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Running Heading: **Germination of two Cunoniaceae species from New Caledonia**

**Methods to promote germination of two Cunoniaceae species, *Cunonia macrophylla* and *Geissois pruinosa*, from New Caledonia.**

**B. FOGLIANI, S. BOURAÏMA-MADJEBI, V. MEDEVIELLE AND R. PINEAU**

L.B.P.V.A. : Laboratoire de Biologie et Physiologie Végétales Appliquées,

Université de la Nouvelle-Calédonie, BP 4477, 98847 Nouméa, New Caledonia

(Email: [bfoglian@univ-nc.nc](mailto:bfoglian@univ-nc.nc))

**Summary**

The response of germination of seeds from two New Caledonian Cunoniaceae, *Geissois pruinosa* Brongniart & Gris, and *Cunonia macrophylla* Brongniart & Gris (which have interesting biological and horticultural properties) to temperature and soaking level was established. It also permitted definition of the temperature range for germination and the thermal optimum. The seeds of these two species possess different germination profiles. Soaking appears to be more important for *C. macrophylla* seeds than for *G. pruinosa* seeds. In order to optimise germination factors, reduce latent time and increase the germination capacity, seeds were exposed to different types of pre-treatment: cold; sulphuric acid; hot water and mechanical scarification. The latter two seem to be the most effective and economic for both species. The other forms of treatment appear ineffective and even harmful.

## Introduction

The Cunoniaceae family occurs largely in the southern hemisphere, in particular in New Caledonia which possesses 91 species of the 300 known. Six genera are inventoried in New Caledonia, *Acsmithia*, *Cunonia*, *Geissois*, *Weinmannia*, *Codia* and *Pancheria*, with the latter two being endemic (Guillaumin, 1948).

*Cunonia macrophylla* Brongniart & Gris belongs to a genus of 25 species (Hoogland *et al.*, 1997), 24 from New Caledonia and 1 from South Africa, *C. capensis*, which is morphologically similar to *C. macrophylla* (Bradford, 1998).

*Geissois pruinosa* Brongniart & Gris belongs to a genus of approximately 18 species distributed in Australia, New Caledonia, Fiji, Vanuatu (New Hebrides) and Santa Cruz (Solomon Islands). About ten of these are inventoried in New Caledonia.

To improve the economic value of natural products extracted from New Caledonian endemic plants, a physiological understanding of the development of these plants with biological activities, from seed to seed, is necessary. For instance, *C. macrophylla* possesses effective antibiotic properties against 5 bacteria, 4 of which are human pathogens (Fogliani *et al.*, 2002a), and also an inhibitory activity against xanthine oxidase (Fogliani *et al.*, 2002b). A chemical study has been carried out to purify molecules of interest and their structure will be soon known. In *G. pruinosa*, fungicidal properties (Fogliani *et al.*, 2002a) and high toxicity against *Artemia salina* (Fogliani *et al.*, 2000) were detected. *G. pruinosa* could also be of economic interest as some species of this genus are exploited for their timber (Timber Research & Development Association, 1979; Lazarides & Hinces, 1993), and in horticulture because of the attractive visual effect of large palm leaves and intense red flowers. Finally *G. pruinosa* appears to be an interesting subject for scientific study as it has a capacity for hyper-accumulating nickel (Jaffre *et al.*, 1979).

Thus, propagation of these two interesting species is essential and production of seedlings would be useful. Little has been reported on the effects of temperature or light on New Caledonian Cunoniaceae species such as *G. pruinosa* and *C. macrophylla* seeds, or their response to water availability. The germination of *Ceratopetalum apetalum* (McGee, 1990) and *Weinmannia racemosa* (Burrows, 1999) have been studied in Australia and New Zealand, respectively. This work on aspects of the germination dynamics of *C. macrophylla* and *G. pruinosa*, in controlled laboratory conditions aimed to optimise their germination.

## **Materials and Methods**

### *Seed collection and storage*

*C. macrophylla* is a small tree with a single, usually unbranched stem. Each year it produces two inflorescences with about 30 fruits each with 15-20 seeds. *G. pruinosa* is a higher tree with branched stem. Number of inflorescences can vary but each is constituted by about

Seeds of *C. macrophylla* and *G. pruinosa* were collected from different mother plants localised on a restricted area of 50m x 50m in the Koghis and Dzumac mountains respectively in March 2000 (figure 1) and herbarium samples were made. As many fruits as possible were collected and packed in paper bags and transported to the “Laboratoire de Biologie et Physiologie Végétales Appliquées” at the University of New Caledonia where they were dried under neon lighting in the laboratory for one week. The seeds obtained after opening the fruit were stored at room temperature in an airtight glass Petri dish until the tests were carried out with a maximum of three months later .

