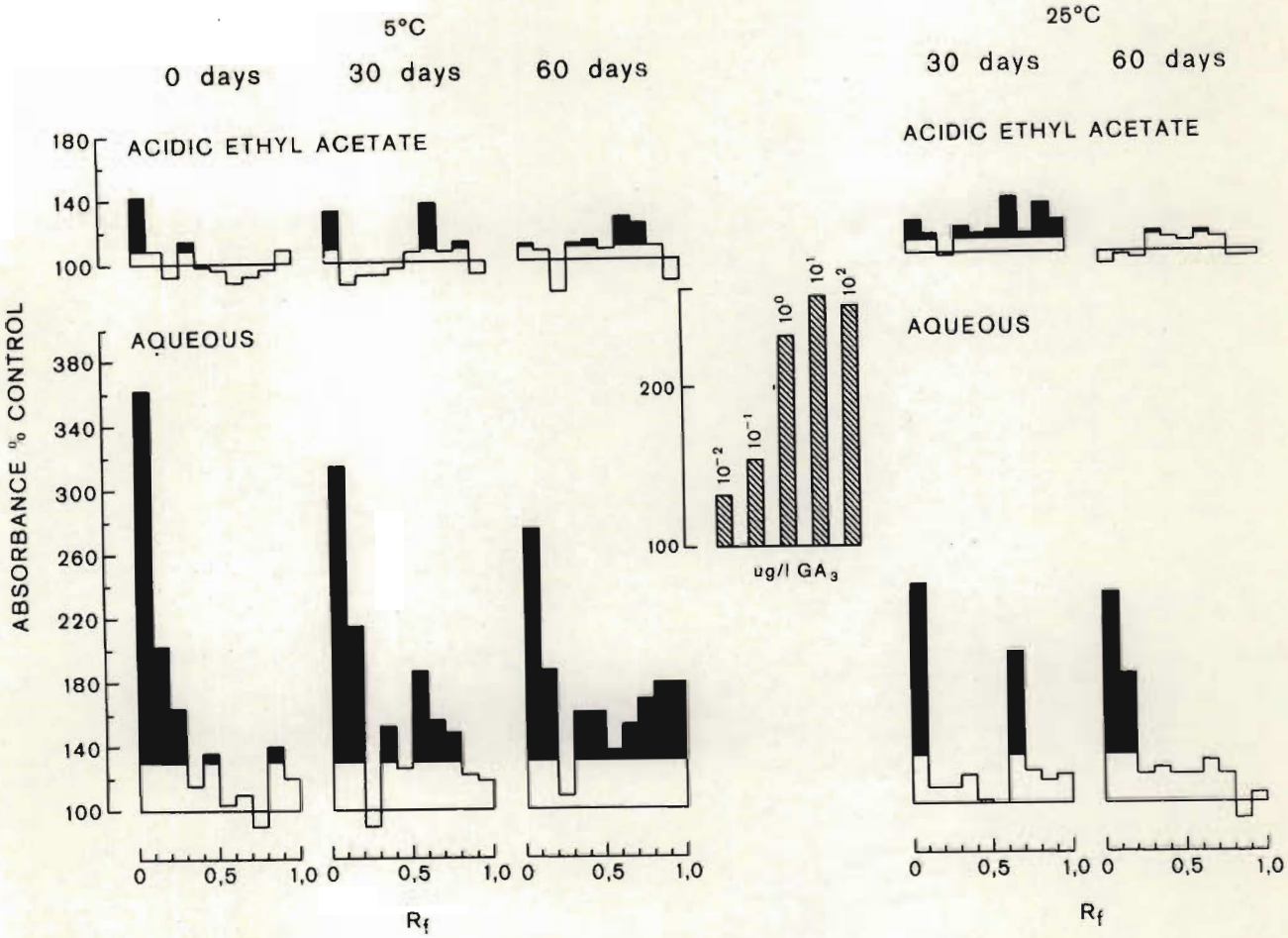



Fig. 5.6

Rumex leaf senescence bioassays of paper chromatograms loaded with acidic ethyl acetate and aqueous fractions of extracts of Leucadendron daphnoides seed incubated at 5°C and 25°C respectively. The equivalent of ten grammes embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and bioassays. Blackened areas represent regions significantly different from the water controls at the 1% level.

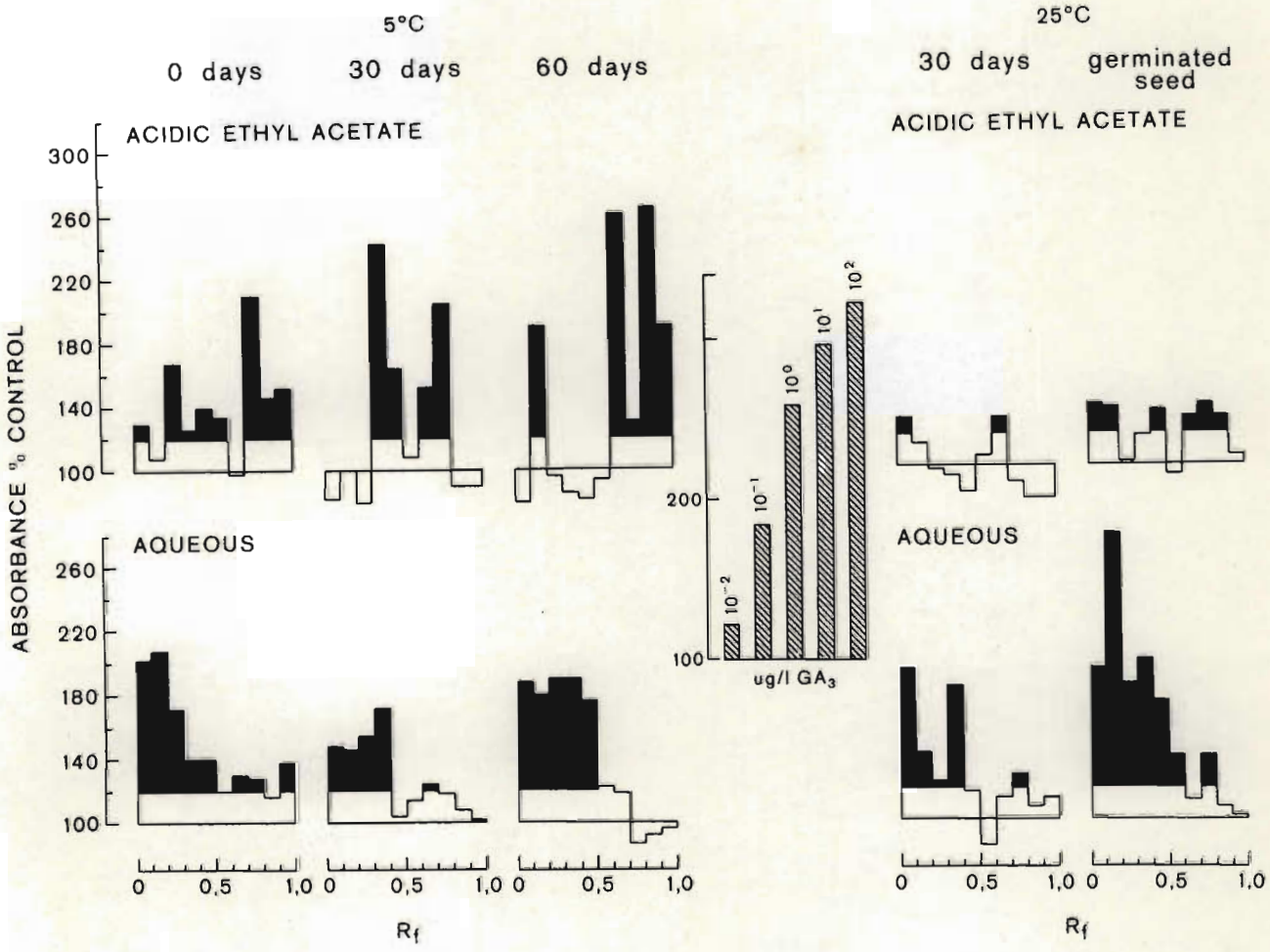



Fig. 5.7

Rumex leaf senescence bioassays of paper chromatograms loaded with acidic ethyl acetate and aqueous fractions of extracts of Protea compacta seed incubated at 5°C and 25°C respectively. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and bioassays. Blackened areas represent regions significantly different from the water controls at the 1% level.

promoters ($R_f$ 0,6 - 1,0).

In Protea compacta (Fig. 5.7) incubation at 5°C resulted in a progressive increase in the level of gibberellin-like activity in the ethyl acetate fraction over a period of 60 days. No clear trend was shown in the levels of activity in the aqueous fraction with incubation at 5°C. Incubation at 25°C resulted in a marked decrease in the level of activity in both fractions. It is interesting to note that germinated seed showed a low level of gibberellin-like substances soluble in ethyl acetate, but a high level in the aqueous fraction.

### 5.2.4 Inhibitor activity

Although there were minor fluctuations in the levels of inhibitors in the acidic ether and aqueous fractions of embryo extracts of Leucadendron daphnoides (Fig. 5.8) and Protea compacta (Fig. 5.9), there were no major trends of changes in inhibitor levels with incubation at either 5°C or 25°C. Germinated seed of Protea compacta showed inhibitor levels no different from the imbibed control seed. Much of the inhibition shown at low $R_f$ values in the aqueous fractions could have been due to poor chromatographic separation of the extracts.

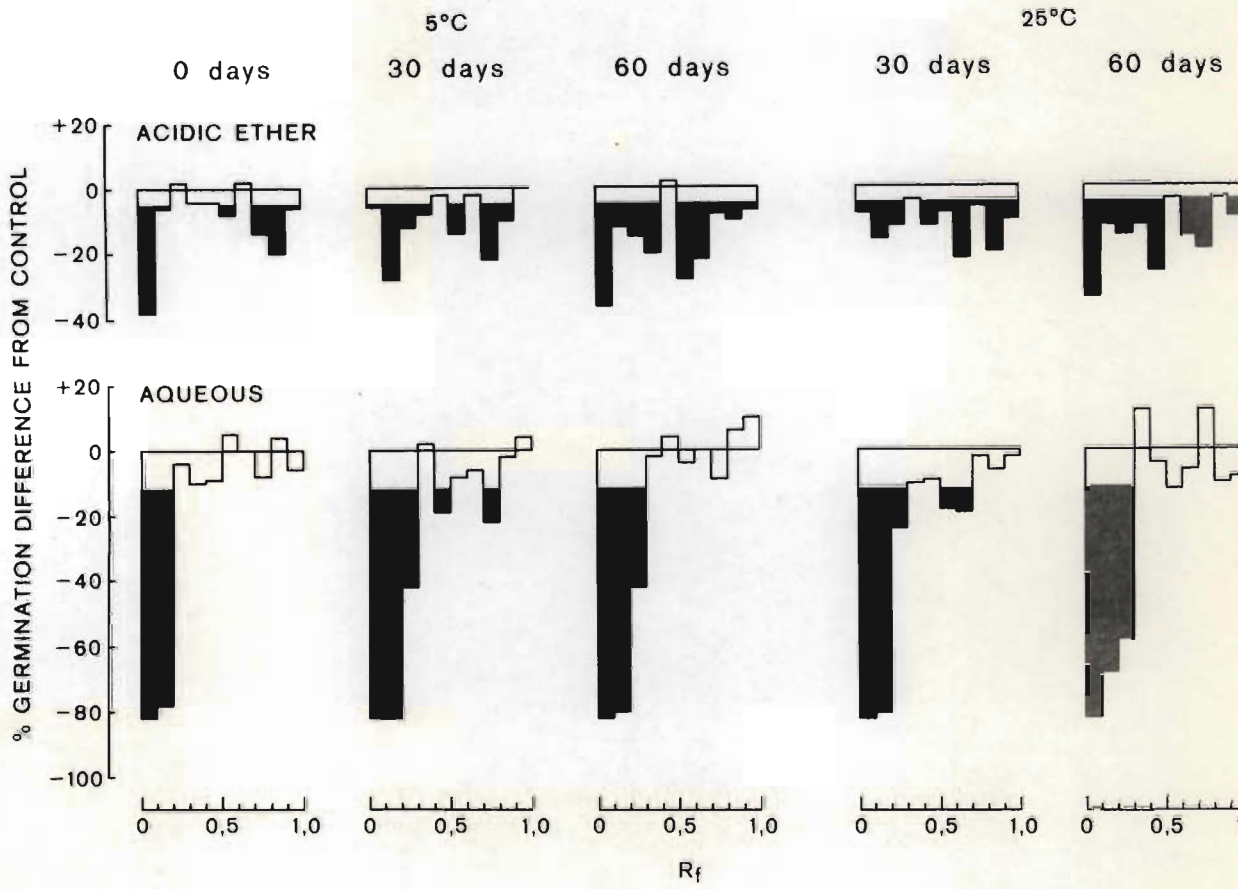



Fig. 5.8

Inhibitors from acidic ether and aqueous fractions of embryo extracts of Leucadendron daphnoides seed incubated at 5°C and 25°C, respectively. Extracts were chromatographed on paper in iso-propanol:ammonia:water (10:1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level.

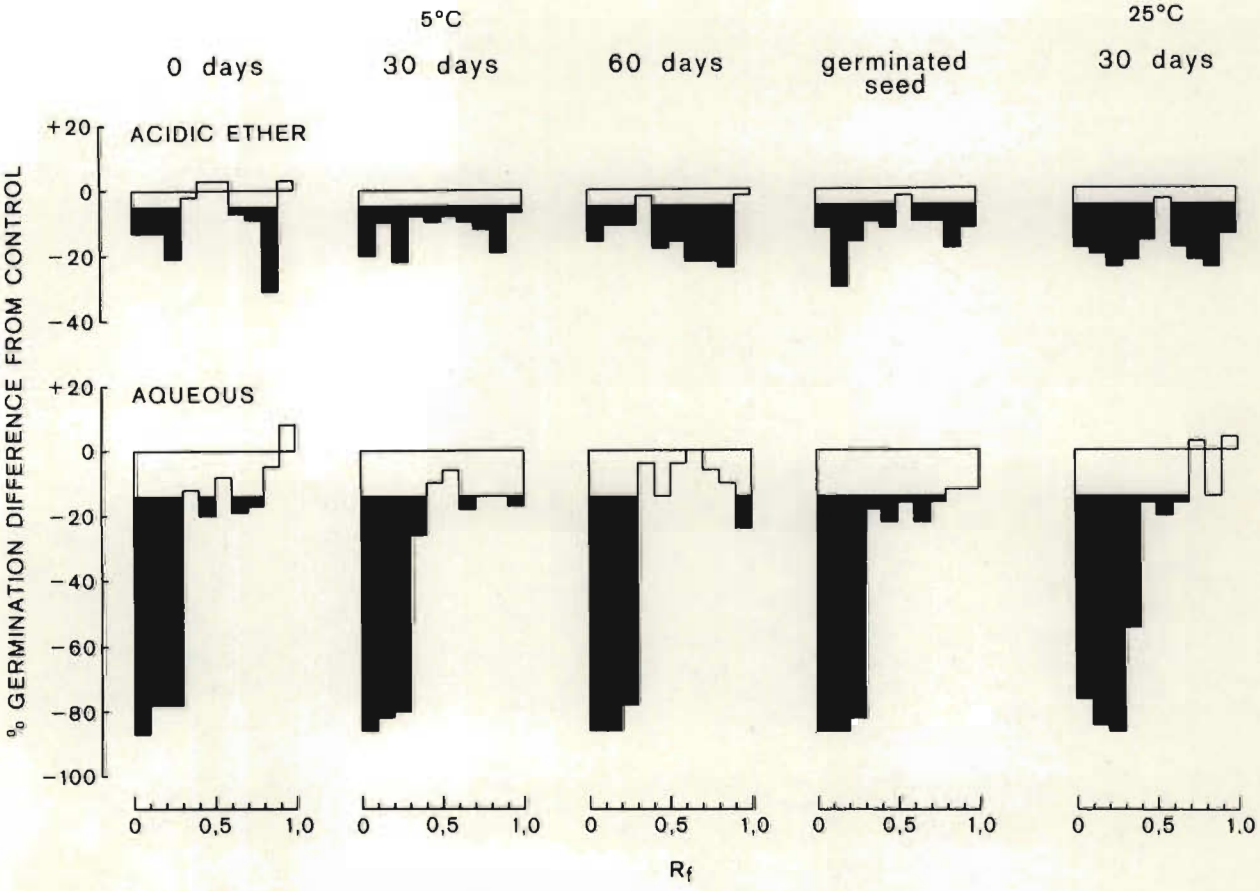



Fig. 5.9

Inhibitors from acidic ether and aqueous fractions of embryo extracts of Protea compacta seed incubated at 5°C and 25°C, respectively. Extracts were chromatographed on paper in iso-propanol:ammonia:water (10:1: v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level.

