Germination of the fruits of *Leucadendron tinctum*

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The pericarp and the embryo contribute to the dormant condition of achenes of *Leucadendron tinctum*. The pericarp apparently mechanically restricts embryo growth and to a lesser extent acts as a barrier to oxygen movement to the embryo. Dormancy is effectively overcome by scarification, stratification and treatment with hydrogen peroxide. Germination was inhibited and cytokinin levels were low in intact achenes subjected to elevated oxygen partial pressures.

S. Afr. J. Bot. 1985, 51: 448 – 452

Die saadhuid en die embrio dra by tot die rustoestand van *Leucadendron tinctum*-vrugte. Die saadhuid beperk waarskynlik embriogroei meganies en beïnvloed tot 'n mindere mate die beweging van suurstof na die embrio. Saadrus kan doeltreffend opgehef word deur skarifisering, stratifisering en behandeling van die vrugte met waterstofperoksied. Ontkieming is geïnhibeer en sitokinienvlakke was laag in heel vrugte wat aan verhoogde suurstof-konsentrasies blootgestel is.

S.-Afr. Tydskr. Plantk. 1985, 51: 448 – 452

Keywords: *Leucadendron*, Proteaceae, seed germination

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Accepted 2 May 1985

Introduction

The fruits of many proteaceous species show poor germination (Van Staden & Brown 1977). Work done on dormant achenes of *Protea compacta* and *Leucadendron daphnoides* (Brown & Van Staden 1973a, 1973b) and *Protea magnifica* (Deall & Brown 1981) has shown that a number of factors may be responsible for imposing dormancy. In *Protea compacta* and *Protea magnifica*, both the pericarp and the embryo contribute to the dormant condition. In *Leucadendron daphnoides* dormancy is imposed by the pericarp which acts as a barrier to oxygen diffusion to the embryo. In *L. daphnoides* incubation of fruits at elevated oxygen partial pressures effectively overcomes dormancy (Brown & Van Staden 1973a). The role of endogenous cytokinins and gibberellins in the breaking of dormancy has been studied in *Leucadendron daphnoides* and *Protea compacta* (Brown 1974) and *Protea magnifica* (Brown & Bouwer 1981, unpublished data). Brown & Van Staden (1975b) considered that in *Leucadendron daphnoides* the role of endogenous cytokinins and gibberellins in dormancy break was different to the roles envisaged by Khan (1971), in that cytokinins, as opposed to gibberellins, had a primary role in dormancy break and that germination depended on a simultaneous increase in the levels of both these hormones. In order to investigate the situation in the genus *Leucadendron* further, another species, viz. *L. tinctum* Williams, was studied.

Materials and Methods

The fruit of *Leucadendron tinctum* is an achene, 9 – 12 mm long and 7 – 8 mm wide. The achenes used in this study were collected from the Lebanon Forest Estate, Houwhoek, Cape and purchased from the Department of Forestry, Pretoria. Achenes were stored dry in sealed polythene bags at 5 °C until required. They were germinated in 9 cm Petri dishes on acid washed sand (40 – 100 mesh) moistened with distilled water. The sand was maintained in a moist condition and care was taken to ensure that no excess water was present, as excessively wet conditions have been shown by Brown & Van Staden (1973a) to inhibit germination of proteaceous fruits. Achenes were incubated under alternating temperatures of 10 °C for 8 h and 20 °C for 16 h. Light (11 Wm^{-2}) was provided, using cool white fluorescent tubes, to correspond with the period of higher temperature. The splitting of the pericarp and the protrusion of the radicle were used as the criterion of germination in the case of intact achenes (see Figure 1). With excised embryos, the positive geotropic curvature of the radicle and the associated greening of the cotyledons were used as the criterion of germination. In studies involving excised