### *Germination assays*

Firstly, seeds were chosen according to a fertility feature characterised by a rounded embryo (figure 2). All selected seeds were disinfected in sodium hypochloride 4%

(48° chlorinated water) for 5 minutes followed by two 5-minute rinses with sterilised distilled water. All assays were carried out in 9 mm diameter airtight autoclaved Petri dishes containing 3 thicknesses of Whatman filter paper. Finally, after the addition of sterilised distilled water, 60 seeds were distributed onto 4 dishes (15 seeds per dish) which were sealed with parafilm and incubated at the desired temperature. A seed was regarded as germinated when the radicle emerged from the seed coat to a length of 1 mm (Istambouli, 1976; Aissa, 1981). Germination was followed for a period of three months but, no germination were observed after 40 days in all cases. For this reason all experimental results were presented from 0 to 40 days. The results are the means of the germination percentages obtained in 4 replicates  $\pm$  standard deviation (SD). The germination rate is expressed as the reciprocal of the time to reach 50% germination (T50) estimated graphically from the cumulative germination.

#### ***Determination of the optimal temperature for germination***

To determine this optimal temperature, the soaking level was fixed at 12 ml of sterilised distilled water and the Petri dishes were incubated at 5, 10, 15, 20, 25, 30, 35 and 40°C.

#### ***Determination of the optimal soaking level for germination***

This was carried out at a temperature of 25°C with quantities of 4, 6, 8, 10 et 12 ml of sterilised distilled water.

#### ***Effect of disinfecting with sodium hypochloride***

This assay was carried out to check if the sodium hypochloride (48° chlorinated water) used to disinfect seeds has an effect on their germination capacity. It was done with disinfected and undisinfected seeds germinated at 25°C and soaked in 12 ml of sterilised distilled water.

### ***Germination at random***

This assay was carried out on unsorted seeds extracted directly from the fruit after collection to give an idea of what happens *in situ*. It was done at a temperature of 25°C, with 12 ml of sterilised distilled water.

### ***Hot water treatment***

To test the effect of hot water on germination capacity, the disinfected seeds were put in small gauze bags, disinfected, then immersed in sterilised distilled water preheated to 30 (**10**), 40 (**11**), 50 (**12**), 60 (**13**), 70 (**14**), 80 (**15**), 90 (**16**) or 100°C (**17**) and left for 12 hours until they cooled to room temperature. They were then germinated at 25°C with 12 ml of sterilised distilled water. Numbers in bold type refer to the treatment numbers in fig. 6.

### ***Sulphuric acid treatment***

Disinfected seeds were treated with concentrated sulphuric acid (95-97%) (**18**) or diluted to 1/2 (**19**), 1/4 (**20**), 1/8 (**21**), and 1/16 (**22**) for 5 minutes. After the treatment, seeds were thoroughly rinsed with sterilised distilled water before being incubated at 25°C with 12 ml of sterilised distilled water.

### ***Cold treatment***

The effect of cold treatment was investigated by immersing disinfected seeds in 10 ml of sterilised distilled water placed in a refrigerator at 4°C for 2 (**3**), 4 (**4**), 7 (**5**), 10 (**6**), 14 (**7**), 21 (**8**) or 28 (**9**) days. The seeds were then germinated at 25°C with 12 ml of sterilised distilled water.

### ***Mechanical scarification (2)***

The effect of mechanical scarification was investigated by carefully removing the tegument of the seed by a circular movement between the fingers. Seeds were then disinfected and germinated at 25°C in 12 ml of sterilised distilled water.

## **Results**

### ***Sensitivity of seeds to temperature***

*G. pruinosa* and *C. macrophylla* seeds responded differently to temperature. In *G. pruinosa*, the seeds started to germinate after 10 to 24 days at temperatures ranging from 15 to 35°C (figure 3a). The highest germination (31%) was obtained at temperatures of 25 and 30°C. At these temperatures, the seeds started to germinate after 10 days and maximal germination was attained after 19 days. At 20 and 35°C, germination started later, beginning at 15 days. At 15°C, it was delayed to 24 days. For these last three temperatures, the maximum of germination was reduced to more than half, between 11 and 13%. No germination occurred after 3 months of investigation at 5, 10 and 40°C. There was a positive correlation between the rate of germination (expressed as the reciprocal of T50) and the temperatures up to the thermal optimum, 0 to 25°C (figure 3c). At higher temperatures, 30 to 40°C, there was also a negative correlation. Using a regression lines method as Özbingöl *et al.* (1998), the thermal optimum is given by their intersection (figure 3c) and was between 27 and 28°C for *G. pruinosa*. Moreover, extrapolation of the same lines to the temperature axis allowed the calculation of the minimal and maximal temperatures for seed germination; which were approximately 9°C and 40°C, respectively.

Germination of *C. macrophylla* started later, between the 15<sup>th</sup> and 24<sup>th</sup> days at temperatures ranging from 15 to 30°C (figure 3b). The highest germination, of the order of 53%, was obtained at temperatures of 20 and 25°C. At these temperatures, seeds started to

germinate after 15 days but in contrast to *G. pruinosa*, germination proceeded regularly until the 38<sup>th</sup> day. The temperature range where germination was possible for *C. macrophylla* seeds was more limited and no germination occurred after 3 months at temperatures under 15°C and over 30°C. At these two temperatures, germination only started after 25 days and continued until the 37<sup>th</sup> day with a smaller maximum germination of 20% at 15°C and 10% at 30°C. Using the same method, figure 3c allowed the determination of the thermal optimum for germination, between 22 and 23°C and also the minimal and maximal temperatures for seed germination that were around 10°C and 35°C respectively for *C. macrophylla*.