## 5.3 Discussion

### 5.3.1 Cytokinin levels

The low germination percentage of unchilled *Protea compacta* seed may be due to the fact that a threshold concentration of endogenous cytokinins is required for radicle elongation. As a result of low temperature treatment the butanol-soluble cytokinins increased significantly after 30 days, a process which appears to be closely correlated with an increased potential for germination. These results are similar to those obtained for the embryo dormant seed of *Acer saccharum* (Van Staden, *et al*. 1972b), and also suggest that the butanol-soluble cytokinins are either synthesized or released from a bound form during chilling. That cytokinins can influence radicle elongation, which in *Protea compacta* is the first visible sign of germination, has been reported by Pinfield and Stobart (1972).

Although coat-imposed dormancy can be overcome by stratification treatments (Wareing and Saunders, 1971), correlations with changes in endogenous cytokinin levels have not previously been reported. In *Leucadendron daphnoides* where dormancy is coat-imposed, high levels of water-soluble cytokinins were found in the unchilled seed. Under low temperature treatments these levels decreased, accompanied by a concomitant increase in

butanol-soluble cytokinins. These changes were paralleled by an increase in germination. Incubation of seed at 25°C brought about a similar pattern of changes, the main difference being that the overall cytokinin levels were lower than those of chilled seed. This suggests that for germination to occur a threshold concentration of butanol-soluble cytokinins is required. The results for Leucadendron daphnoides suggest that the effect of chilling on this coat-imposed dormant seed is to bring about an interconversion of cytokinins, rather than a de novo synthesis, as apparently occurs in embryo-dormant seed of Acer saccharum (Van Staden, et al. 1972b) and seed of Protea compacta.

In both Leucadendron daphnoides and Protea compacta no cytokinin activity could be detected in seed that had germinated. This seems to indicate that the compounds which accumulate prior to any visible sign of germination, are rapidly metabolized once growth is resumed. Germination in both species is apparently closely related to changes in levels of butanol-soluble cytokinins. The fact that the seed of Leucadendron daphnoides contains high levels of water-soluble cytokinins yet remains predominantly dormant would suggest that these ribotides are physiologically less active than their corresponding free bases and ribosides.

5.3.2 Gibberellin levels

According to Wareing (1969) there is considerable evidence to suggest that in some seeds the level of gibberellins increases with stratification. This was found to occur in seed of Protea compacta stratified at 5°C, where the level of gibberellin-like activity increased progressively over a period of 60 days. In seed of Leucadendron daphnoides there was only a slight increase in the level of activity after 30 days at 5°C. In Acer saccharum, Webb et al. (1973a) observed a peak of gibberellin-like activity after 40 days stratification at 5°C. Sinska and Lewak (1970) observed a peak of gibberellin activity in apple seeds corresponding to $GA_4$ after a similar period of stratification. In both these species it was reported that application of $GA_3$ overcame dormancy. In Protea compacta stratification at 5°C for 30 days resulted in approximately 50% increase in germination and the application of $GA_3$ (10 mg/l) resulted in a 20% increase in germination. It thus appears that in Protea compacta endogenously produced gibberellins may be more effective in breaking dormancy than applied $GA_3$ or that there was insufficient penetration of $GA_3$ into the seeds to promote germination more effectively. Alternatively, as applied cytokinins gave 37% increase in germination and butanol-soluble cytokinins increased four-fold with stratification at 5°C, it may indicate that

cytokinins are more important in promoting germination than are gibberellins. In Leucadendron daphnoides both stratification for 30 days and the application of $GA_3$ (10 mg/l) gave an increase in germination of 50%, indicating that seeds of this species may have a lower gibberellin requirement for germination.

In Acer saccharum, the optimum temperature for germination ($5^{o}C$) coincided with the stratification temperature (Webb et al. 1973a). In studies of Leucadendron daphnoides and Protea compacta, after stratification at $5^{o}C$, seeds were germinated under alternating temperatures of $10^{o}C$ and $20^{o}C$ (1.2). Although it was not investigated, there is the possibility that significant changes in gibberellin levels may have occurred once seeds were transferred to the higher temperatures. Bradbeer (1968) found that in hazel there was only a small increase in gibberellin levels during chilling at $5^{o}C$ and it was only when seeds were transferred to a temperature suitable for germination ($20^{o}C$) that the gibberellin levels increased markedly.

Although gibberellin levels have been observed to change during or after stratification in some species, in others, such as Prunus avium (Proctor and Dennis, 1968) and Acer pseudoplatanus (Webb et al. 1973b) no detectable changes were observed during stratification.

The Rumex leaf senescence bioassay has been

successfully used by Grausland (1972), Browning (1973) and others. It has been criticized on the grounds that substances other than gibberellins, e.g. peptones (Reynolds, 1969) and cytokinins (Goldthwaite and Laetsch, 1968), will also retard leaf senescence. The possibility of peptones being present in any of the seed extracts was regarded as remote. There is the possibility, however, that endogenous cytokinins may have influenced the bio-assay results, as Van Staden (1973) has shown that a considerable proportion of the cytokinins in plant extracts may pass into the ethyl acetate fractions at low pH values. Cytokinins would also be present in the aqueous fractions. Whyte and Luckwill (1966) did not obtain senescence inhibition by kinetin but, according to Goldthwaite and Laetsch (1968), this was probably due to the use of insufficiently concentrated solutions. The latter workers found that nearly-saturated solutions of kinetin (approx. 40 mg/l) caused substantial retardation of chlorophyll loss. Lower concentrations of kinetin were in some cases also effective. Benzyladenine was also effective (1,0 - 500µM) and at short times of incubation was nearly as effective as $GA_3$ in retarding chlorophyll loss.

In the present investigation peak gibberellin-like activity, as shown in the Rumex assay, was sometimes correlated with the peak in cytokinin activity (Fig. 5.10,

Leucadendron daphnoides seed after 30 days incubation at 5°C) and thus cytokinins might be suspected of being partly responsible for senescence retardation. In contrast, it seems unlikely that cytokinins were responsible for senescence retardation where levels of gibberellin-like activity continued to rise as cytokinin levels dropped (Fig. 5.11, Protea compacta seed between 30 and 60 days incubation at 5°C).

### 5.3.3 Inhibitor levels

According to Wareing and Saunders (1971) the available evidence for the role of inhibitors in seed dormancy is far from conclusive at present. Substances extracted from the seed of many species have been shown to inhibit germination of test seeds or the growth of Avena coleoptiles in bioassays. Their occurrence in seeds does not in itself necessarily imply that they play a functional role in seed dormancy under normal conditions. In order to establish a role it is necessary to take into account various types of evidence, such as correlations between the levels of inhibitors and the state of dormancy (Wareing, 1965). No such correlations were shown when seed of both species was incubated at 5°C or 25°C. Chilling at 5°C for 30 days brought about a considerable improvement in germination in both species, but there were no major changes in levels of

inhibitors. On the other hand, incubation at 25°C depressed germination and again inhibitor levels did not change significantly.

The present findings that the levels of inhibitors (which include phenolic compounds) did not drop during stratification contrasts with the results of Monin (1967, 1968 as quoted by Wareing and Saunders, 1971) who found that the inhibitory activity in Euonymous europaeus, thought to be due to a phenolic compound (p-coumaric acid), declined during chilling. A decrease in the level of endogenous inhibitors during stratification has also been reported in Prunus avium (Lipe and Crane, 1966) and Juglans regia (Martin, Mason, and Forde, 1969). In Fraxinus excelsior the levels of water-soluble inhibitors do not seem to change markedly with stratification (Villiers and Wareing, 1965). However, in the same species a drop in ether-soluble inhibitors has been shown to occur during stratification (Kentzer, 1966 as quoted by Webb et al. 1973a). In Fraxinus americana (Sondheimer, Tzou and Galson, 1968), apple (Rudnicki, 1969) and Acer saccharum (Webb et al. 1973a), where ABA appears to be a major component of the inhibitor fraction, a drop in inhibitor levels during chilling has been reported. On the other hand, the levels of an unidentified inhibitory substance did not change during chilling in hazel and beech (Frankland and Wareing, 1966).

5.3.4 Relative levels of hormones

If endogenous inhibitors do indeed play a role in the control of seed dormancy, then it would seem likely that they interact with growth promoters such as gibberellins and cytokinins in such control (Wareing, Van Staden and Webb, 1973). If germination is regulated by the relative levels of promoters and inhibitors (Villiers and Wareing, 1960; Amen, 1968), then maximum germination would be expected where promoter levels are high relative to inhibitor levels. In both species the level of inhibitors extracted from embryos did not change significantly with incubation at 5°C or 25°C. In Leucadendron daphnoides (Fig. 5.10) the level of butanol-soluble cytokinins increased markedly with chilling at 5°C for 30 days, whereas the levels of gibberellin-like substances did not change appreciably. This treatment gave maximum germination which was 50% higher than in control seed (Fig. 5.1). Incubation at 25°C (Fig. 5.10) gave a similar trend of changes in promoter levels, except that the levels of butanol-soluble cytokinins were 20% lower than in seed chilled at 5°C. Germination was markedly depressed at the higher temperature. The results suggest that a threshold level of butanol-soluble cytokinins may be of importance in promoting germination. The results for this species do not support the hypothesis of Kahn (1971) that gibberellins

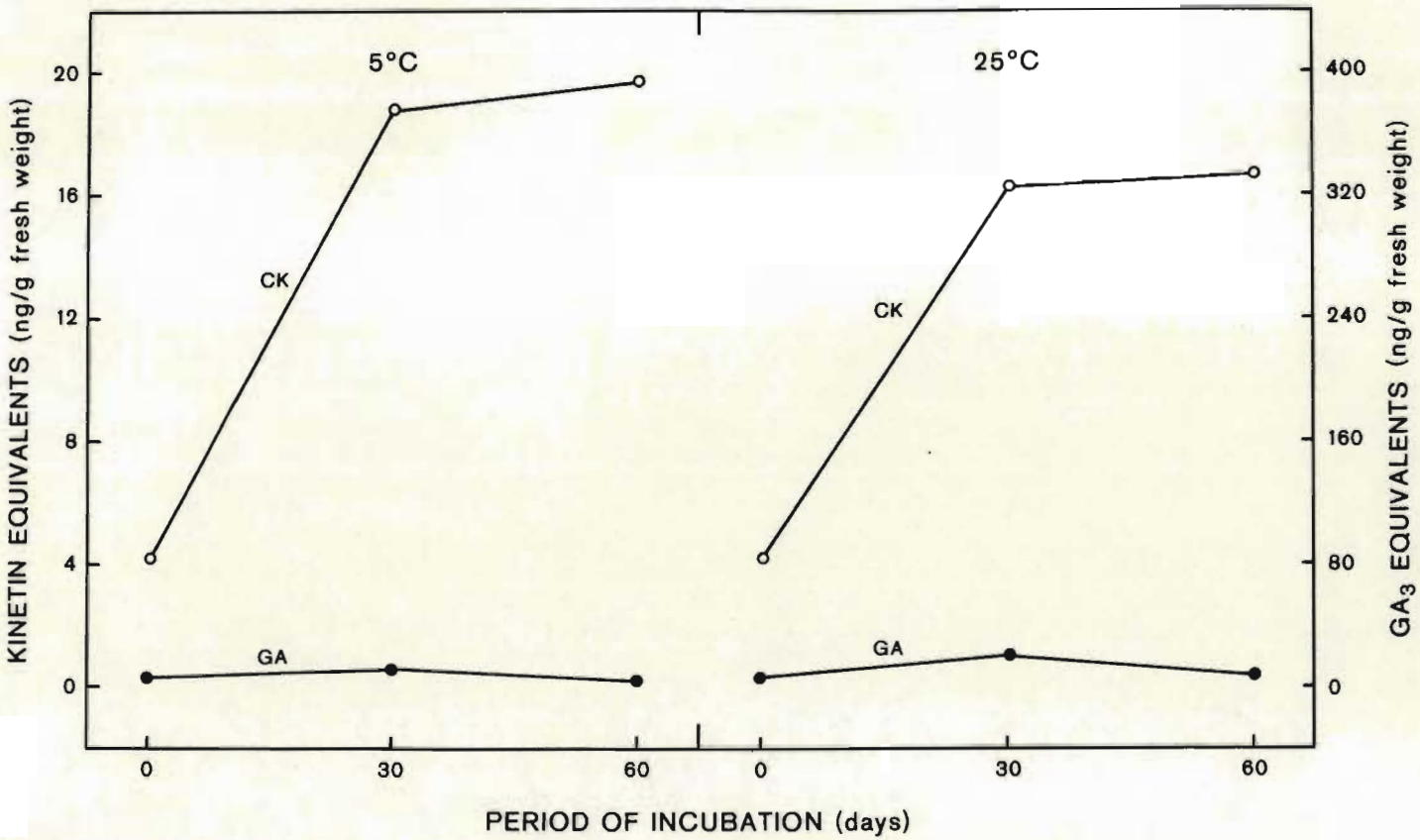



Fig. 5.10

The effect of incubation at 5°C and 25°C on the levels of butanol-soluble cytokinins and acidic gibberellin-like substances in seed of Leucadendron daphnoides. O Butanol-soluble cytokinins measured by the soybean callus bioassay (CK). ● Acidic gibberellin-like substances measured by the Rumex leaf senescence retardation bioassay (GA).

play the primary role in promoting germination and that the roles of cytokinins and inhibitors are essentially "permissive" and "preventive", respectively. According to this hypothesis the main role of cytokinins is to remove the block to germination imposed by inhibitors and thus to enable the gibberellin-mediated processes of germination to be completed. In Leucadendron daphnoides seed, butanol-soluble cytokinins appear to play the primary role in promoting germination. Gibberellins appear to play a lesser role, as very little change in acidic gibberellin levels was recorded. The low percentage germination at 25°C may be partly due to the loss of viability brought about by accelerated respiration at this temperature, as was suggested by Simmonds and Dumbroff (1974) for seed of Acer saccharum incubated at 20°C.