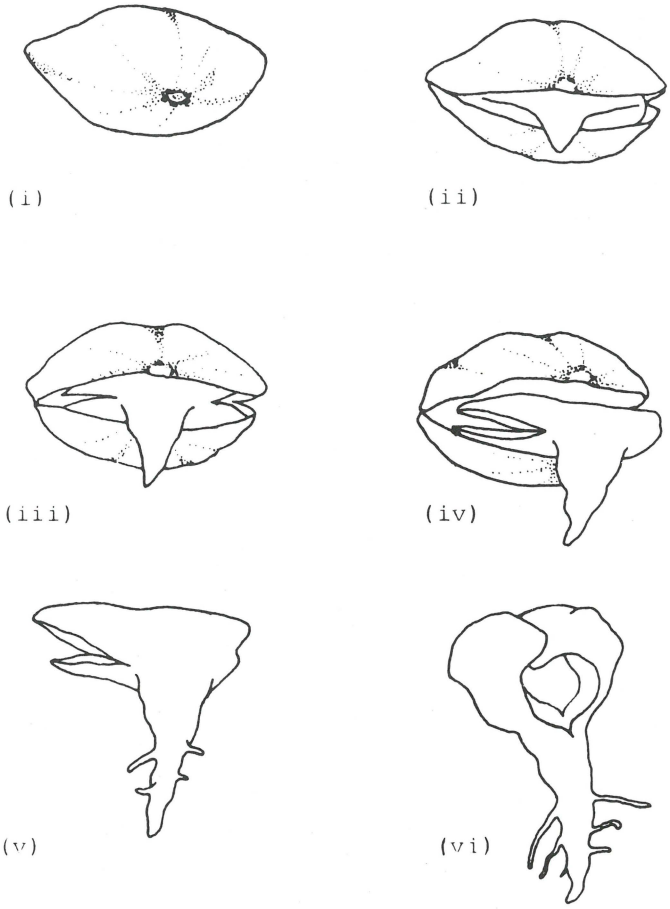

Figure 1 Mode of germination of achenes of *L. tinctum*. (i) Dry achene; (ii) cracking of the pericarp, due to cotyledon expansion enabling protrusion of the radicle; (iii) elongation of the radicle; (iv) shedding of pericarp; (v) seedling having shed pericarp; (vi) opening out of the cotyledons and further seedling development (x4).

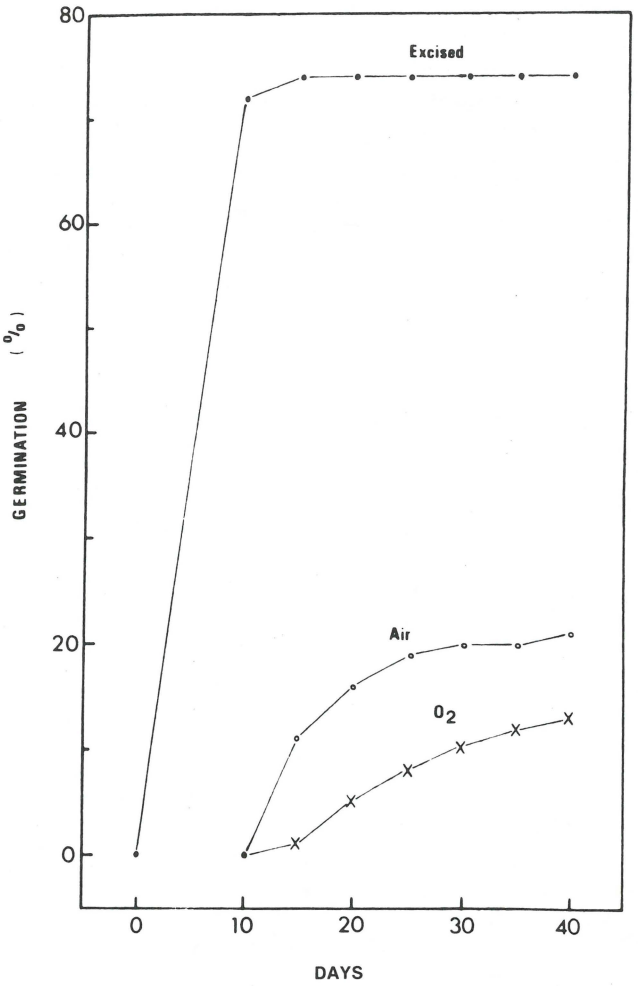

Figure 2 Germination of intact achenes of *L. tinctum* incubated in air and in elevated oxygen partial pressures, in comparison with germination of excised embryos in air. Figures represent germination means (percentages) of three trials of 50 achenes each, over a period of 40 days.

embryos, both the hard pericarp and the membranous testa were removed. Unless otherwise stated, there were five replicates of each treatment (i.e. 50 achenes) and each trial was carried out three times. The results are given as mean percentage of these three trials.