#### ***Sensitivity of seeds to soaking level***

At 25°C, the amount of water supplied did not affect the time of onset of germination for *G. pruinosa* seeds as it started between the 10<sup>th</sup> and 11<sup>th</sup> day (figure 4a). In contrast, in *C. macrophylla* seeds, as less water was supplied, time before onset of germination increased; from 12 days with 12 ml of water to 21 days with 4 ml (figure 4b).

Germination of *G. pruinosa* seeds was less in 4 ml (24%) than at higher volumes of water (31%). In *C. macrophylla*, the maximum germination appeared to be proportional to the volume of water.

#### ***Effects of disinfecting by using sodium hypochloride***

Observation of the curves of cumulated germination according to time, for the two Cunoniaceae species (figure 5) showed that disinfecting with sodium hypochloride at 4%, had no notable effect on the germinating capacity of the seeds, as the maximum level of germination varied from 31 to 33% for *G. pruinosa* and from 51 to 53% for *C. macrophylla*. Only the latent period for the species *C. macrophylla* was affected by this treatment as it decreased from 19 to 15 days, while the species *G. pruinosa* only varied by one day.

However, observation during handling showed this treatment had a notable effect in decreasing the amount of contamination by bacteria and fungi.



### ***Germination at Random***

The presence of a large quantity of infertile seeds within the fruit of each species (figure 2), led us to carry out this test in order to determine the maximum germination likely to be obtained *in situ*. This rate is 11% for both species.

### ***Effects of Hot Water Treatment***

Pre-treatment of *G. pruinosa* seeds with water heated from 30 to 60°C had the effect of increasing the germination capacity and notably decreasing the latent period (figure 6a). The highest germination of 43% was obtained with water at 40°C. The latent time decreased from 4 to 2 days. Finally it is important to note that the time necessary to reach the maximum germination increased proportionally with water temperature. No germination was noted in treatment with water temperatures over 60°C.

In contrast to the *G. pruinosa* seeds, the germination capacity of *C. macrophylla* seeds was only optimised by treatment with water heated to between 30 and 50°C (figure 6b) and germination occurs with water temperatures of 70°C. The highest germination of 70% was obtained with water at 40°C as *G. pruinosa* seeds. The latent time was little affected by water temperatures between 30 and 40°C but increased proportionally with water temperature over 40°C.

### ***Effects of sulphuric acid treatment***

Whatever the species considered, it clearly appeared that the type of sulphuric acid treatment which we applied to *G. pruinosa* and *C. macrophylla* seeds was unfavourable for their germination. In both cases the maximum germination capacity was affected (figure 7). For *C. macrophylla*, this capacity was less affected than for *G. pruinosa* as the only treatment which made it drop to 4% is the acid diluted to 1/2. No germination was detected using pure sulphuric acid for 5 minutes whatever the species considered.

### ***Effects of Cold Treatment***

The maximum germination capacity of *G. pruinosa* seeds decreased when they were pre-treated at a temperature of 4°C for a period of between 2 and 14 days (figure 8a). If this period was for 21 or 28 days, this capacity increased considerably compared with the control as it went up to 38% and 42% respectively. The latent time was also affected by this long treatment and decreased by 3 days.

As far as the *C. macrophylla* seeds were concerned, only a cold treatment of 14 days allowed an improvement in the maximum germination capacity which only increased by 4% (figure 8b). All of the other periods of exposure to cold tested had a negative effect on germination.

### ***Effects of Mechanical Scarification***

Mechanical scarification allowed a considerable increase in the maximum of germination of the *G. pruinosa* and *C. macrophylla* seeds which went from 31% to 40% and from 53% to 82% respectively (figure 9). This is proof of the efficiency of such treatment in spite of the damage which could be caused to the embryo. The latent time was only very slightly affected.

### **Discussion**

This study of two New Caledonian Cunoniaceae species which belong to two different genera confirm how much the response of seeds to different treatment can vary within the same family. *G. pruinosa* seeds germinate at temperatures between 15 and 35°C, with an optimum established between 27 and 28°C, while *C. macrophylla* seeds germinate at temperatures between 15 and 30°C, with an optimum established between 22 and 23°C. Below 20°C and above 30°C, the rate of germination is greatly reduced for both species. Germination was not detected at temperatures of 5 and 40°C in either species. The minimal germination temperature for both species was established as close to 9-10°C. The maximal

temperatures are different, as that of *G. pruinosa* is estimated to be 40°C compared with 35°C for *C. macrophylla* seeds.

Their reaction to water is also different. It appears to be clear that for the two species, volumes of between 8 and 12 ml of sterile distilled water lead to the best germination, however, it is noted that the *C. macrophylla* seeds are more sensitive to variation in the quantity of water. This is another argument in favour of an appropriate soaking level and temperature to realise the germination of a batch of seeds.