In Protea compacta there was an increase in the level of acidic gibberellin-like substances between 30 and 60 days of chilling (Fig. 5.11). Although not as marked as in Leucadendron daphnoides, there was nevertheless a significant increase in the level of butanol-soluble cytokinins after 30 days at 5°C. This peak level of cytokinins was correlated with maximum germination which was 50% higher than that of the control seed. Incubation at 25°C (Fig. 5.11) resulted in a drop in the levels of both butanol-soluble cytokinins as well as

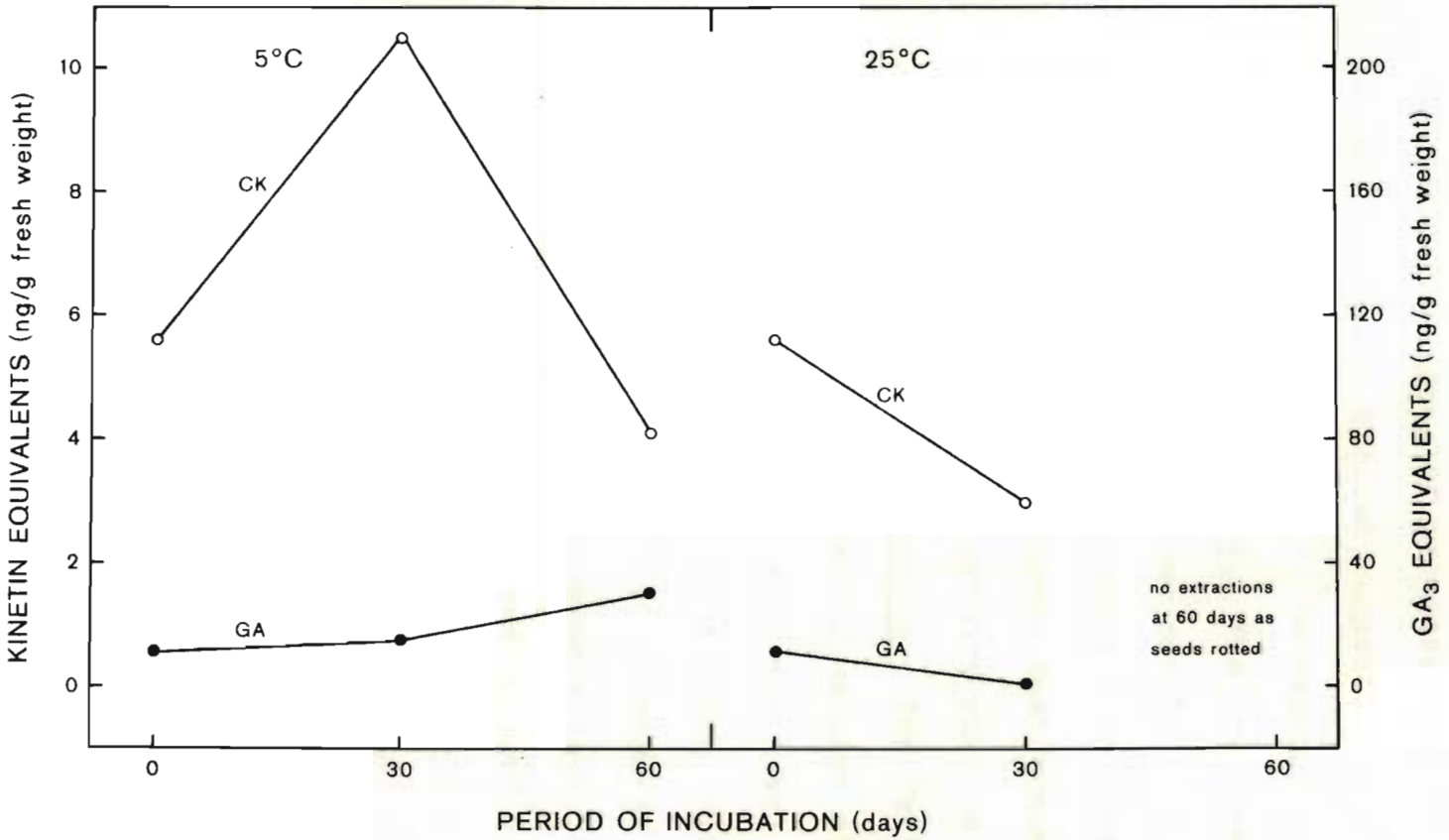



Fig. 5.11

The effect of incubation at 5°C and 25°C on the levels of butanol-soluble cytokinins and acidic gibberellin-like substances in seed of Protea compacta. O Butanol-soluble cytokinins measured by the soybean callus bioassay (CK). ● Acidic gibberellin-like substances measured by the Rumex leaf senescence retardation bioassay (GA).

# CHAPTER 6

## CHANGES IN ENDOGENOUS HORMONE LEVELS DURING INCUBATION IN AIR, OXYGEN AND NITROGEN

Seed dormancy in Leucadendron daphnoides is apparently due to the restricting effect of the seed coat on oxygen diffusion to the embryo. Treatments that increased the availability of oxygen to the embryo, viz., scarification (3.1) and high oxygen tensions (3.4), increased germination at least three-fold. Interference with oxygen diffusion or its elimination, reduced germination to levels well below those of the controls. In Protea compacta the seed coat appears to mechanically restrict radicle elongation and to a lesser extent to retard oxygen uptake. High oxygen tensions (3.4) also resulted in increases in germination, but this increase was not as marked as in Leucadendron daphnoides.

The effects of oxygen in overcoming seed dormancy have been attributed to its influence on inhibitor levels. Wareing and Foda (1957) and Black and Wareing (1959) reported that high oxygen tensions were required for the inactivation of inhibitors in seeds of Xanthium and Betula, respectively. Edwards (1968) suggested that under conditions of restricted oxygen supply an inhibitor was produced in the embryo of charlock (Sinapsis arvensis).

Although the effect of oxygen may be on inhibitor levels in some cases, it is equally possible that oxygen may be directly responsible for increasing the production of promoters which stimulate germination. The effect of incubation in air, oxygen and nitrogen on the levels of endogenous cytokinins, gibberellin-like substances and germination inhibitors was investigated.

## 6.1 Materials and Methods

Seeds of Leucadendron daphnoides and Protea compacta were imbibed in distilled water for 24 hours and then placed on moist filter paper in 500 ml flasks. The flasks were evacuated and then flushed with air, oxygen or nitrogen. The gases were renewed daily and care was taken not to exceed atmospheric pressure. The flasks were incubated in a germinator with temperatures alternating between 10°C for 8 hours and 20°C for 16 hours (1.2). After incubation, ten gramme samples of embryo material from non-germinated seed were extracted and assayed for cytokinins, gibberellin-like substances and germination inhibitors, as previously described (5.1). In Leucadendron daphnoides, where germination usually commenced after 5 - 10 days incubation, seed was incubated for 0, 5, 10 and 20 days. In Protea compacta seed was incubated for 0, 10, 15 and 20 days, as germination did not usually commence until after 10 - 15 days incubation. Germination

data was obtained from seed samples incubated in air, oxygen and nitrogen in a separate set of flasks. Results were based on at least two replicates of each experiment done at different times.

## 6.2 Results

### 6.2.1 Germination

Germination of Leucadendron daphnoides seed was significantly increased by high oxygen tensions (Fig. 6.1). The maximum rate of germination was recorded 15 - 25 days after incubation was commenced. Although incubation in oxygen also brought about an increase in the germination of seed of Protea compacta (Fig. 6.1) the effect was not as marked. Most seed, however, also germinated 15 - 25 days after incubation was started. No germination occurred where seeds were incubated in nitrogen.

### 6.2.2 Cytokinin activity

As was shown previously, dormant seed of Leucadendron daphnoides imbibed for 24 hours contained a relatively high level of water-soluble cytokinins and a low level of butanol-soluble cytokinins (Fig. 6.2). The slow moving, water-soluble compounds separated at $R_f$ 0,2 and 0,3 in iso-propanol:ammonia:water (10:1:1 v/v).

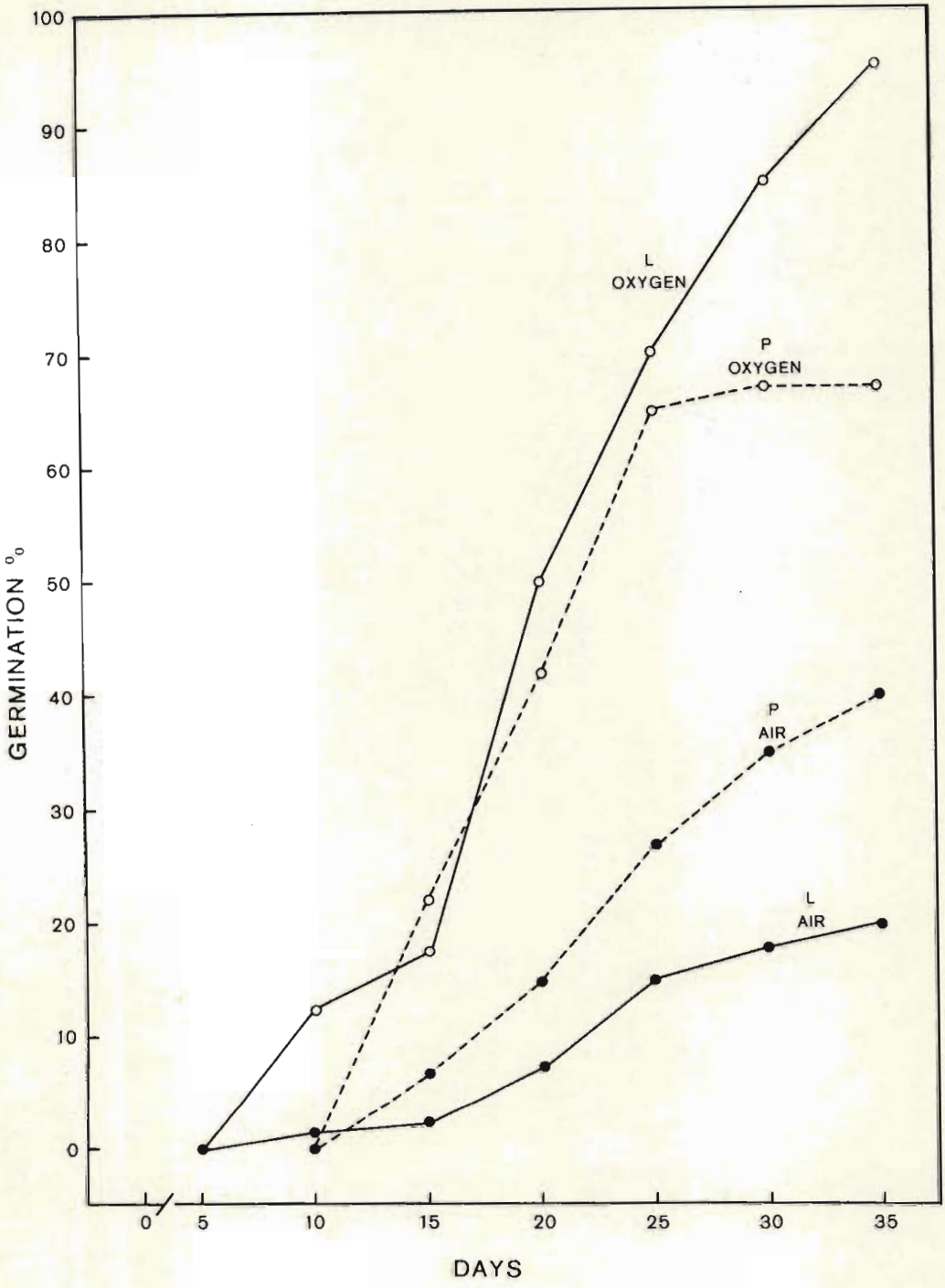



Fig. 6.1

The effect of oxygen and air on the germination of seed of Protea compacta (P) and Leucadendron daphnoides (L). Figures are means of 250 seeds. No germination occurred in nitrogen.

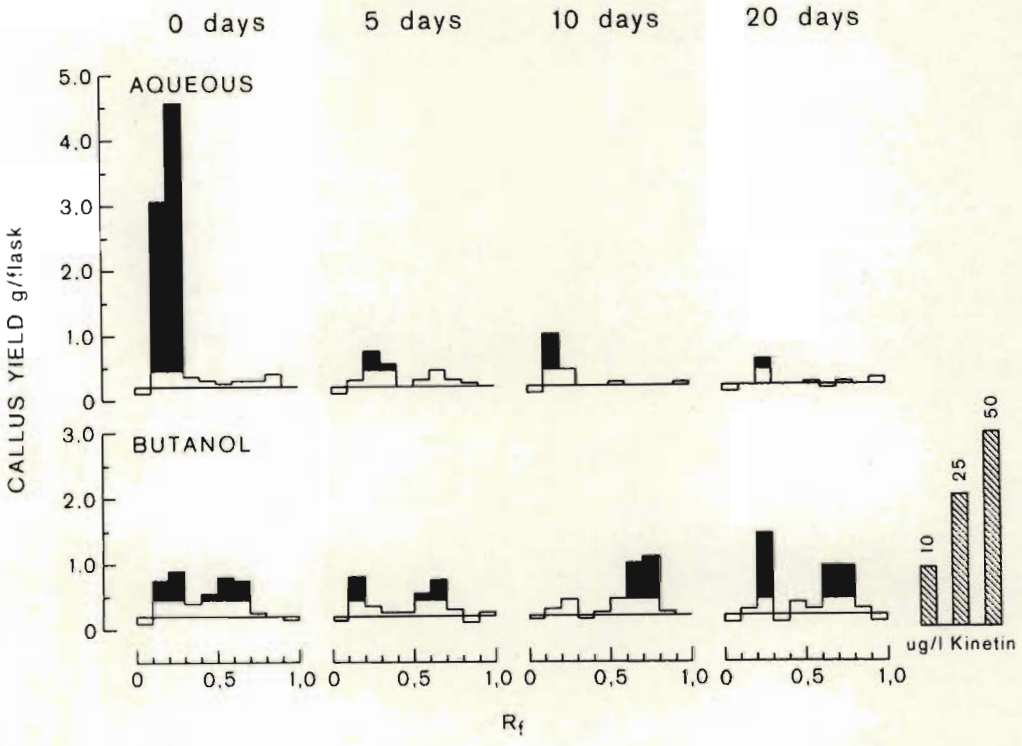



Fig. 6.2

Soybean callus assays of paper chromatograms loaded with aqueous and butanol extracts of Leucadendron daphnoides seed, incubated in air. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia: water (10:1:1 v/v). The values presented are the means of two separate extractions and assays. Blackened areas represent regions significantly different from the controls at the 1% level.

After five days of incubation in air, a significant decrease in the levels of water-soluble cytokinins was recorded and they became almost undetectable after 20 days. The level of butanol-soluble cytokinins dropped after the first five days of incubation, but subsequently increased and at 20 days reached a level 15% higher than in seed imbibed for 24 hours. These changes in butanol-soluble cytokinin levels after incubation in air were accompanied by a low level of germination.

The levels of water-soluble cytokinins of seed incubated in oxygen showed the same pattern as those incubated in air (Fig. 6.3). In the presence of oxygen, however, the level of butanol-soluble cytokinins started to increase after five days and reached a peak after ten days; that is, prior to the occurrence of any visible sign of germination. The peak level which represented a four-fold increase in the level of butanol-soluble cytokinins (calculated as kinetin equivalents) was subsequently followed by a very marked increase in germination. The fact that the butanol-soluble cytokinin levels were low after 20 days incubation can probably be attributed to the fact that the ungerminated seed was extracted after the majority of seed had already germinated. Most of the cytokinin activity present in the butanol extracts was due to fast moving compounds which occurred at

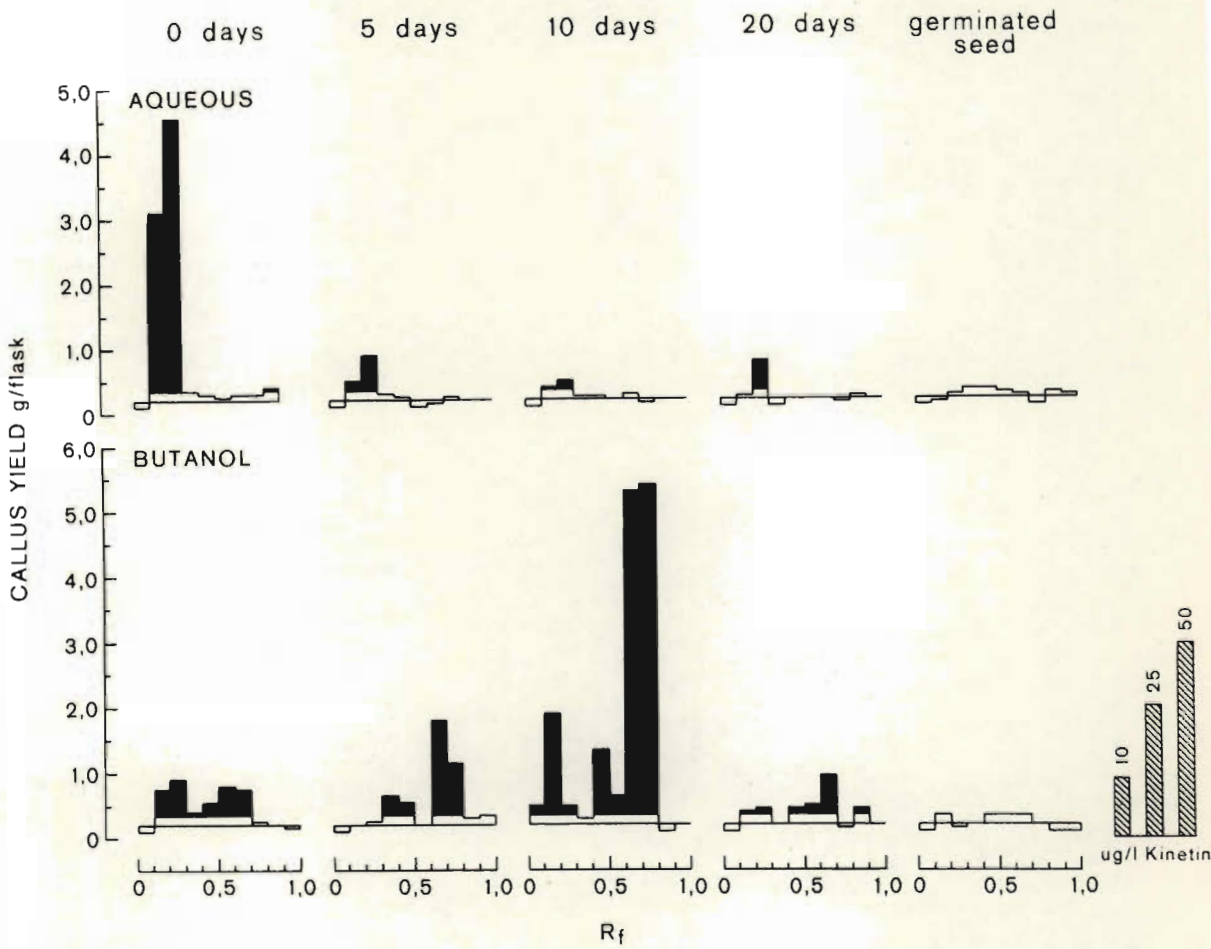



Fig. 6.3

Soybean callus assays of paper chromatograms loaded with aqueous and butanol extracts of Leucadendron daphnoides seed, incubated in oxygen. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia: water (10:1:1 v/v). The values presented are the means of two separate extractions and bioassays. Blackened areas represent regions significantly different from the controls at the 1% level.