The levels of endogenous cytokinins were investigated in intact achenes and excised embryos incubated in air, and in intact achenes incubated in elevated oxygen partial pressures. The intact achenes and excised embryos were incubated on moist acid-washed sand in large trays covered with polythene film. Samples of 10 g fresh mass of embryo material were taken at 0, 3, 6, 9 and 12 days after the start of imbibition. Intact achenes were sampled after 0, 6, 12 and 18 days. For oxygen treatments, intact achenes were incubated in 500 cm^3 Erlenmeyer flasks, flushed with oxygen every two days. Flasks were kept sealed with rubber bungs and petroleum jelly. Samples for extraction were taken 0, 6, 12 and 18 days after the start of imbibition. On the removal of intact achenes from incubation in air or oxygen, the embryos were excised and 10 g (fresh mass) samples of embryos were used for cytokinin extraction. The sampling times were based on the rates of germination obtained under the various treatments (Figure 2). Cytokinins were extracted using the methods of Van Staden (1976) and the extracts were then strip-loaded onto Whatman no. 1 chromatography paper. Extracts were chromatographed using iso-propanol:25% ammonium hydroxide:water (10:1:1 v/v). The chromatograms were oven-dried at 25 °C for 24 h and then assayed for cytokinin activity using the soybean callus bioassay of Miller (1963).

Results and Discussion

In a preliminary trial only 20% of the achenes germinated under standard incubation conditions. The presence of a hard, woody pericarp which is a characteristic of the achenes of many *Leucadendron* species, suggested that dormancy might be coat-imposed. Dormancy might therefore be overcome by treatments that weaken or break the barrier imposed by the pericarp. One sample of achenes of *L. tinctum* was mechanically scarified midway between the 'radicle' and 'stylar' ends, using sandpaper. In another sample, the embryos were excised from the covering structures. The results in Table 1 show that both scarification and embryo excision markedly improved germination and indicate that dormancy is apparently imposed by the pericarp.

Where substances present within the pericarp and testa are inhibitory to embryo growth, leaching of the seed often leads to improved germination (Khan 1977). Achenes of *L. tinctum* were leached for 3 and 6 days respectively, under a fine spray of water for 4 h daily. The achenes were suspended on cheese cloth and maintained in a moist condition throughout the treatment period. The results in Table 2 indicate that leachable inhibitors are apparently not involved in the dormant condition of achenes. Leaching for 3 days did not significantly improve germination and leaching for 6 days depressed germination. The leaching treatment results for achenes of *L. tinctum* support the contention of Brown & Van Staden (1973a, 1975a) and Van Staden & Brown (1977) that inhibitors

Table 1 Germination of achenes of *L. tinctum* after scarification of the pericarp and embryo excision. Figures represent means (± SE) of three trials of 50 achenes each, after 40 days

Treatment	Germination %
Intact achenes (control)	20 ± 3
Scarified achenes	84 ± 5
Excised embryos	74 ± 7

Table 2 Germination of achenes of *L. tinctum* after being leached in water for 4 h daily. Figures represent means (± SE) of three trials of 50 achenes each, after 40 days

Treatment	Germination %
No leaching (control)	20 ± 4
Leaching for 3 days	24 ± 6
Leaching for 6 days	8 ± 2

Table 3 Germination of achenes of *L. tinctum* incubated in air, oxygen and nitrogen. Figures represent means (± SE) of three trials of 50 achenes each, after 40 days