In these favourable conditions established, at 25°C with 12 ml of sterile, distilled water, it seemed interesting to determine the maximal germination capacity obtained by exposing the unsorted seeds coming directly from a bunch of fruit collected from the mother plants. Counting the number of seeds which appear to be fertile, compared with the total number of seeds gives a percentage of 35% for *G. pruinosa*, compared with 20% for *C. macrophylla*. However, it clearly seems that the lower number of seeds which appear to be fertile established for *C. macrophylla* is compensated by a higher germination, which therefore means there are almost equal chances of seeing as many *G. pruinosa* plants as *C. macrophylla* plants growing in the natural environment with germination rates close to 11%.

Observation of the cumulated germination curves of these two species reveals that they belong to two types of theoretical germination curves (Côme, 1982). In fact *G. pruinosa* seeds germinate rapidly and more synchronous while *C. macrophylla* seeds germinate over a longer time period, with both species having seeds which don't germinate.

In order to optimise germination, we attempted to eliminate possible tegumentary inhibitions, or embryonic dormancy using several pre-treatments. It clearly appears that mechanical scarification is a simple and efficient method which leads to a considerable increase in germination capacity for *G. pruinosa*, and a greater increase for *C. macrophylla* (figure 10). This is even more interesting because the two species don't really have a thick tegument layer (figure 2) which would justify the use of this method which is often used for

seeds with this characteristic (Tibagu & Odén, 2001; El-Siddig *et al.*, 2001). This fact is confirmed by the harmful effect of pre-treatment with pure or diluted sulphuric acid, which seems to provoke irreversible lesions on the embryo, as it leads to a lower germination rate than the control (figure 10). Hypothesis about the effect of mechanical scarification on seeds include better uptake of water, easier emergence of the radicle by weakening the structure of the seminal tegument, or the elimination of phenolic components. The coat of *C. macrophylla* is known to be rich in tanins (Fogliani, 1997), which could have the effect of limiting the supply of oxygen to the embryo (Côme, 1970).

The use of hot water as pre-treatment has been recognised as improving germination for leguminous species with a thick tegument (Tibagu & Odén, 2001). Our study shows that it also allows optimisation of germination for the two species of Cunoniaceae, but this is at lower temperatures, especially 40°C. In the case of *G. pruinosa*, this treatment is equivalent, indeed better than mechanical scarification, while for *C. macrophylla*, it appears to be less favourable. At water temperatures above 60°C, germination of *G. pruinosa* seeds is non-existent, and very weak for *C. macrophylla* seeds, which no longer germinate above that. These high temperatures probably damage the embryo. In comparison with leguminous seeds which do not germinate in water temperatures above 80°C and whose seed coat is very thick, resistant to acid, and requires considerable mechanical scarification, Cunoniaceae seeds, whose tegument is fine, seem proportionally resistant to high temperatures.

Cold treatment seems paradoxical for seeds from species which are of subtropical origin, however temperatures around 5 to 10°C are recorded in New Caledonia, at night during the months of July and August. In the case of *G. pruinosa*, the longer the exposure to cold, the greater the maximal germination capacity, but only treatment for a period of more than 21 days allows an improvement in comparison with the control (figure 10). This rate is the same as that obtained by mechanical scarification or pre-treatment with water heated to 40°C. On the contrary, for *C. macrophylla*, this treatment is not notably advantageous,

because even for 14 days of cold treatment, the germination obtained of 58% is almost the same as that of the control.

### **Recommendations**

In conclusion, this original study of two New Caledonian Cunoniaceae species has enabled us to determine the impact of certain factors on germination of *G. pruinosa* and *C. macrophylla* seeds in controlled laboratory conditions. Other factors such as light and oxygen have not yet been analysed. This study also revealed the presence of tegumentary and embryonic dormancy, which require further study. But it is now possible to select the most efficient pre-treatment to optimise germination of these two seed types within the framework of a large scale improvement of the economic value of these species for their biological properties or horticultural exploitation. Thus, mechanical scarification, or pre-treatment with water heated to 40°C appear to be the most advantageous methods as they allow a considerable increase in the germination of these seeds at a reasonable price. However, if we have a better grasp of what treatment to use to make the seeds of the two species studied more apt to germinate, the real reasons for this improvement should be researched in induced metabolic events. Consequently, we can hope that they will permit a better understanding of the intimate mechanisms implied in the decisive passage of the embryo of these seeds from a slowed down life to active growth. An investigation of other types of pre-treatment could be of real interest, especially in the case of *C. macrophylla* for which it would be convenient to reduce the long time necessary to reach maximum germination.