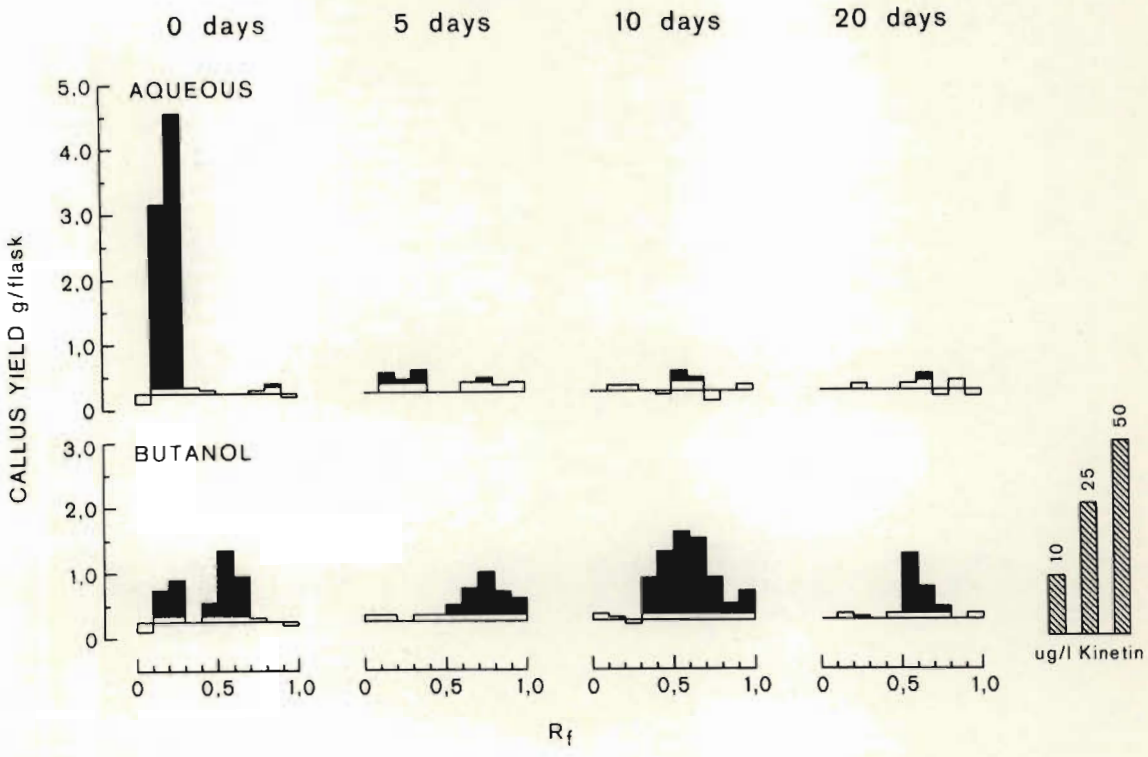



Fig. 6.4

Soybean callus assay of paper chromatograms loaded with aqueous and butanol extracts of Leucadendron daphnoides seed incubated in nitrogen. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol: ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and bioassays. Blackened areas represent regions significantly different from the controls at the 1% level.

$R_f$ 0,7 and 0,8.

In the presence of nitrogen, the levels of water-soluble cytokinins were almost undetectable after ten days incubation (Fig. 6.4). The level of butanol-soluble cytokinins remained virtually unchanged with incubation in nitrogen and no germination occurred.

As reported earlier, seed of Protea compacta imbibed for 24 hours showed no significant cytokinin activity in the aqueous fraction (Figs. 6.5, 6.6 and 6.7). The butanol fraction did, however, yield significant activity. The $R_f$ values of this activity corresponded with the $R_f$ values (0,6 - 0,7) of authentic zeatin and zeatinriboside. The levels of activity in the aqueous fractions increased slightly with incubation in all three gases. The levels of butanol-soluble cytokinins in seed incubated in air, oxygen and nitrogen all dropped after ten days incubation. The levels increased again, however, with further incubation and all reached a peak after 15 days. In oxygen the peak represented an increase of 16% in the level of butanol-soluble cytokinins when compared with the control (calculated as kinetin equivalents). The peak in air represented a decrease of 20% when compared with the level of the control, and in nitrogen the decrease was 40%. The peak level of butanol-soluble cytokinins in oxygen corresponded with the beginning of the period in which the maximum rate of germination

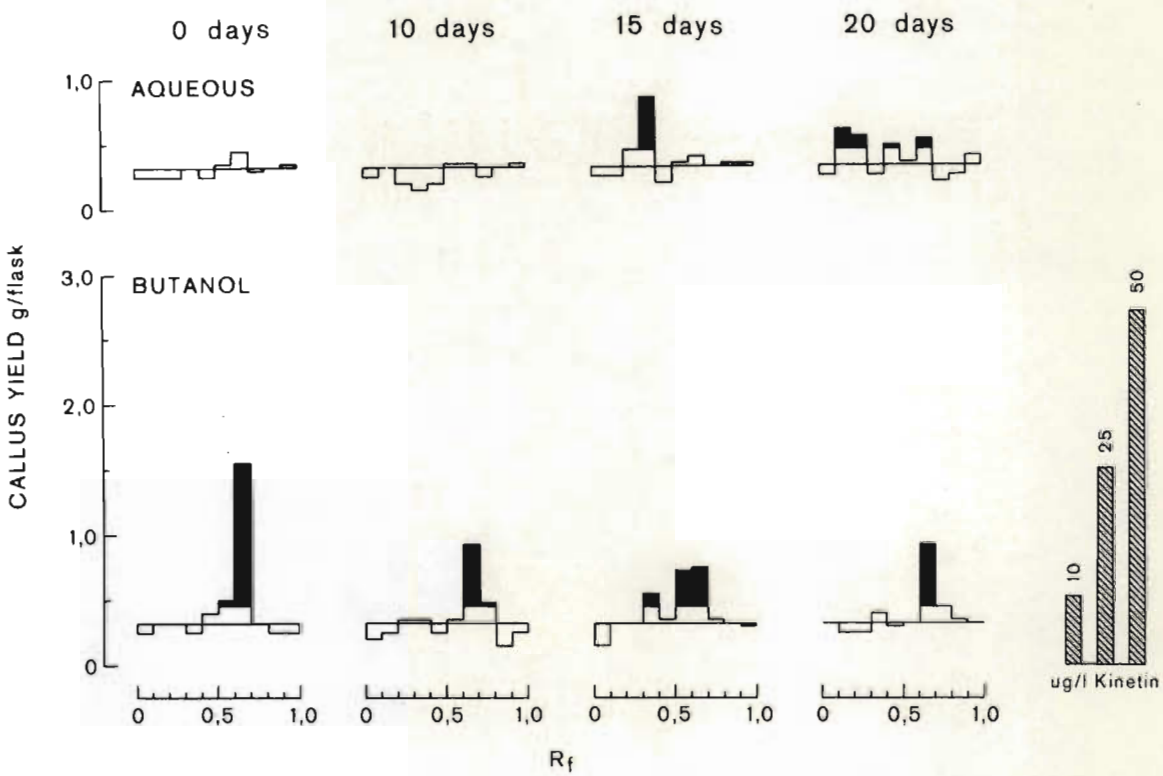



Fig. 6.5

Soybean callus assays of paper chromatograms loaded with aqueous and butanol extracts of Protea compacta seed incubated in air. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and assays. Blackened areas represent regions significantly different from the controls at the 1% level.

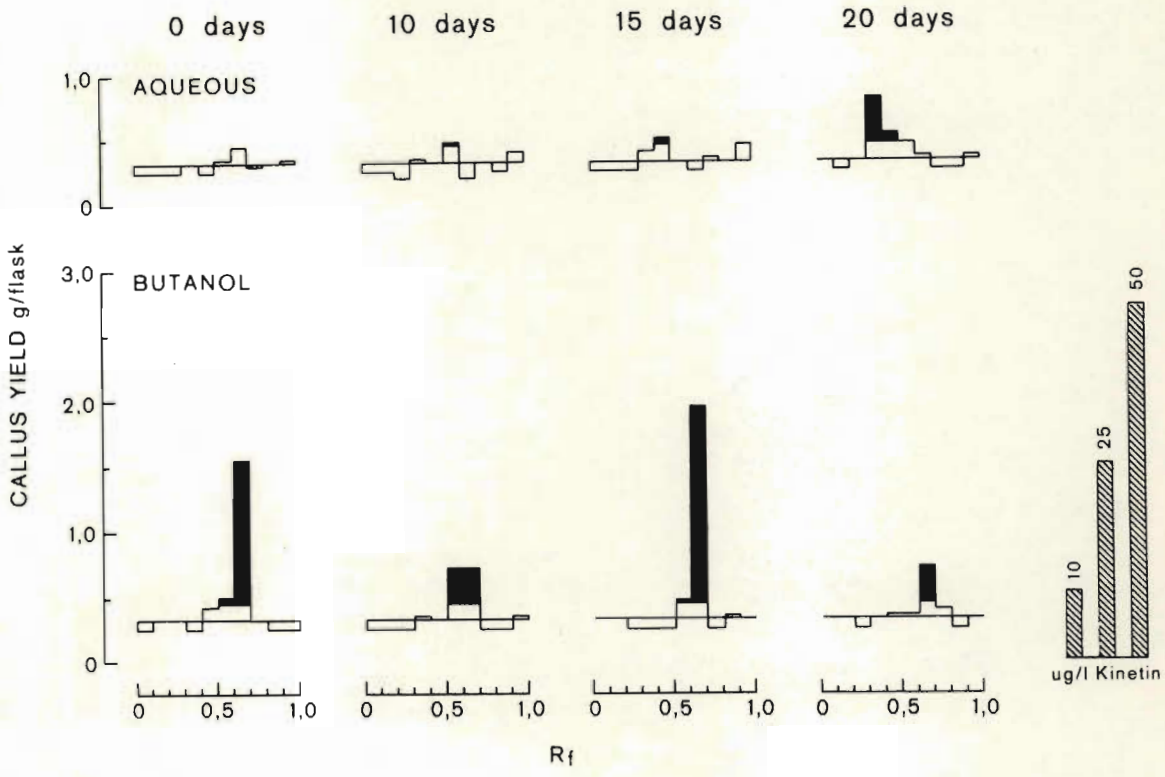



Fig. 6.6

Soybean callus assays of paper chromatograms loaded with aqueous and butanol extracts of Protea compacta seed incubated in oxygen. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and assays. Blackened areas represent regions significantly different from the controls at the 1% level.

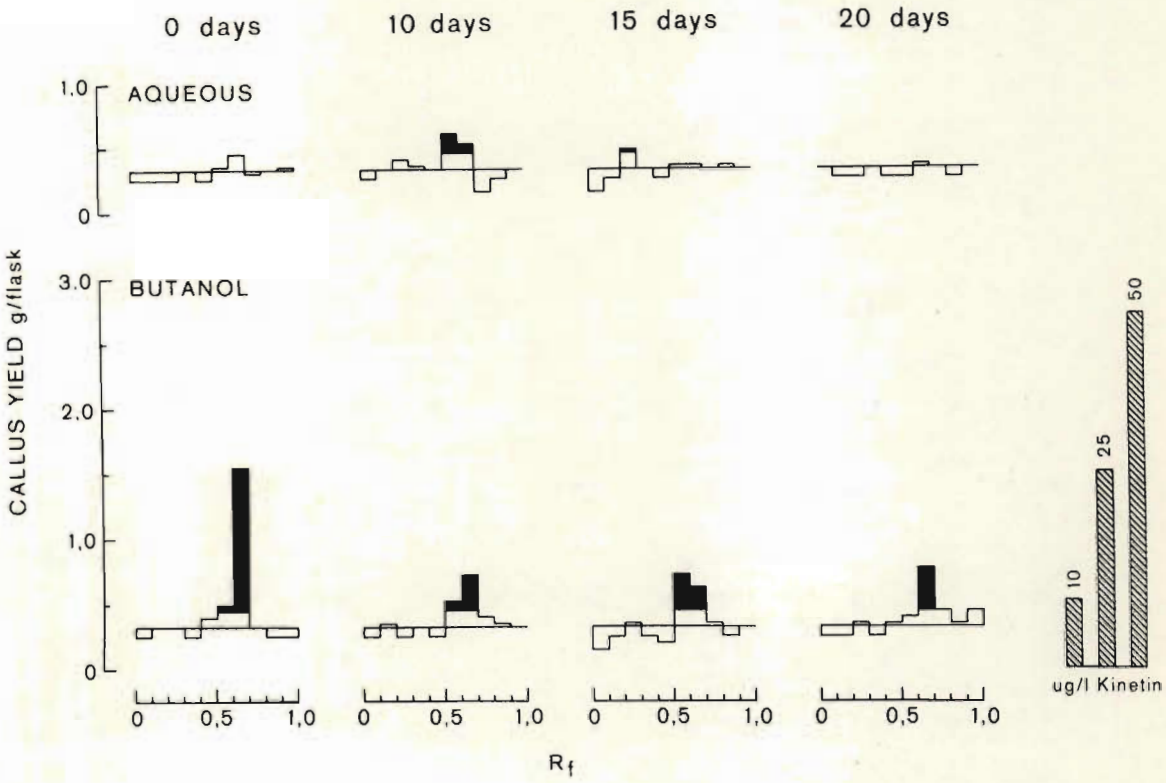



Fig. 6.7

Soybean callus assyas of paper chromatograms loaded with aqueous and butanol extracts of Protea compacta seed incubated in nitrogen. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia:water (10:1:1 v/v). The values presented are means of two separate extractions and assays. Blackened areas represent regions significantly different from the controls at the 1% level.

occurred (Fig. 6.1).