Treatment	Germination %
Air (control)	21 ± 4
Oxygen	13 ± 2
Nitrogen	9 ± 7

Table 4 Germination of achenes of *L. tinctum*, excised after 40 days incubation of intact achenes in oxygen. Figures represent means (± SE) of three trials of 50 achenes each, after 20 days

Treatment	Germination %
Excised after O_2 incubation	85 ± 3
Excised from untreated achenes (control)	75 ± 9

Table 5 Germination of excised embryos of *L. tinctum* incubated in air and oxygen. Figures represent means (± SE) of three trials of 50 achenes each, after 20 days

Treatment	Germination %
Air	92 ± 8
Oxygen	66 ± 10

Table 6 Germination of achenes of *L. tinctum* after being imbibed in hydrogen peroxide (H_2O_2) solutions of 24 h. Figures represent means (± SE) of three trials of 50 achenes each, after 40 days

Treatment	Germination %
Control)	16 ± 4
10% H_2O_2 (v/v)	54 ± 4
30% H_2O_2 (v/v)	4 ± 4

are not responsible for the dormancy of proteaceous fruits.

In *Leucadendron daphnoides* dormancy is imposed by the pericarp which apparently restricts oxygen diffusion to the embryo. Incubation of achenes in elevated oxygen partial pressures was shown to improve germination significantly (Brown & Van Staden 1973a). Achenes of *L. tinctum* were therefore incubated in either nitrogen or oxygen (medical grade), care being taken to ensure that the pressure in the flasks never exceeded atmospheric pressure. The results in Table 3 suggest that elevated oxygen partial pressures have an inhibitory effect on germination, this being contrary to the situation in *L. daphnoides*, as reported by Brown & Van Staden (1973a). The inhibition in *L. tinctum* is apparently not due to impurities in the oxygen as 78% germination of *L. daphnoides* fruits was obtained using this oxygen source (Brown, unpublished data). Germination in the nitrogen treatment was low, but not as low as that reported for *Leucadendron daphnoides* and *Protea compacta* (Brown & Van Staden 1973a). The low germination in nitrogen flasks is probably related to the very small amount of oxygen present.

The detrimental effect of oxygen on germination of *L. tinctum* achenes was investigated further. Embryos from intact achenes which had been treated to elevated oxygen partial pressures were excised and incubated under standard conditions. The results in Table 4 show that the oxygen treatment did not have a permanent detrimental effect on the embryos from intact achenes. The effect of the oxygen treatment on the germination of excised embryos was then investigated. Excised embryos were incubated either in air or under elevated oxygen partial pressures. The results in Table 5 show that fewer embryos germinated in oxygen than in air and once again indicate that elevated oxygen levels have an inhibitory effect on germination.

The effect on germination of imbibing intact achenes in hydrogen peroxide (H_2O_2) was then investigated. Hydrogen peroxide, in the presence of peroxidase and catalase enzymes, which are present in most plant tissues (Thimann 1977), reacts to release oxygen. Germination of achenes of *Leucospermum cordifolium* (Proteaceae) was improved following imbibition in H_2O_2 solutions (Brits & Van Niekerk 1976). Achenes of *Leucadendron tinctum* were imbibed in H_2O_2 {10% (v/v) or 30% (v/v)} for 24 h. They were then washed in running water for 5 min and incubated under standard conditions. The results in Table 6 show that 10% H_2O_2 markedly improved germination, but 30% H_2O_2 had an inhibitory effect. The response of achenes to H_2O_2 suggests that the embryo may respond to a certain level of oxygen supplied by H_2O_2. However, if the concentration of H_2O_2 is too high this is either toxic or the higher level of oxygen supplied, is inhibitory to germination.