### **Acknowledgements**

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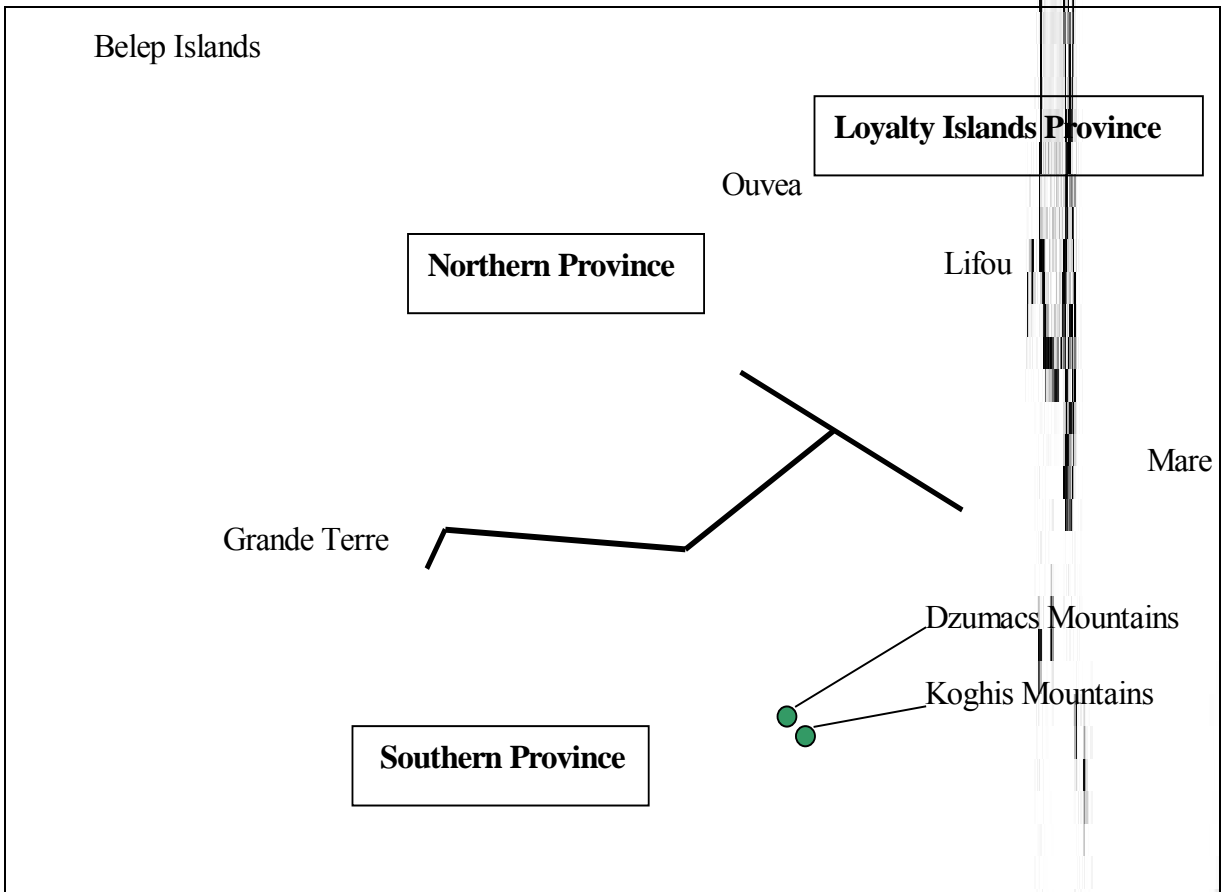
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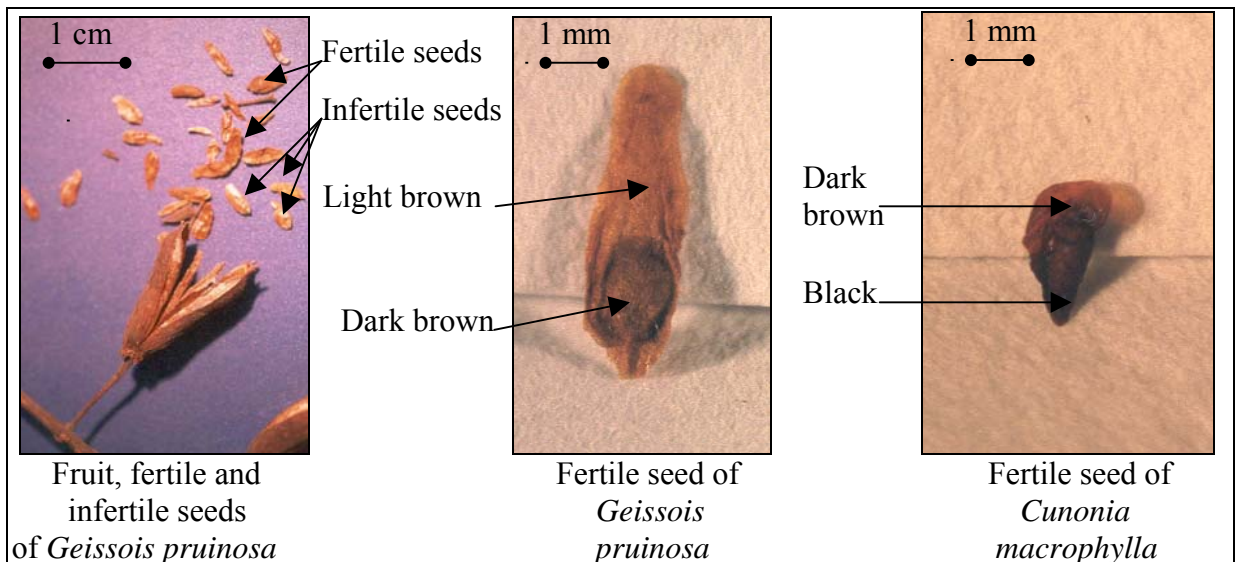
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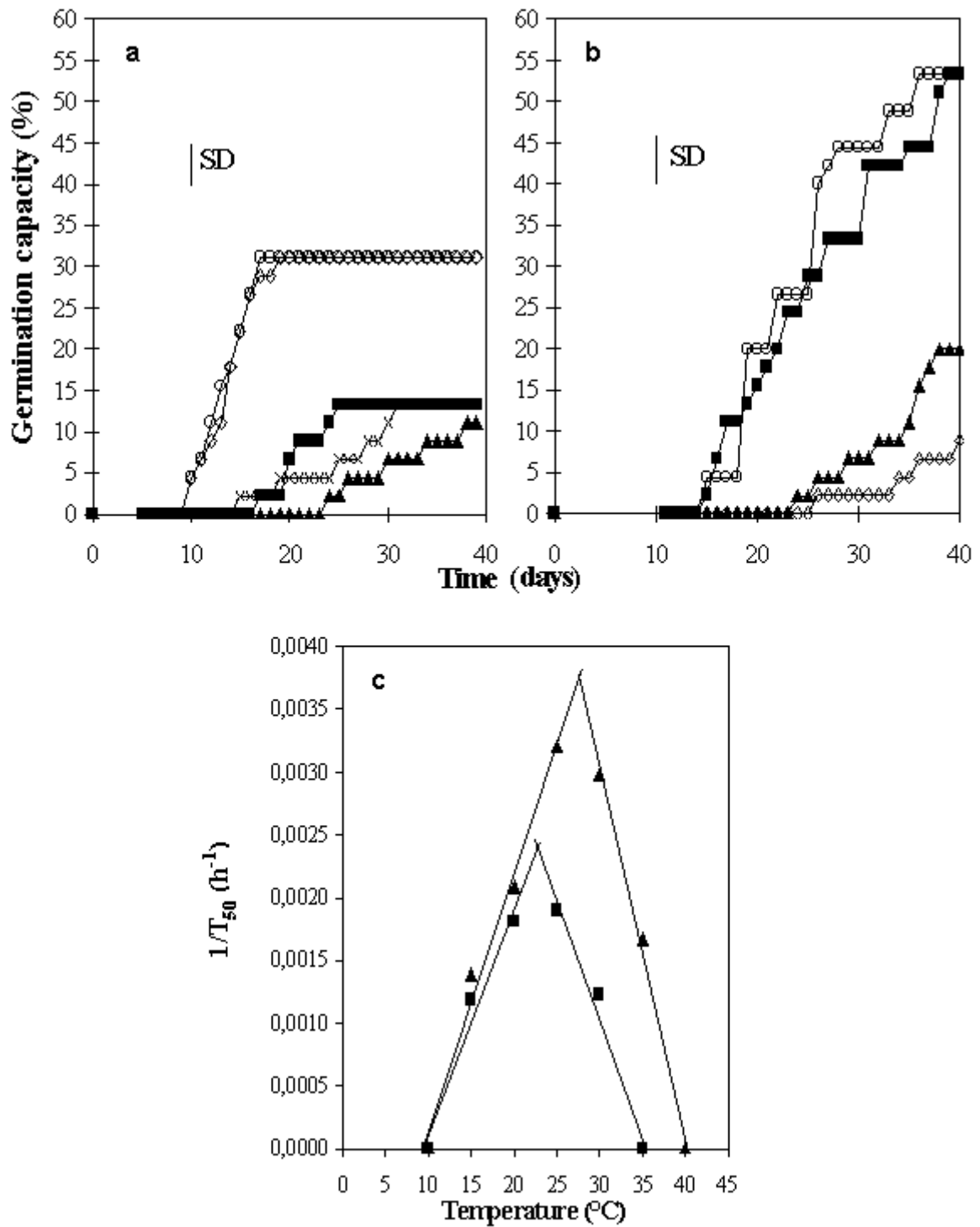