6.2.3 Gibberellin-like activity

After being imbibed for 24 hours seed of Leucadendron daphnoides showed a relatively low level of activity in the acidic ethyl acetate fraction and a high level of activity in the aqueous fraction (Fig. 6.8). With incubation in air there was a relatively marked increase in the level of activity in the ethyl acetate fraction over 20 days, but only a slight increase in the level of activity in the aqueous fraction. Seed germination (Fig. 6.1) was slow in air and the final percentage after 35 days incubation was low (20%). With incubation in oxygen (Fig. 6.9), the level of activity in the ethyl acetate fraction increased very markedly and reached a peak after ten days. On the basis of $GA_3$ equivalents the peak level of activity in oxygen was twice as high as the peak level in air. In oxygen the level of activity in the aqueous fraction increased considerably and reached a peak after five days incubation. Seed germination (Fig. 6.1) was markedly increased by incubation in oxygen with the maximum rate occurring between 15 and 25 days after the commencement of the experiment. The activity in the ethyl acetate fractions from seed incubated in nitrogen (Fig. 6.10) showed similar trends to those shown by seed incubated in oxygen. The levels of activity were,

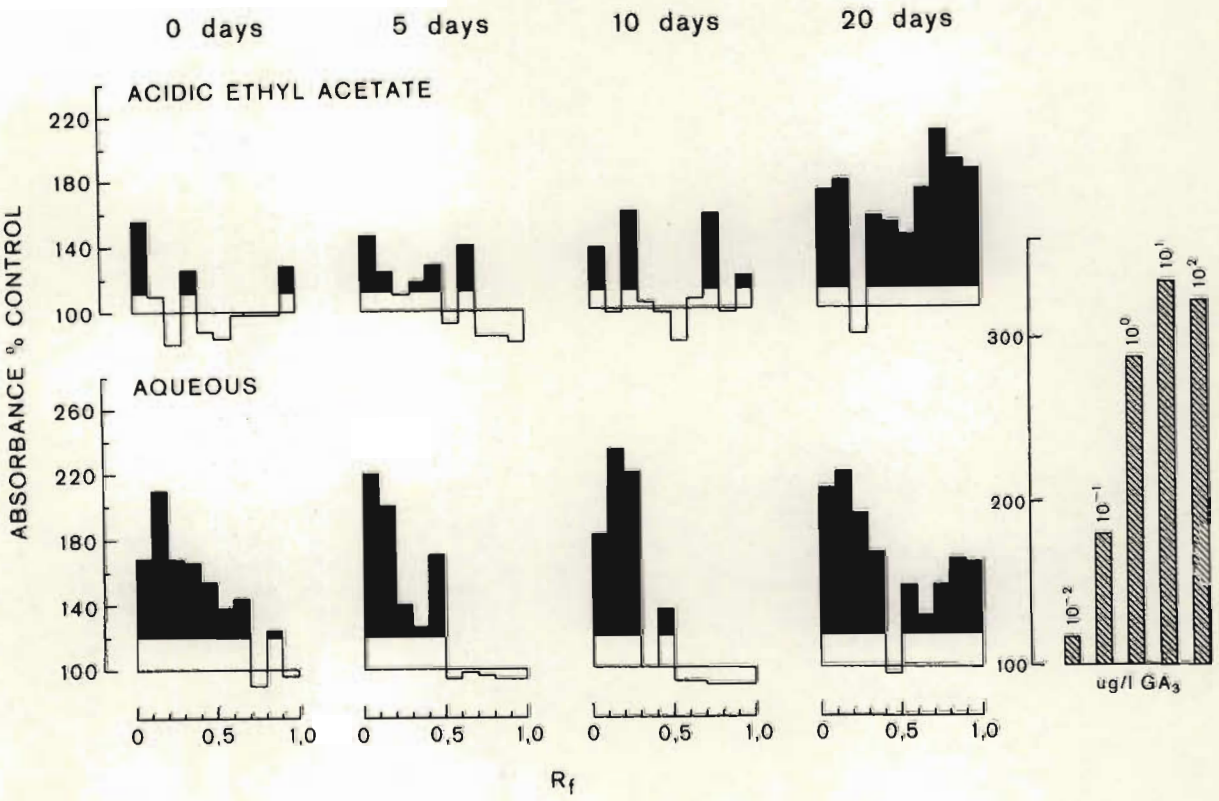



Fig. 6.8

Rumex leaf senescence bioassays of paper chromatograms loaded with acidic ethyl acetate and aqueous fractions of extracts of Leucadendron daphnoides seed incubated in air. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and assays. Blackened areas represent regions significantly different from the water controls at the 1% level.

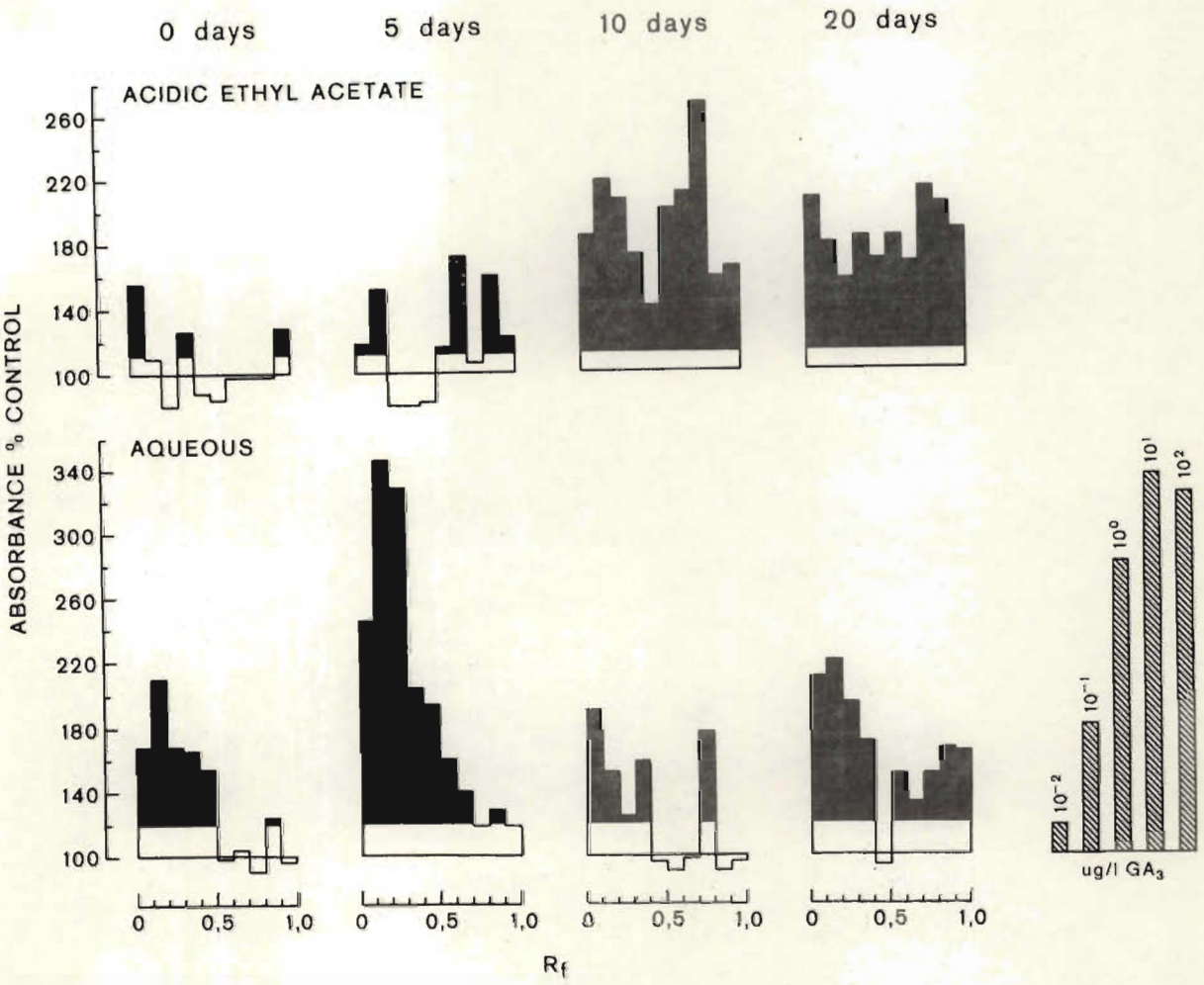



Fig. 6.9

Rumex leaf senescence bioassays of paper chromatograms loaded with acidic ethyl acetate and aqueous fractions of extracts of Leucadendron daphnoides seed incubated in oxygen. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in isopropanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and assays. Blackened areas represent regions significantly different from the water controls at the 1% level.

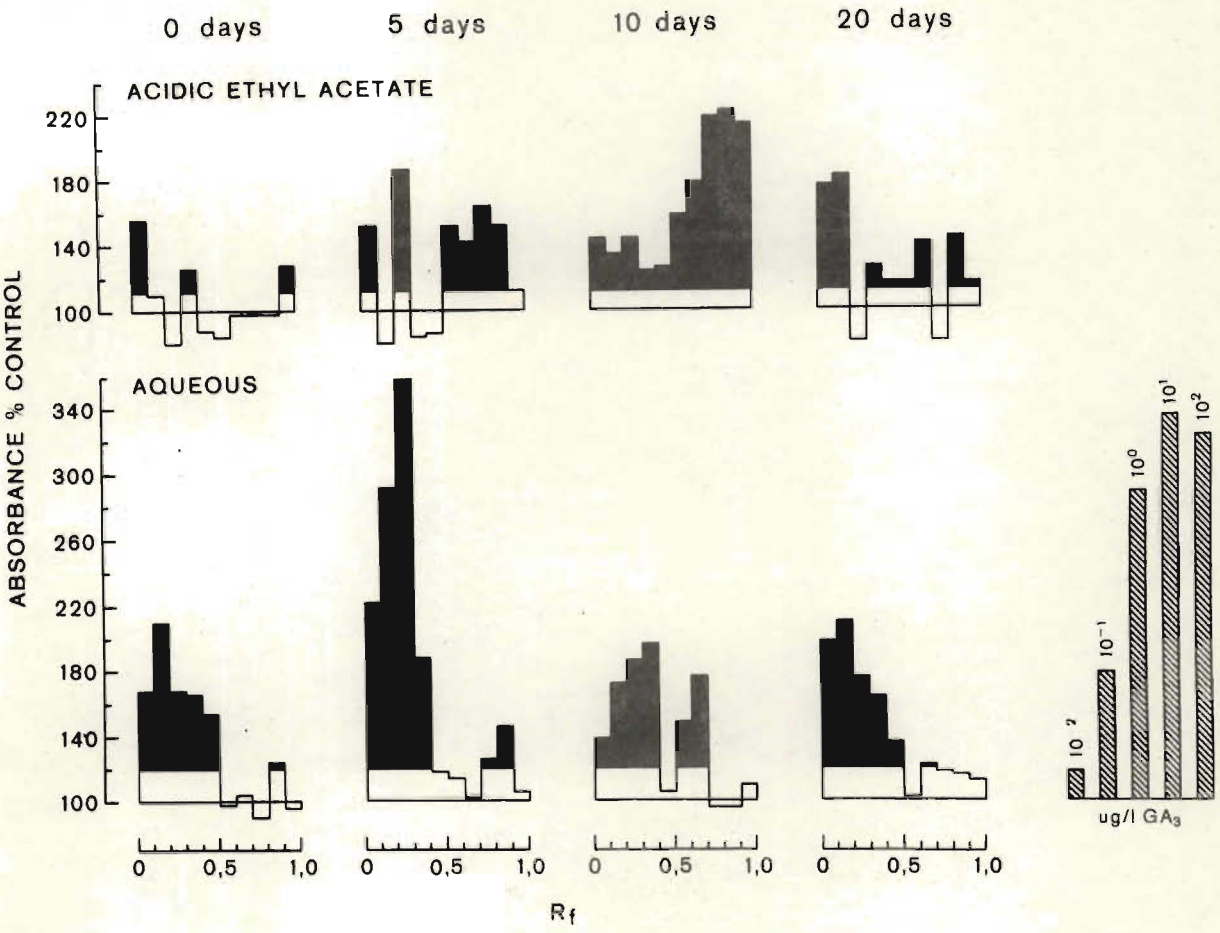



Fig. 6.10

Rumex leaf senescence bioassays of paper chromatograms loaded with acidic ethyl acetate and aqueous fractions of extracts of Leucadendron daphnoides seed incubated in nitrogen. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in isopropanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and assays. Blackened areas represent regions significantly different from the water controls at the 1% level.

however, considerably lower in nitrogen-incubated seed. In the aqueous fraction the trends and the levels of activity were virtually the same in both oxygen and nitrogen. No germination, however, took place at all with seed incubated in nitrogen.

In seed of Protea compacta imbibed for 24 hours there was also a relatively low level of activity in the acidic ethyl acetate fraction and a considerably higher level of activity in the aqueous fraction (Fig. 6.11). With incubation in air, the level of activity in the ethyl acetate fraction increased progressively and reached a peak at 15 days. In the aqueous fraction, however, the activity decreased over a period of 20 days to a level approximately one third of that of the control. Germination in air was initially slow, but increased substantially between 15 and 25 days after the commencement of the experiment (Fig. 6.1). With incubation in oxygen (Fig. 6.12) there was a similar pattern of activity in the acidic ethyl acetate fractions, with a peak at 15 days. The pattern of activity in the aqueous fraction, however, was different. Here the level of activity dropped initially at five days, but then increased and reached a peak at 15 days. Germination was increased by incubation in oxygen (Fig. 6.1) with the maximum rate being recorded between 15 and 25 days after commencement of the experiment; i.e., during the period immediately

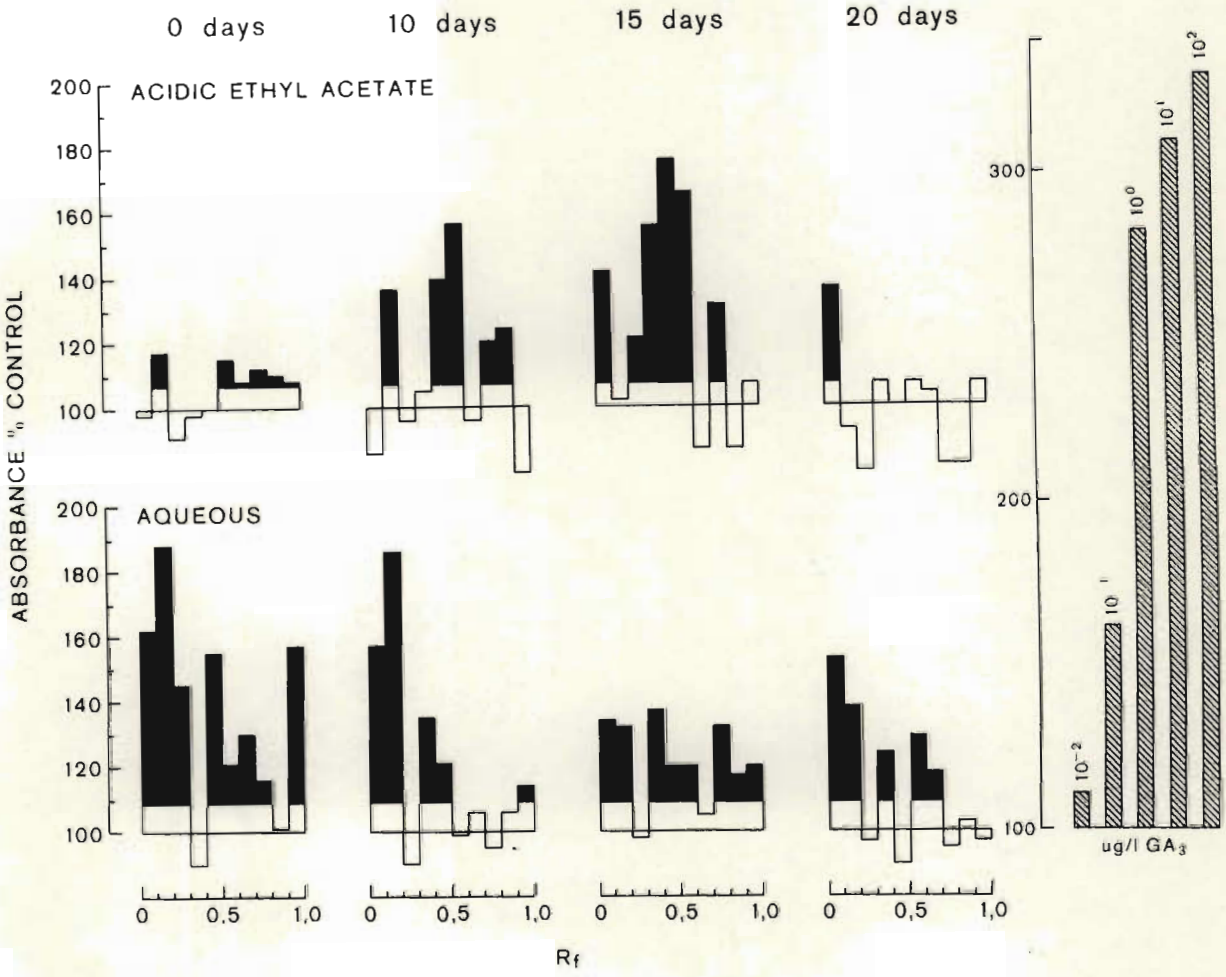



Fig. 6.11

Rumex leaf senescence bioassays of paper chromatograms loaded with acidic ethyl acetate and aqueous fractions of extracts of Protea compacta seed incubated in air. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and assays. Blackened areas represent regions significantly different from the water controls at the 1% level.