To get an indication of whether the hard, woody pericarp of the achenes of *L. tinctum* was mechanically restricting embryo growth, achenes were scarified midway between the 'radicle' and 'stylar' ends. The scarified surface was

immediately covered with a thick layer of anhydrous lanolin to restrict the free movement of oxygen, whilst the scarification released the possible mechanical restriction to cotyledon expansion. The results in Table 7 again show that scarification significantly improves germination. There is, however, no difference between the final germination percentage of achenes in which the scarified surface was covered with lanolin and those where the surface was left uncovered. These results suggest that the dominant influence of the pericarp is to restrict embryo growth and this restriction is reduced by mechanical scarification. However, the rate of germination of achenes in which the scarified surface was covered with lanolin was significantly slower than that of achenes in which the surface was left uncovered. The latter results suggest that the pericarp possibly also restricts the diffusion of oxygen to the embryo.

Table 7 Germination of achenes of *L. tinctum* with the scarified surface covered with lanolin. Figures represent means (± SE) of three trials of 50 achenes each, after 40 days

Treatment	Germination %
Intact achenes (control)	20 ± 4
Scarified achenes	84 ± 5
Scarified achenes and lanolin	80 ± 4
Excised embryos covered with lanolin	84 ± 7

Table 8 Germination of achenes of *L. tinctum* following incubation under moist conditions at 5 °C and 25 °C. Figures represent means (± SE) of three trials of 50 achenes each, 40 days after achenes were transferred to standard germination conditions

Treatment	Time (Days)	Germination %
Incubation at 5 °C	30	32 ± 7
	60	52 ± 5
Incubation at 25 °C	30	8 ± 4
	60	6 ± 8

Exposure of imbibed seeds to low temperatures is often effective in overcoming both embryo and coat imposed dormancy, this effect being related to hormonal, metabolic and morphological changes which take place in the embryo during the low temperature treatment (Lewak & Rudnicki 1977). In *Protea compacta* both the embryo and the pericarp contribute to the dormant condition and stratification was found to markedly improve germination (Brown & Van Staden 1973a). Achenes of *L. tinctum* were imbibed and incubated at 5 °C and 25 °C for periods of 30 and 60 days. After the treatment periods, the achenes were incubated under standard conditions. Incubation at 5 °C for 30 and 60 days gave significantly higher germination than incubation at 25 °C for the same period (Table 8). The fact that stratification improves germination suggests that factors within the embryo contribute to the dormant condition. Stratification apparently increases the ability of the cotyledons to split the pericarp, this being related to hormonal and metabolic changes occurring in the embryo during stratification (Brown & Van Staden 1973a, 1973b).

The role of the embryo in dormancy was investigated further by studying the changes in embryo cytokinin levels occurring during the incubation of excised embryos and intact achenes in air and under elevated partial pressures of oxygen. Germination results are given in Figure 2. Both intact achenes and excised embryos imbibe most water during the first 72 h of incubation (unpublished data). The results in Figures 3 & 4 show that there was an increase in the levels of endogenous cytokinins during this period. This suggests that cytokinins are involved in the early stages of the germination of achenes of *L. tinctum*, as has been postulated in other proteaceous species (Brown & Van Staden 1973b, 1975b). One cannot determine whether the cytokinins are directly involved with the 'triggering' of germination or whether changes in levels

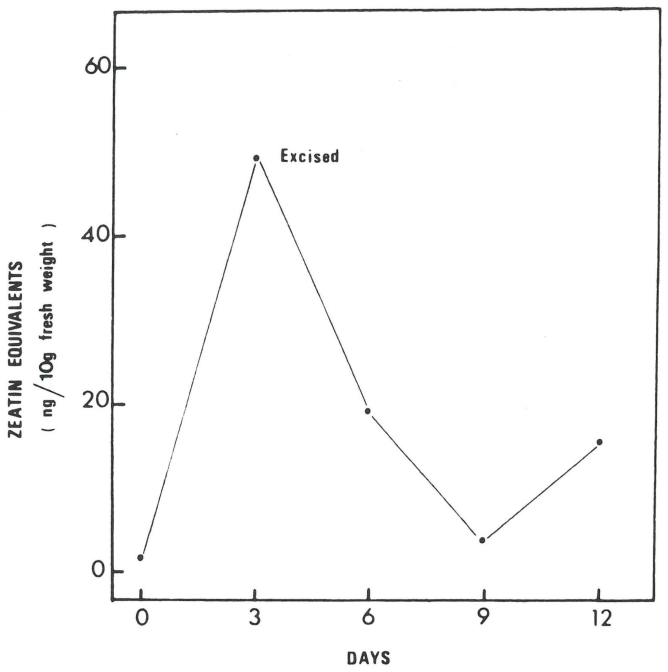

Figure 3 The cytokinin levels of excised embryos of *L. tinctum* after removal of the pericarp and incubation in air. Figures represent total cytokinin content of embryo material, expressed as zeatin equivalents (ng/10 g fresh mass).