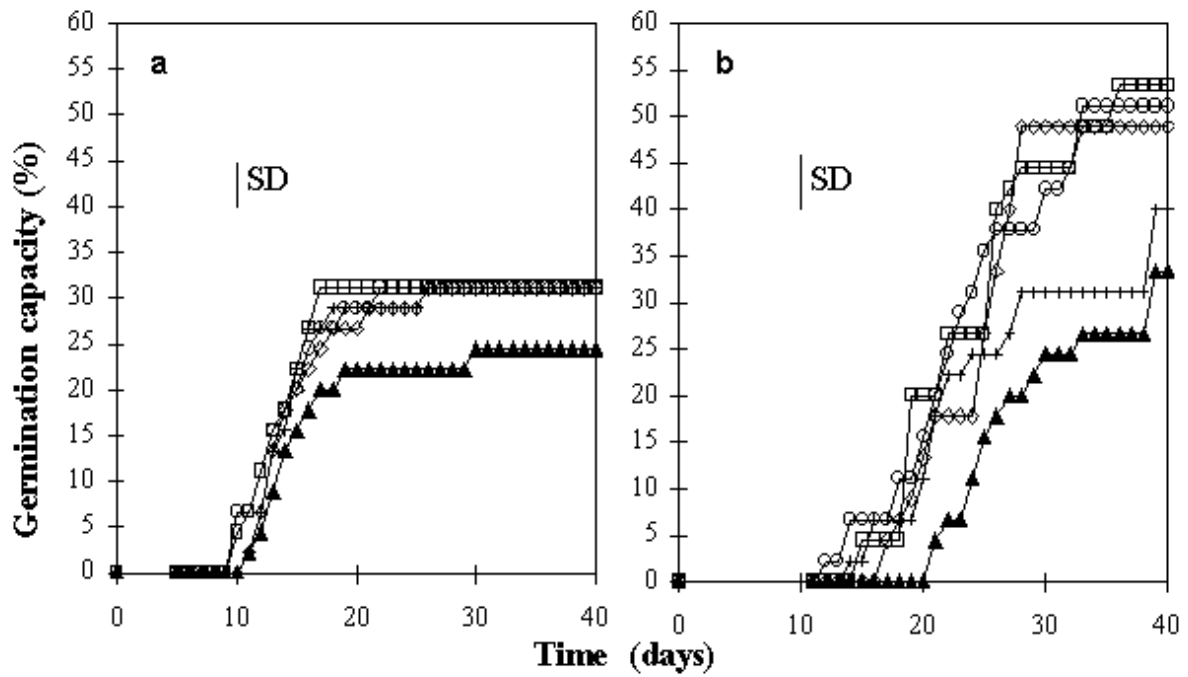
**Figure 1.** Map of New Caledonia showing its 3 provinces and location of *Geissois pruinosa* (Dzumacs Mountains) and *Cunonia macrophylla* (Koghis Mountains) seed collection sites.