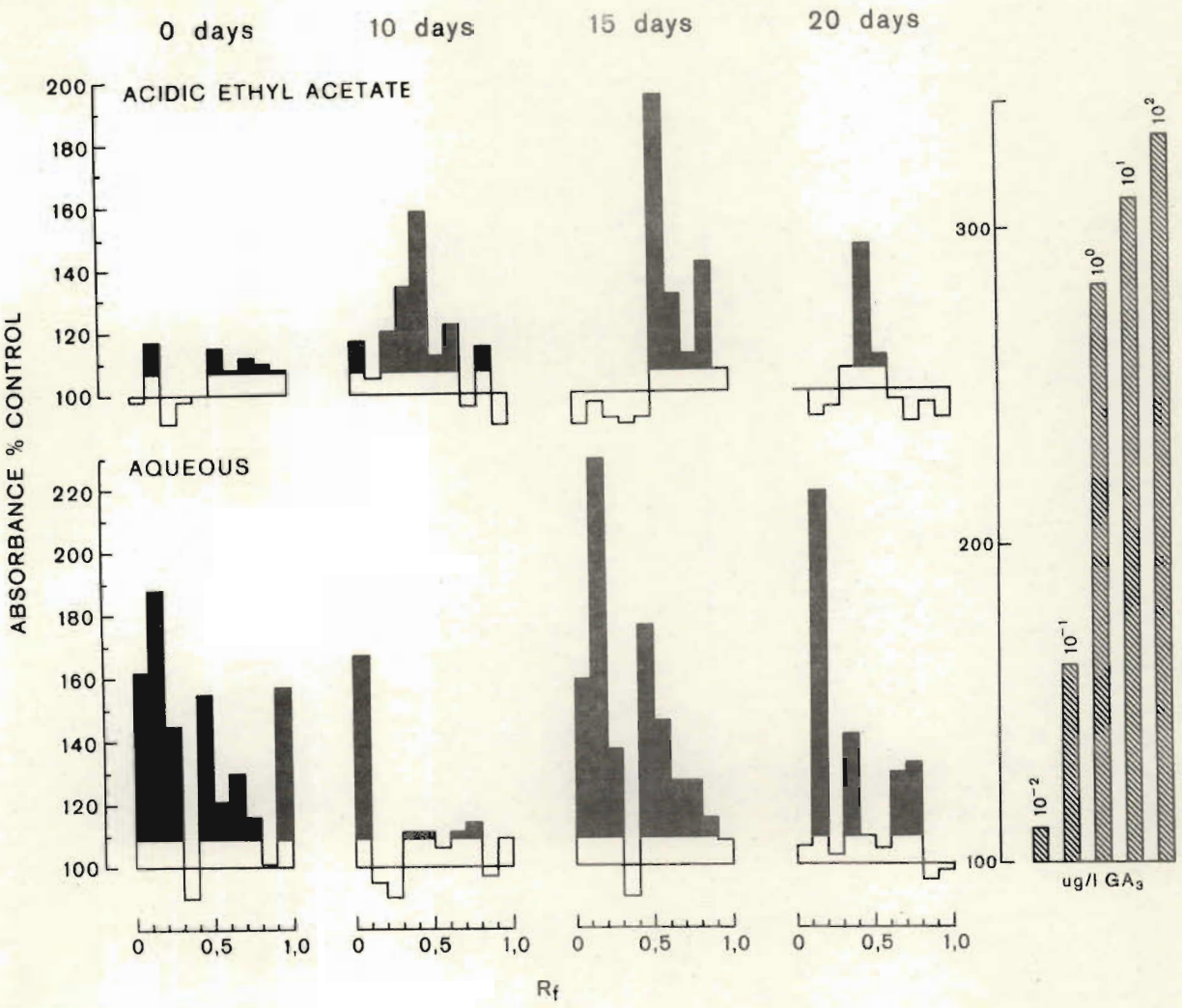



Fig. 6.12

Rumex leaf senescence bioassays of paper chromatograms loaded with acidic ethyl acetate and aqueous fractions of extracts of Protea compacta seed incubated in oxygen. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in isopropanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and assays. Blackened areas represent regions significantly different from the water controls at the 1% level.

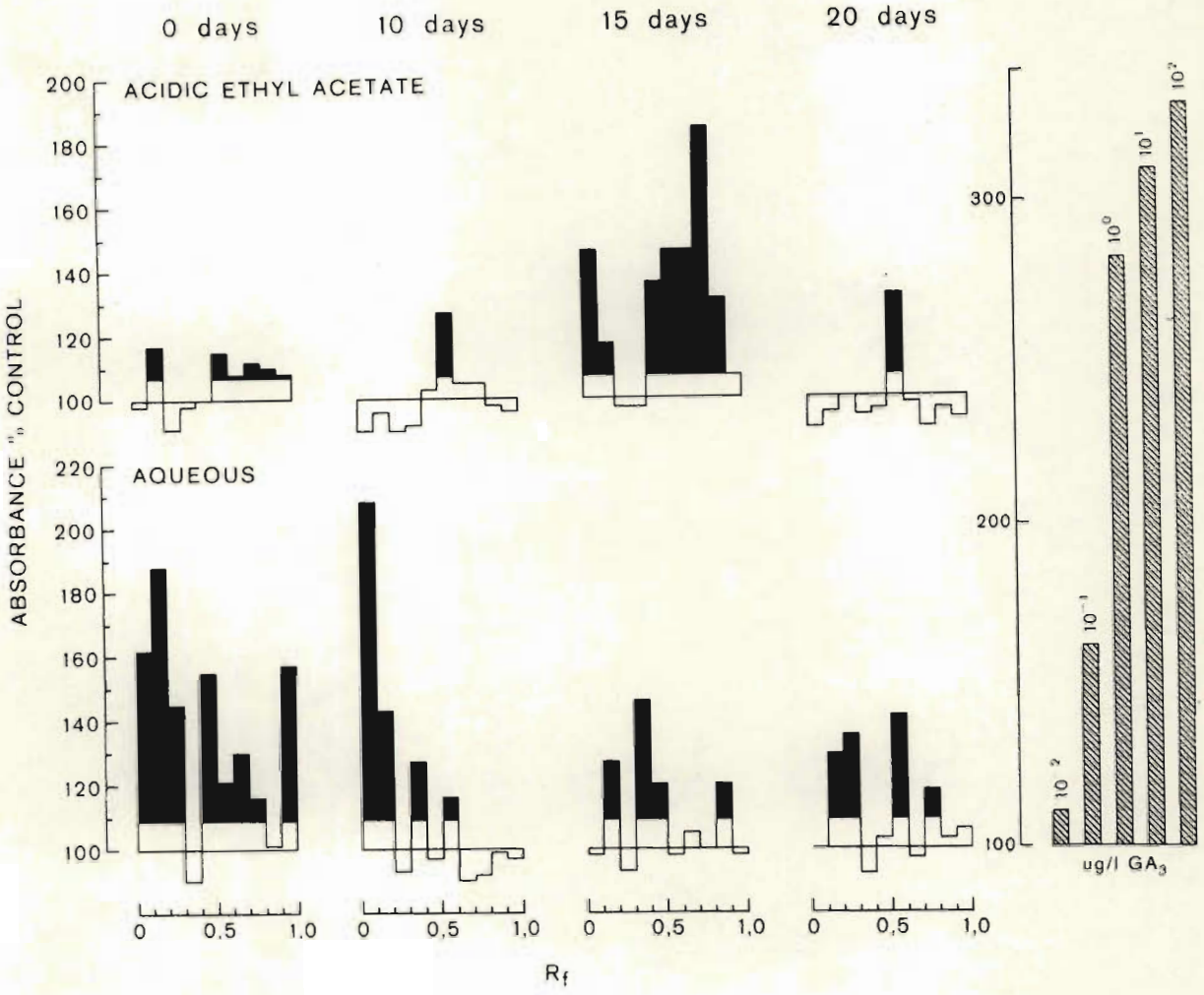



Fig. 6.13

Rumex leaf senescence bioassays of paper chromatograms loaded with acidic ethyl acetate and aqueous fractions of extracts of Protea compacta seed incubated in nitrogen. The equivalent of ten grammes of embryo material was chromatographed on Whatman No. 1 paper in iso-propanol:ammonia:water (10:1:1 v/v). The values presented are the means of two separate extractions and assays. Blackened areas represent regions significantly different from the water controls at the 1% level.

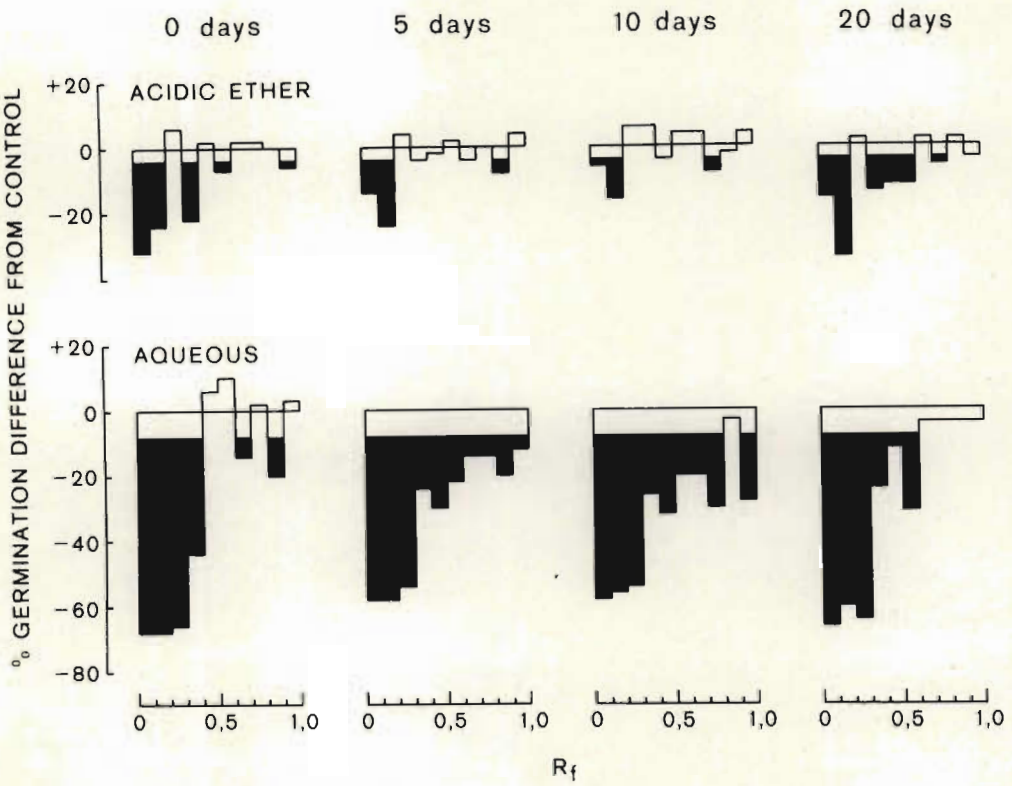



Fig. 6.14

Inhibitors from acidic ether and aqueous fractions of embryo extracts of Leucadendron daphnoides seed incubated in air. Extracts were chromatographed on paper in iso-propanol:ammonia: water (10:1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level.

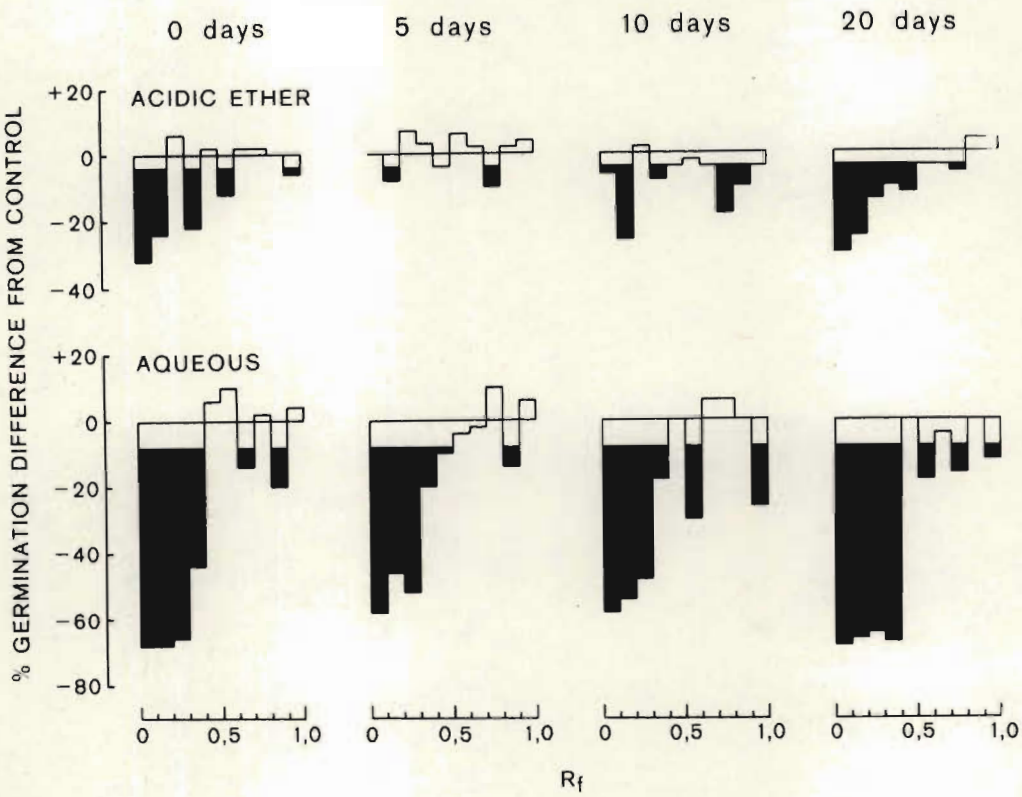



Fig. 6.15

Inhibitors from acidic ether and aqueous fractions of embryo extracts of Leucadendron daphnoides seed incubated in oxygen. Extracts were chromatographed on paper in iso-propanol:ammonia: water (10:1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level.

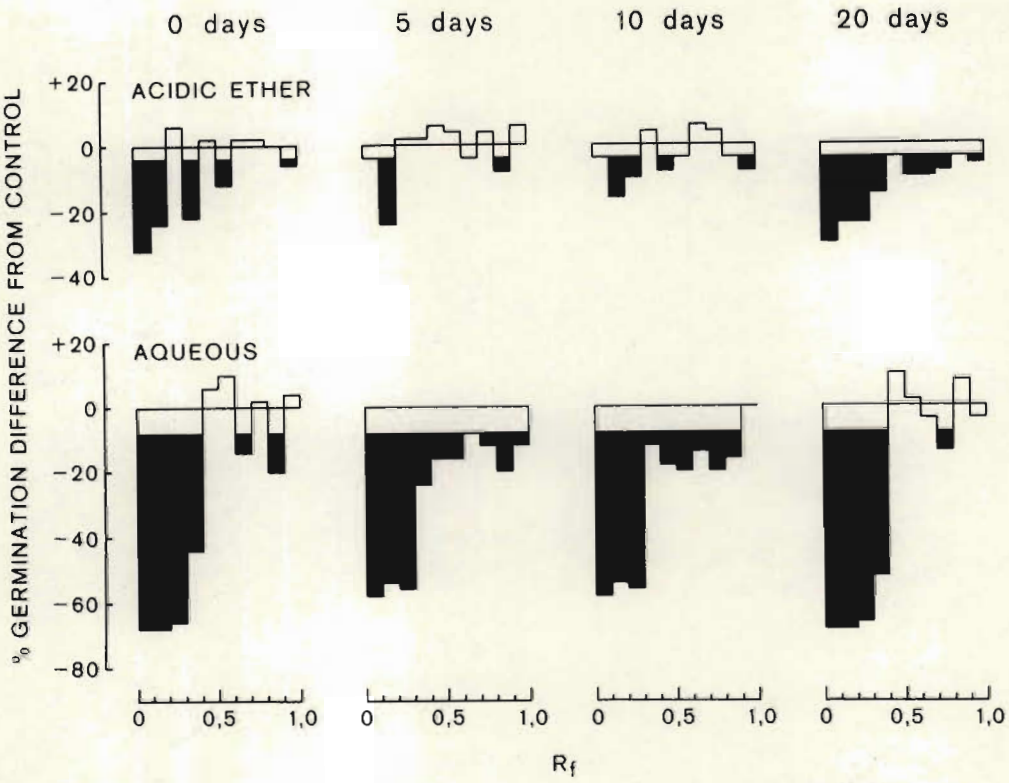



Fig. 6.16

Inhibitors from acidic ether and aqueous fractions of embryo extracts of Leucadendron daphnoides seed incubated in nitrogen. Extracts were chromatographed on paper in iso-propanol:ammonia: water (10:1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level.