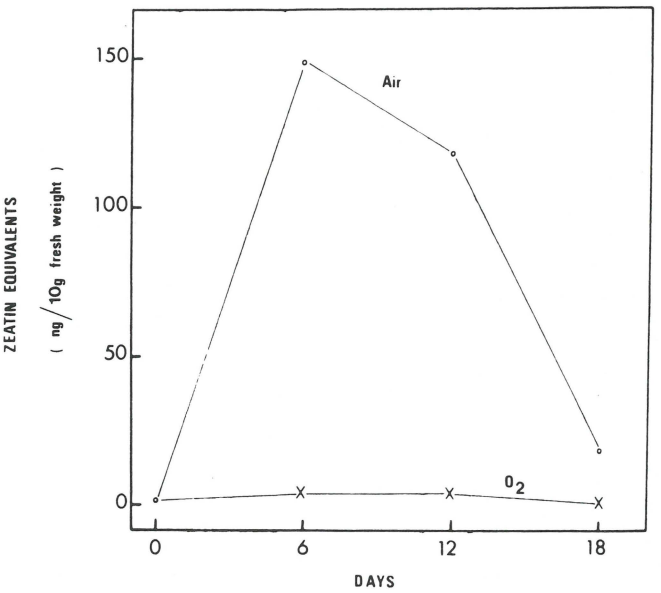

Figure 4 The cytokinin levels of embryos of *L. tinctum*, after incubation of intact achenes in air and in elevated oxygen partial pressures. Figures represent total cytokinin content of embryo material, expressed as zeatin equivalents (ng/10 g fresh mass).

are merely secondary phenomena associated with the initial stages of germination. A decrease in cytokinin levels occurred during visible germination (Figures 3 & 4) suggesting that the cytokinins were utilized rapidly. Elevated levels of cytokinins were associated with the germination of excized embryos and intact achenes incubated in air (Figures 3 & 4). Incubation in air which resulted in 21% germination reflected higher levels of cytokinins, on the basis of zeatin equivalents than excised embryos in which germination was 74%. It is possible that when the restrictive covering structures are removed, cytokinin levels associated with cotyledon expansion need not be so high in order to promote germination, as would be the case in intact achenes. The high level of cytokinins associated with the relatively poor germination in air (Figures 2 & 4) indicates that cytokinins alone are not able to overcome dormancy effectively. This may correspond to the situation in *L. daphnoides* where a high percentage germination was correlated with a simultaneous increase in the levels of gibberellin-like substances and cytokinins (Brown & Van Staden 1975b). A study of the gibberellin levels in germinating achenes of *L. tinctum* is obviously necessary to determine whether the same situation exists.

Contrary to the situation in *L. daphnoides*, elevated oxygen levels depressed the levels of endogenous cytokinins (Figure 4). This was correlated with the low germination percentage in intact achenes incubated in oxygen (Figure 2). The fact that a low percentage germination was correlated with a very low level of cytokinins, would support the idea that cytokinins have a primary role in breaking dormancy in *L. tinctum*, as is the case suggested for fruits of *L. daphnoides* (Brown & Van Staden 1975b). The fact that inhibitors do not appear to be involved in the dormancy of *L. tinctum* achenes (Table 2) further suggests that the role of cytokinins in breaking dormancy is not merely a 'permissive' one in overcoming the effect of inhibitors, as postulated by Khan (1971).

Acknowledgement

The financial assistance of the Council for Scientific and Industrial Research is gratefully acknowledged.

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