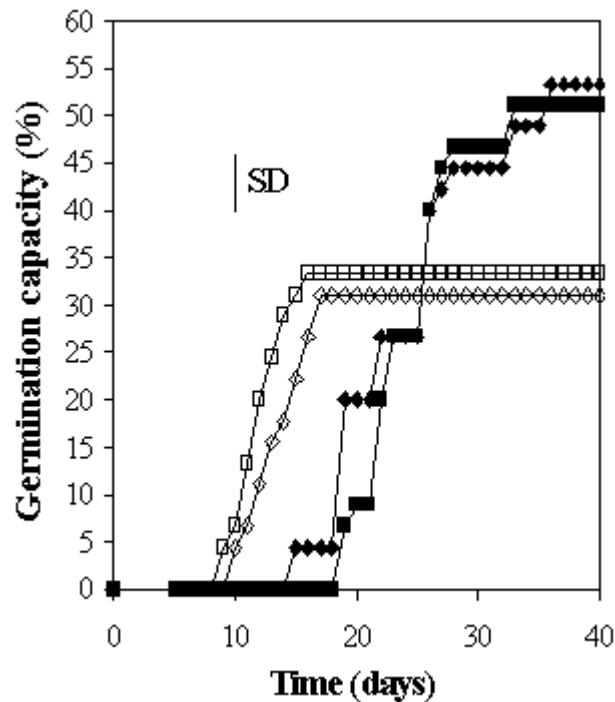
**Figure 2.** Photographs of *Geissois pruinosa* and *Cunonia macrophylla* seeds