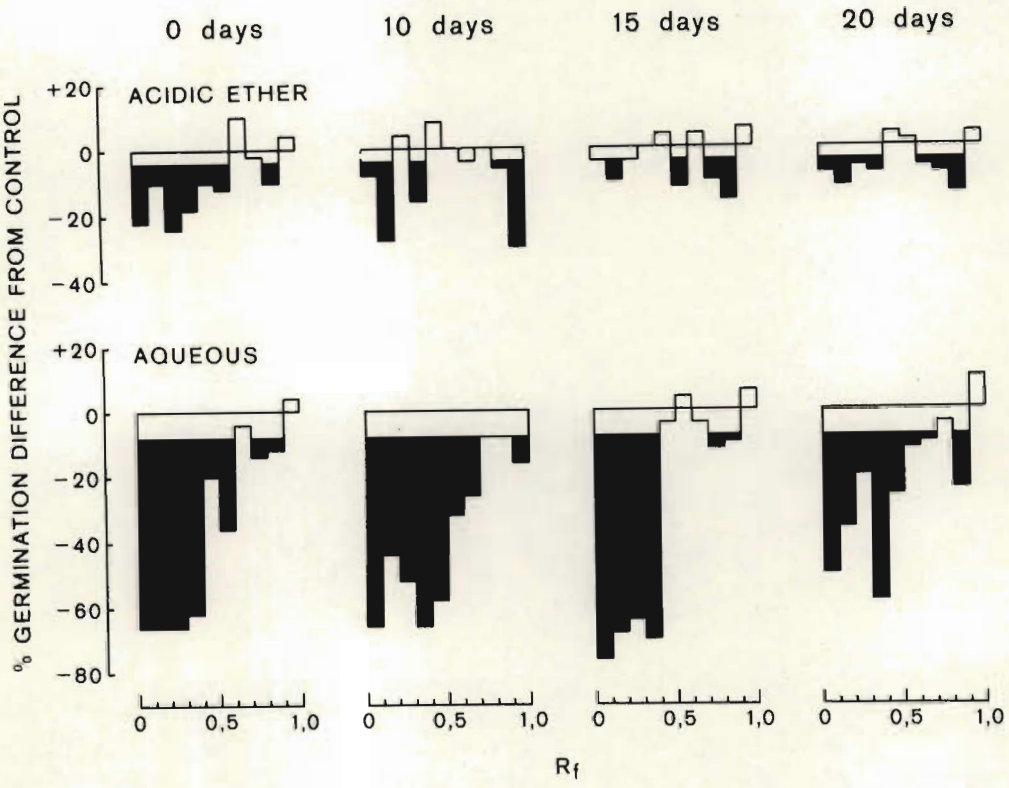



Fig. 6.17

Inhibitors from acidic ether and aqueous fractions of embryo extracts of Protea compacta seed incubated in air. Extracts were chromatographed on paper in iso-propanol:ammonia:water (10:1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level.

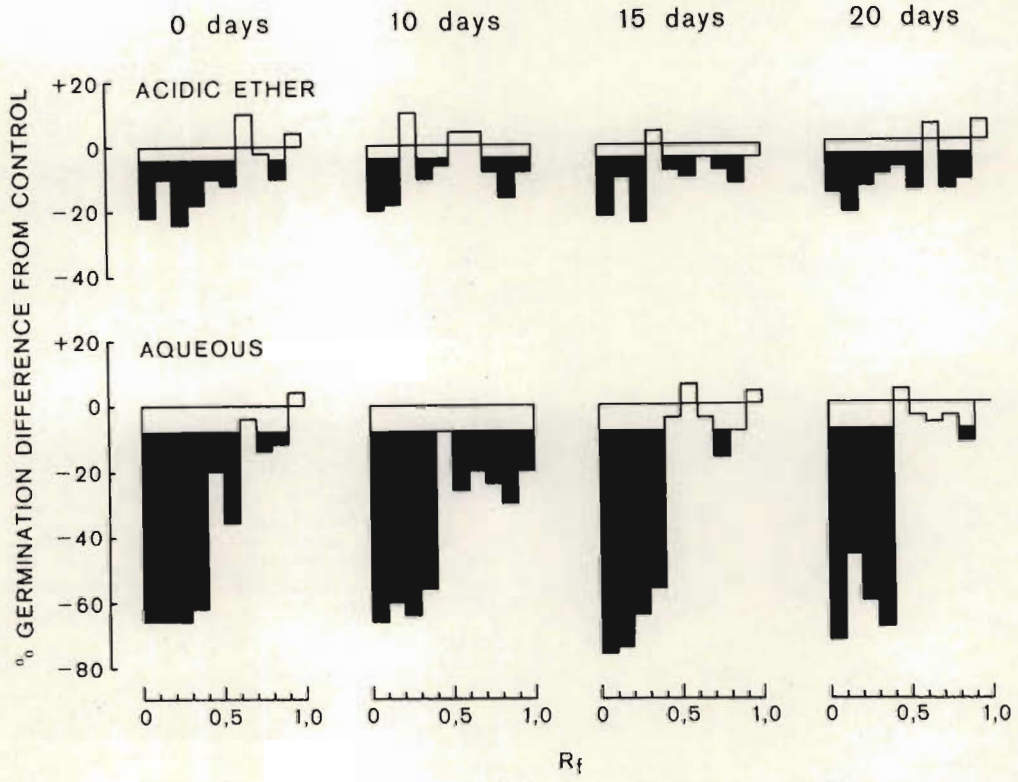



Fig. 6.18

Inhibitors from acidic ether and aqueous fractions of embryo extracts of Protea compacta seed incubated in oxygen. Extracts were chromatographed on paper in iso-propanol:ammonia:water (10:1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level.

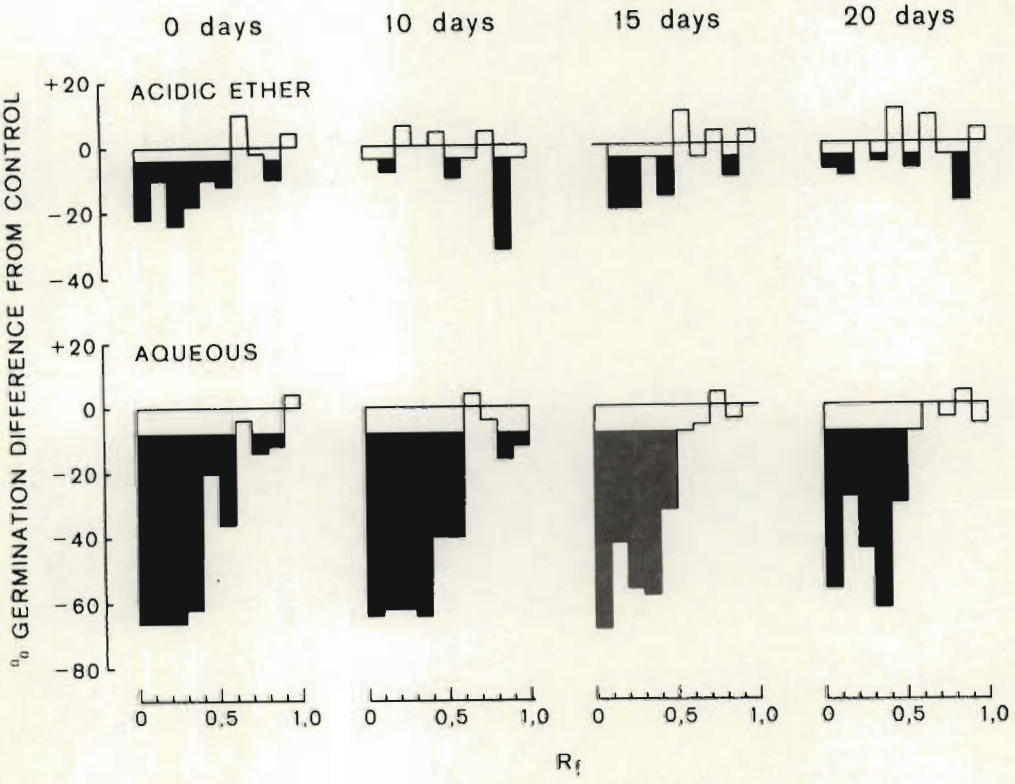



Fig. 6.19

Inhibitors from acidic ether and aqueous fractions of embryo extracts of Protea compacta seed incubated in air. Extracts were chromatographed on paper in iso-propanol:ammonia:water (10:1:1 v/v) and assayed with the Grand Rapids lettuce seed germination bioassay. Shaded areas represent differences significant from water controls at the 1% level.

following the peak of gibberellin activity. Incubation in nitrogen (Fig. 6.13) gave virtually the same trends in the ethyl acetate and aqueous fractions as did incubation in air. No germination, however, was recorded during incubation in nitrogen.

### 6.2.4 Inhibitor activity

Although there was a certain amount of variation in the number of bands of inhibition and their position on chromatograms, with incubation in air, oxygen and nitrogen, there were no major trends of changes in inhibitor levels in the acidic ether and aqueous fractions of embryo extracts of Leucadendron daphnoides (Figs. 6.14, 6.15 and 6.16) and Protea compacta (Figs. 6.17, 6.18, 6.19). The relatively high level of inhibition at low $R_f$ values in the aqueous fractions of both species, suggests again that the chromatographic separation of the water-soluble inhibitors was poor.

## 6.3 Discussion

### 6.3.1 Cytokinin levels

Oxygen tensions higher than occur under normal atmospheric conditions resulted in an increase in butanol-soluble cytokinins in Leucadendron daphnoides

seed. The highest level of activity was recorded after ten days. This was immediately prior to any visible sign of germination, a process which apparently starts with an expansion of the cotyledons (2.1). That cytokinins affect this growth response is well documented (Ikuma and Thimann, 1963; Letham, 1971). Incubating seed in air resulted in 20% of the seeds germinating compared to 95% under high oxygen tensions. As there was very little change in butanol-soluble cytokinin levels in seed incubated in air, the overall difference in germination between the two treatments could probably be explained on the basis of very few seeds in air actually attaining a threshold concentration of butanol-soluble cytokinins required for germination. Evidence for the existence of such a threshold requirement of cytokinin for the germination of Leucadendron daphnoides seed was obtained in the stratification experiments. As applied cytokinins are capable of improving germination (3.5) and treatments that increase germination are paralleled by an increase in butanol-soluble cytokinins, it would appear as if the failure of seed to germinate is due to insufficient amounts of these promoters being available.

The present results recorded over much shorter intervals than the stratification treatments (5.2.2), support the postulation that an interconversion from

water-soluble to butanol-soluble cytokinins is required for germination. However, they indicate that this conversion does not take place directly. Imbibition and the activation of enzymes, which is generally accepted to occur subsequently, apparently leads to the disappearance of the water-soluble cytokinins. As very little cytokinin activity could be detected after five days incubation in air and oxygen and after ten days in nitrogen, it would appear as if intermediate compound(s), not very active in the soybean assay, are formed. Under certain conditions these are then converted to butanol-soluble cytokinins. It thus appears as if in the intact seed the embryos can generate sufficient energy in air and nitrogen to facilitate the breakdown of water-soluble cytokinins, but not for the production of butanol-soluble cytokinins. The fact that increasing the kinetic energy by incubating seed at 25°C, does lead to higher butanol-soluble cytokinin levels, although not sufficient for germination (5.2.2) supports this suggestion.

High oxygen tensions had a less marked effect on germination and cytokinin levels in Protea compacta. The levels of butanol-soluble cytokinins dropped during the first ten days of incubation in air, oxygen and nitrogen. Thereafter, the levels increased again relative to the ten day levels, each reaching a peak at 15 days. However, whereas the peak level in oxygen represented

an increase of 16% over the level in the control, the peaks in air and nitrogen were 20% and 40% below the control, respectively. The increase in level of butanol-soluble cytokinins in oxygen occurred immediately prior to a period in which the maximum rate of germination occurred. Sixty-seven percent of the seeds germinated in oxygen, whereas 40% germinated in air and no germination took place in nitrogen.

It has been postulated that a threshold level of butanol-soluble cytokinins is required for germination. The higher overall butanol-soluble cytokinin level in oxygen may be due to a larger proportion of seeds reaching this threshold in oxygen than occurs in air. The still lower levels of butanol-soluble cytokinins in seed incubated in nitrogen suggest that still fewer (if any) seeds reach the threshold level for germination.

In the histograms showing water-soluble cytokinin levels (Figs. 6.5, 6.6 and 6.7) there are indications that the butanol-soluble cytokinins were not completely removed in the partitioning procedure.

### 6.3.2 Gibberellin levels

In Leucadendron daphnoides the level of activity in the acidic ethyl acetate fraction after incubation in oxygen for ten days was more than twice that in air after 20 days (Fig. 6.20). In air the

increase in acidic gibberellin-like substances which represented an eight-fold increase over the level in the control was accompanied by a slight increase in the rate of germination (Fig. 6.1). In oxygen, however, the 17-fold increase in acidic gibberellin-like substances after ten days occurred immediately prior to a period during which germination occurred at a very rapid rate. Also of significance was the fact that the final germination percentage in oxygen was 500% higher than in air. It appears that following imbibition and the accompanying activation of enzymes that is generally accepted to occur at this stage, a rise in the level of acidic gibberellin-like substances occurs even in the absence of oxygen (Fig. 6.20). No germination occurs in the absence of oxygen possibly because of the absence of metabolic activities which require energy from respiration. In air, where due to the nature of the seed coat, there is apparently only limited oxygen penetration and probably limited respiration, there was only 20% germination. In high oxygen tensions there is evidence to suggest that sufficient oxygen reaches the embryo and this apparently considerably enhances the production of acidic gibberellin-like substances. The high oxygen tensions would also be expected to promote respiration and indirectly metabolic activities dependant on energy from respiration. These effects of high oxygen tensions could possibly explain

the five-fold increase in germination.

Incubation of seed of Protea compacta in air, oxygen and nitrogen resulted in a similar trend in the levels of activity in the acidic ethyl acetate fraction in each gas. A peak of activity was shown in each gas at 15 days. The level of activity in the aqueous fractions dropped with incubation, except in the case of oxygen where it increased and reached a peak at 15 days. This latter peak, together with the peaks in the ethyl acetate fractions of seed incubated in air and oxygen, all occurred immediately prior to the period during which the majority of seeds germinated. Although a peak of activity in the ethyl acetate fraction also occurred with incubation in nitrogen, no germination occurred.

### 6.3.3 Inhibitor levels

The incubation of seed in air, oxygen and nitrogen produced no evidence to suggest that the inhibitors extracted from embryos are involved in the regulation of germination. Levels of inhibitors extracted from embryos were the same both in treatments which gave maximum germination and those in which no germination occurred. No evidence was obtained for an oxidative breakdown of inhibitors as suggested for seeds of Xanthium (Wareing and Foda, 1957) and Betula (Black and Wareing, 1959).

The results support the findings of the leaching experiments and stratification experiments that, if inhibitors extracted from embryos do play a role in imposing dormancy, it is not an overriding one. This is in agreement with the report that the poor germination of seed of Leucospermum cordifolium cannot be directly attributed to the presence of inhibitors (Van Staden and Brown, 1973). Embryo extracts of this species, which had previously been shown to inhibit lettuce and cress seed germination (Brown and Van Staden, 1971), did not have any effect on germination when applied to excised embryos.

Seed coat extracts of Leucadendron daphnoides and Protea compacta showed a number of bands of inhibition when assayed with the lettuce seed germination bioassay (Figs. 4.1 - 4.5). These inhibitors may indirectly influence germination in intact seed, especially if they are phenolic compounds which are known to act as oxygen binders in the seed coat (Côme and Tissaoui, 1973).

### 6.3.4 Relative levels of hormones

The levels of inhibitors in embryo extracts of both species did not change during incubation in air, oxygen or nitrogen. When seed of Leucadendron daphnoides was incubated in air, the level of acidic gibberellin-like substances rose appreciably between 10 and 20 days

incubation (Fig. 6.20). After an initial drop, the level of butanol-soluble cytokinins increased again to a level slightly higher than that of the imbibed control seed. Germination in air was initially very slow (Fig. 6.1) and the slight increase in the rate of germination after 15 days was correlated with the simultaneous increase in levels of both promoters. This probably indicates that, in air, promoter levels in relatively few seeds increased sufficiently to bring about germination.

Incubation in oxygen brought about a very marked simultaneous increase in the levels of butanol-soluble cytokinins and acidic gibberellin-like substances (Fig. 6.20). The levels of both promoters reached a peak after ten days incubation, i.e., immediately prior to the period during which the majority of seeds germinated (Fig. 6.1). The marked drop in levels of promoters between 10 and 20 days in oxygen could be due to the fact that once a large proportion of the seeds had germinated, those seeds remaining and which were used for extraction at 20 days, generally had a low germination potential.

The peak of cytokinin activity obtained with incubation in oxygen (Fig. 6.20) corresponded very closely, numerically, to the peak obtained after stratification at 5°C (Fig. 5.10), when calculated on the basis of kinetin equivalents. This again suggests that a threshold level of cytokinins is required for germination.

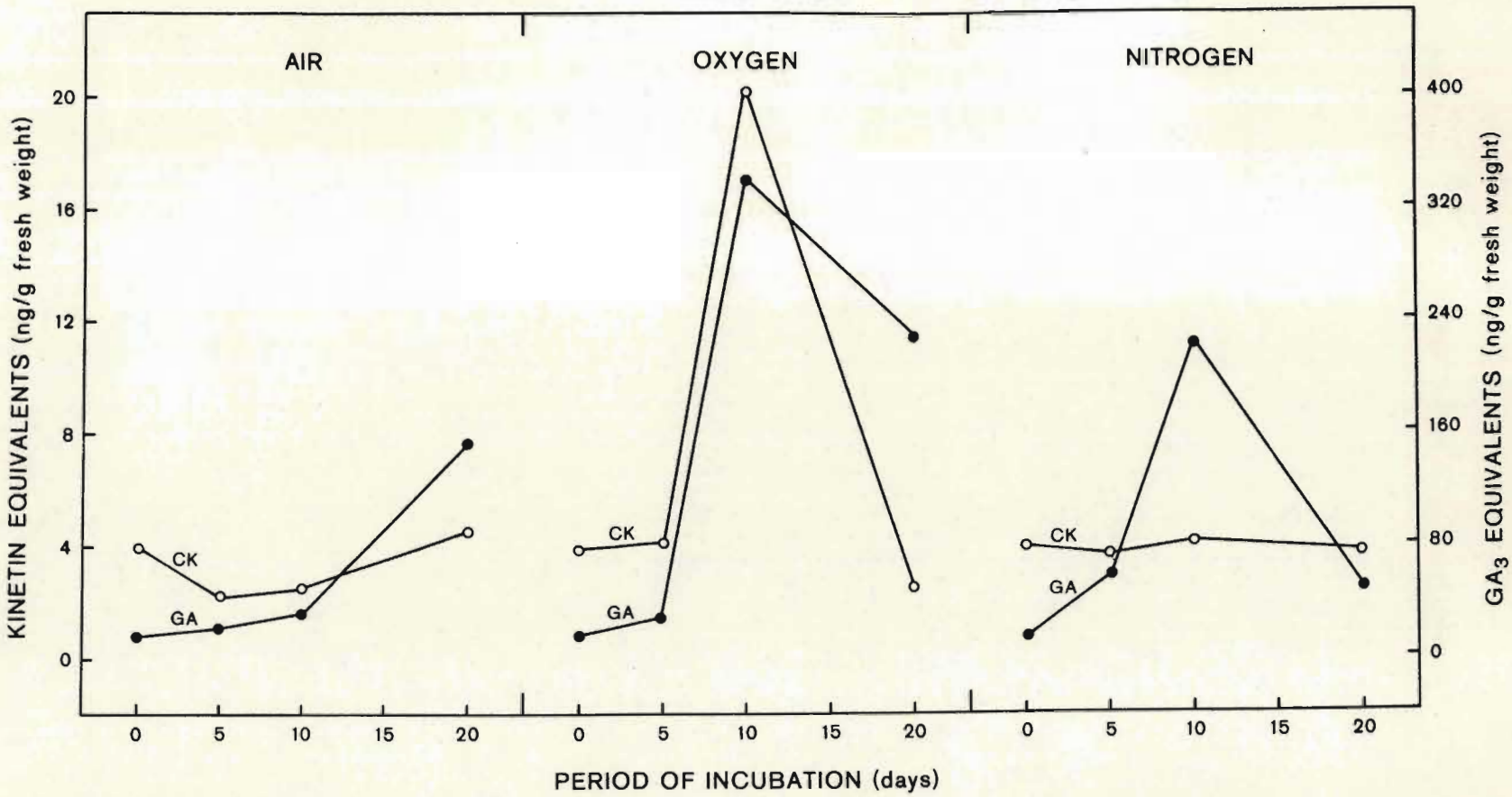



Fig. 6.20

The effect of incubation in air, oxygen and nitrogen on the level of butanol-soluble cytokinins and acidic gibberellin-like substances in seed of Leucadendron daphnoides. O Butanol-soluble cytokinins measured by the soybean callus bioassay (CK). ● Acidic gibberellin-like substances measured by the Rumex leaf senescence retardation bioassay (GA).

Stratification at 5°C resulted in a 50% increase in germination whereas incubation in oxygen brought about a 500% increase. The greater effectiveness of high oxygen tensions in promoting germination, appears to be due to the enhancing effect it has on levels of gibberellin-like promoters. The peak of gibberellin-like activity after ten days incubation in oxygen was more than 30 times the level of the peak reached after 30 days stratification at 5°C (Calculated on the basis of $GA_3$ equivalents).

These results suggest again that the role of butanol-soluble cytokinins in promoting germination of Leucadendron daphnoides seed is more than merely a "permissive" one. In this situation, contrary to the views of Khan (1971) that cytokinins by themselves do not have any profound effects on seed germination, it appears that they play the primary role in promoting germination. The role of gibberellins in promoting germination does not appear to be a primary one as postulated by Khan (1971), but their presence appears to have an additive effect in the presence of a threshold level of cytokinins.

Further evidence for the suggested roles of cytokinins and gibberellins is given by the results of incubation in nitrogen. Levels of butanol-soluble cytokinins were low and although levels of acidic gibberellin-like substances increased, no germination occurred. The

increases in levels of acidic gibberellin-like substances apparently occur following imbibition even in the absence of oxygen. However, appreciable increases in levels of butanol-soluble cytokinins appear to depend on the presence of oxygen. As suggested previously, it is possible that in air and nitrogen the energy generated in seed is sufficient only for the conversion of water-soluble cytokinins to intermediate compounds, which are apparently not very active in the soybean assay. In oxygen, however, sufficient energy is apparently generated to enable the conversion of these intermediate compounds to butanol-soluble cytokinins to take place.

Maximum germination does not appear to depend on phasic changes in promoter levels, but rather on whether the increase in the level of acidic gibberellin-like substances coincides with the increase in the level of butanol-soluble cytokinins.

Inhibitor levels did not show any major quantitative changes when seed of Protea compacta was incubated in air, oxygen or nitrogen. The overall trends of promoter levels was the same in all three gases (Fig. 6.21). However, butanol-soluble cytokinins again appeared to play the primary role in promoting germination, with acidic gibberellin-like substances being of lesser importance Maximum germination (67%) which occurred with incubation in oxygen was correlated with the highest level of

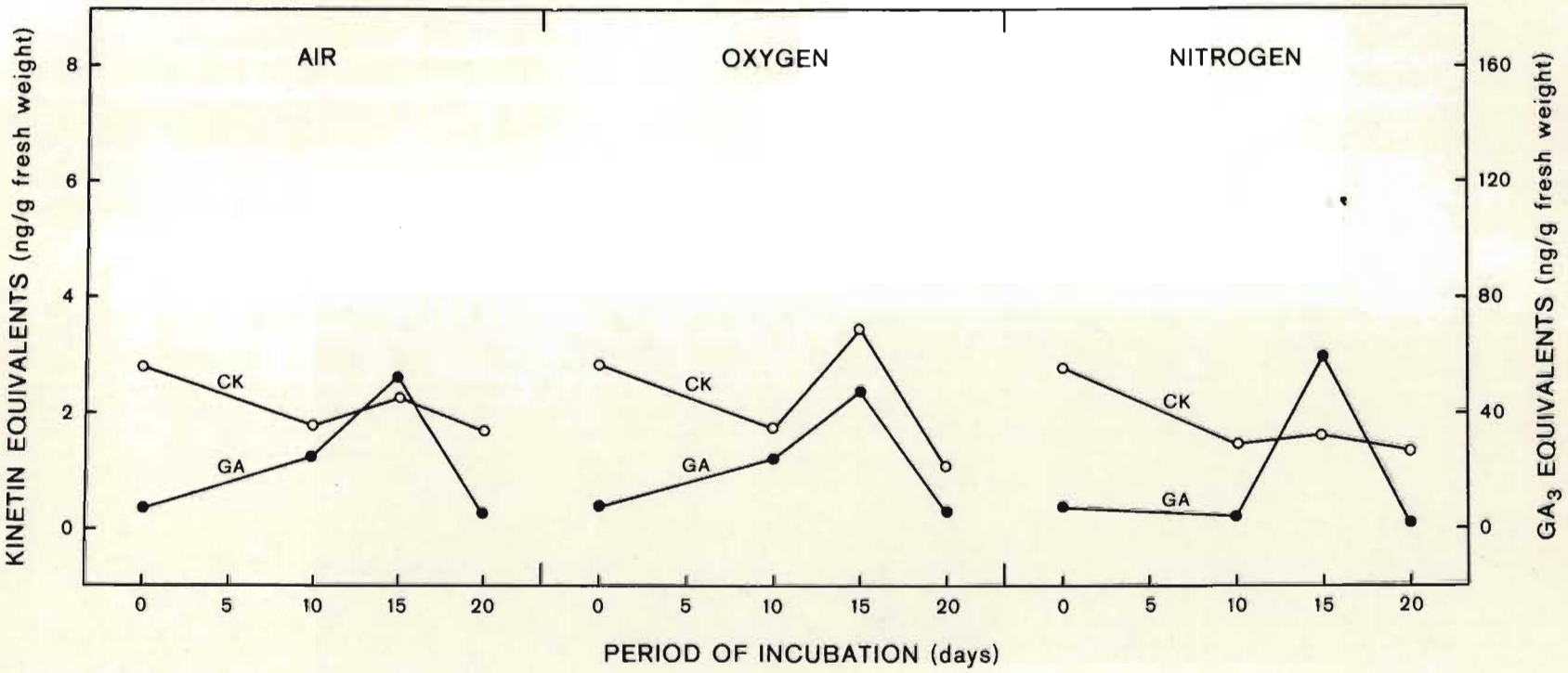



Fig. 6.21

The effect of incubation in air, oxygen and nitrogen on the level of butanol-soluble cytokinins and acidic gibberellin-like substances in seed of Protea compacta. O Butanol soluble cytokinins measured by the soybean callus bioassay (CK). ● Acidic gibberellin-like substances measured by the Rumex leaf senescence retardation bioassay (GA).

butanol-soluble cytokinins and a relatively high level of acidic gibberellin-like substances. Levels of acidic gibberellin-like substances were equally high in air and nitrogen. However, the level of butanol-soluble cytokinins in air, where 40% of seeds germinated, was higher than in nitrogen where no germination was recorded.

It appears that the changes in levels of acidic gibberellin-like substances which occur during incubation are not dependant on the presence of oxygen. As mentioned previously, the level of water-soluble cytokinins is low in seed of Protea compacta and the increase in butanol-soluble cytokinins which is correlated with an increase in germination, probably occurs as a result of de novo production. It is possible that this de novo production is enhanced directly or indirectly by high oxygen tensions and this increased production promotes germination.

When seed was incubated at 5°C changes in promoter levels suggested that maximum germination might depend on a phasic increase in the level of butanol-soluble cytokinins followed by an increase in acidic gibberellin-like substances.

Where seed was incubated in different gases the experiment was conducted at higher temperatures which alternated between 10°C (16 hours) and 20°C (8 hours). Under these conditions, much higher levels of acidic gibberellin-like substances were produced in all gases

and the peak levels occurred at the same time during incubation as those of the butanol-soluble cytokinins. This suggests that maximum germination does not depend on a phasic change of promoter levels, but primarily on the level of butanol-soluble cytokinins.

Different batches of Protea compacta seed were used in the temperature and gas incubation experiments. This probably explains why the absolute levels of butanol-soluble cytokinins and gibberellin-like substances differed in the different samples of imbibed seed.

As the seeds were incubated in closed flasks, it is possible that ethylene accumulation may have influenced germination. Although ethylene has been reported to be involved in dormancy of seeds of a number of species, for example, peanuts (Ketring and Morgan, 1971) and clover seeds (Esashi and Leopold, 1969b), no attempt was made to investigate the effects of ethylene on seed germination in the proteaceous species, because of the lack of suitable facilities for such work.

## SUMMARY OF CONCLUSIONS

The mode of germination of the two species differs. In Protea compacta the first visible sign of germination is the protrusion of the radicle through the hard, woody pericarp. In Leucadendron daphnoides the hard, woody pericarp splits first, probably as a result of cotyledon expansion and this is then followed by the protrusion of the radicle.

In Protea compacta both the coat and embryo apparently contribute to the dormant condition. The coat appears to mechanically restrict radicle elongation and to a lesser extent to retard oxygen uptake. These effects were overcome to a greater or lesser degree by scarification, high oxygen tensions and stratification. The effect of stratification may be due to physical changes in the covering structures enabling the radicle to emerge more readily, or to chemical changes in the embryo enabling the radicle to develop sufficient thrust to pierce the coat.

In Leucadendron daphnoides the seed coat apparently imposes dormancy by acting as a barrier to oxygen diffusion to the embryo. Scarification treatments which broke that barrier, as well as high oxygen tensions applied to intact seed, resulted in significant increases in germination. Stratification also improved germination

and its effect may be an indirect one brought about by the influence of low temperatures on oxygen diffusion to the embryo.

In both species, the application of exogenous gibberellic acid, kinetin and benzyladenine increased germination.

Although inhibitors could be leached from intact seed, no evidence could be found that they were actually involved in the regulation of germination or that their leaching is a pre-requisite for germination. In addition the covering structures did not appear to restrict the leaching of such compounds from the embryo. An attempt to characterize the inhibitors, indicated that the major one present behaved chemically and physically in a manner similar to coumarin. A number of other unidentified inhibitors were also shown to be present in some extracts. Further investigation of extracts of Protea compacta seed showed no evidence for the presence of ABA in detectable quantities.

When seed of both species was incubated at different temperatures (5°C and 25°C) and in different gases (air, oxygen, nitrogen) there was no major quantitative change in the level of inhibitors extracted from embryos. It appears reasonable to assume that if inhibitors do have a role in regulating germination, it is not an overriding one. Dormancy appears to be due to

a lack of promoters rather than to the presence of inhibitors.

In Leucadendron daphnoides the effectiveness of chilling and oxygen incubation in improving germination is apparently mediated through their effect in increasing the levels of growth promoters. Chilling resulted in a marked increase in the level of butanol-soluble cytokinins and a slight increase in the level of acidic gibberellin-like substances. Incubation in oxygen resulted in a similar increase in the level of butanol-soluble cytokinins. However, in addition, it resulted in a simultaneous increase in the level of acidic gibberellin-like substances, thirty times greater than was obtained with chilling. Whereas chilling resulted in a 50% increase in germination, oxygen resulted in a 500% increase in germination. The results suggest that butanol-soluble cytokinins play the primary role in promoting germination and that acidic gibberellin-like substances have an additive effect in the presence of a threshold level of cytokinins. These results do not agree with the hypothesis of Khan (1971) that gibberellins play the primary role and cytokinins essentially a "permissive" role in the regulation of germination. Levels of butanol-soluble cytokinins were low in seed incubated in nitrogen. Although there was a considerable increase in the level of acidic gibberellin-like

substances, no germination occurred. Whereas the increase in levels of acidic gibberellin-like substances apparently occurs following imbibition even in the absence of oxygen, the production of butanol-soluble cytokinins in appreciable quantities apparently requires the presence of oxygen. Maximum germination does not appear to depend on phasic changes in promoter levels, but rather on whether the increase in the level of acidic gibberellin-like substances coincides with the increase in the level of butanol-soluble cytokinins.

In Protea compacta the effectiveness of chilling and oxygen incubation in improving germination by approximately 50% is apparently mediated through their effect in increasing levels of butanol-soluble cytokinins. The latter promoter apparently plays the primary role in promoting germination. The levels of acidic gibberellin-like substances which increased with incubation even in the absence of oxygen appear to be of lesser importance in influencing germination. Maximum germination does not appear to depend on the phasic change in promoter levels but primarily on the level of butanol-soluble cytokinins.

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