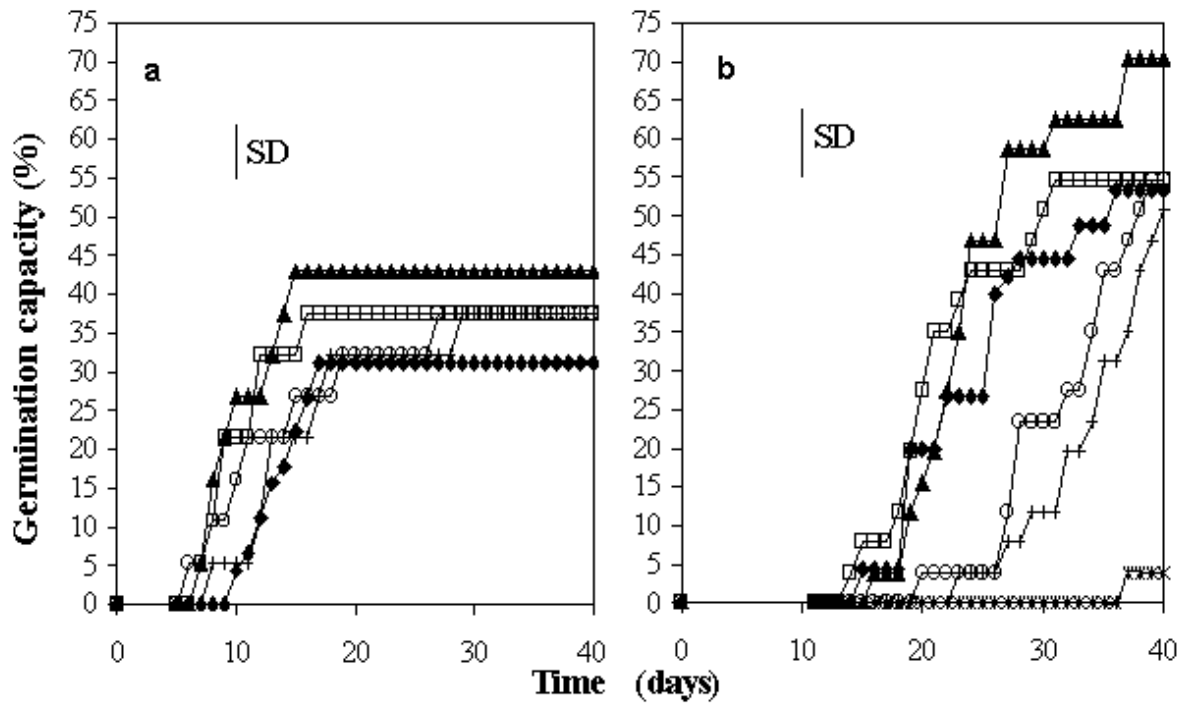
**Figure 3.** Effects of temperature on the germination of *Geissois pruinosa* (a, c) and *Cunonia macrophylla* (b, c). a and b : time course of germination at 15°C (▲), 20°C (■), 25°C (○), 30°C (◇), 35°C (×). Means of 4 replicates. Vertical bar denotes the largest SD. c : Effect of temperature on the germination rate expressed as the reciprocal of time to obtain 50% germination ( $T_{50}$ ) for *G. pruinosa* (▲) and *C. macrophylla* (■); regression coefficients of the lines were 0.985 and -0.995 for *G. pruinosa* and 0.968 and -0.972 for *C. macrophylla*.



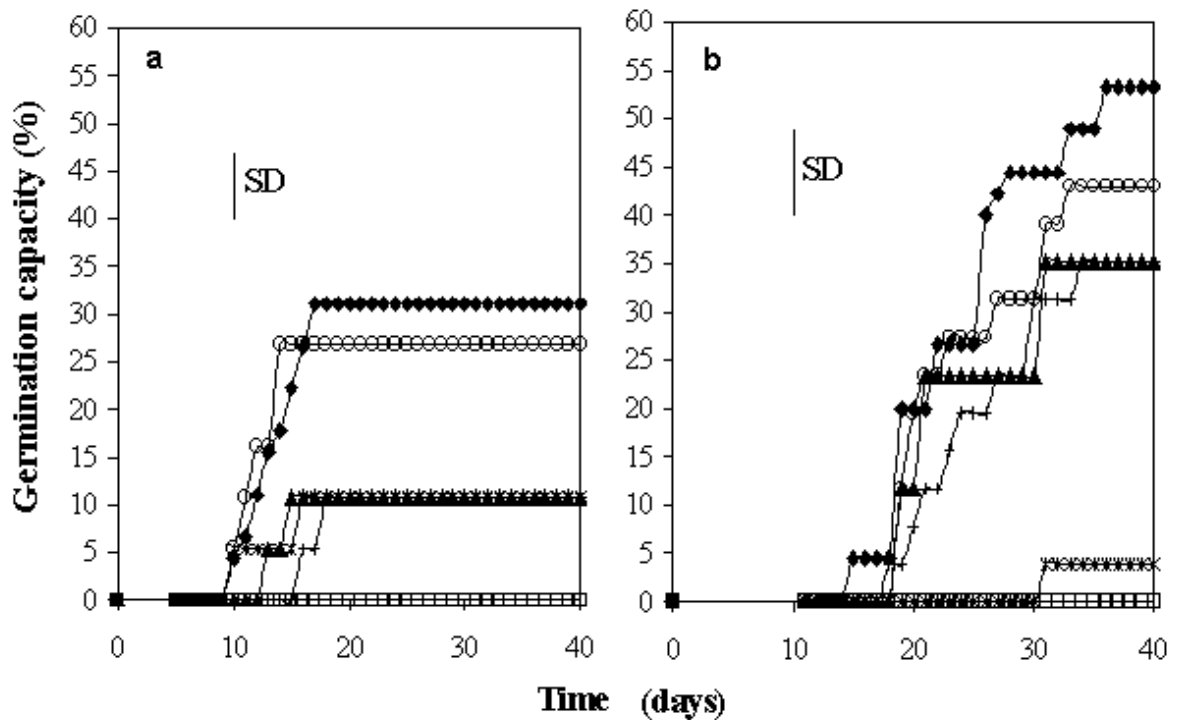
**Figure 4.** Effects of soaking level on the germination of *Geissos pruinosa* (a) and *Cunonia macrophylla* (b). Time course of germination with 4 ml (▲), 6 ml (+), 8 ml (◇), 10 ml (○), 12 ml (□) of sterilised distilled water at 25 °C. Means of 4 replicates. Vertical bar denotes the largest SD.



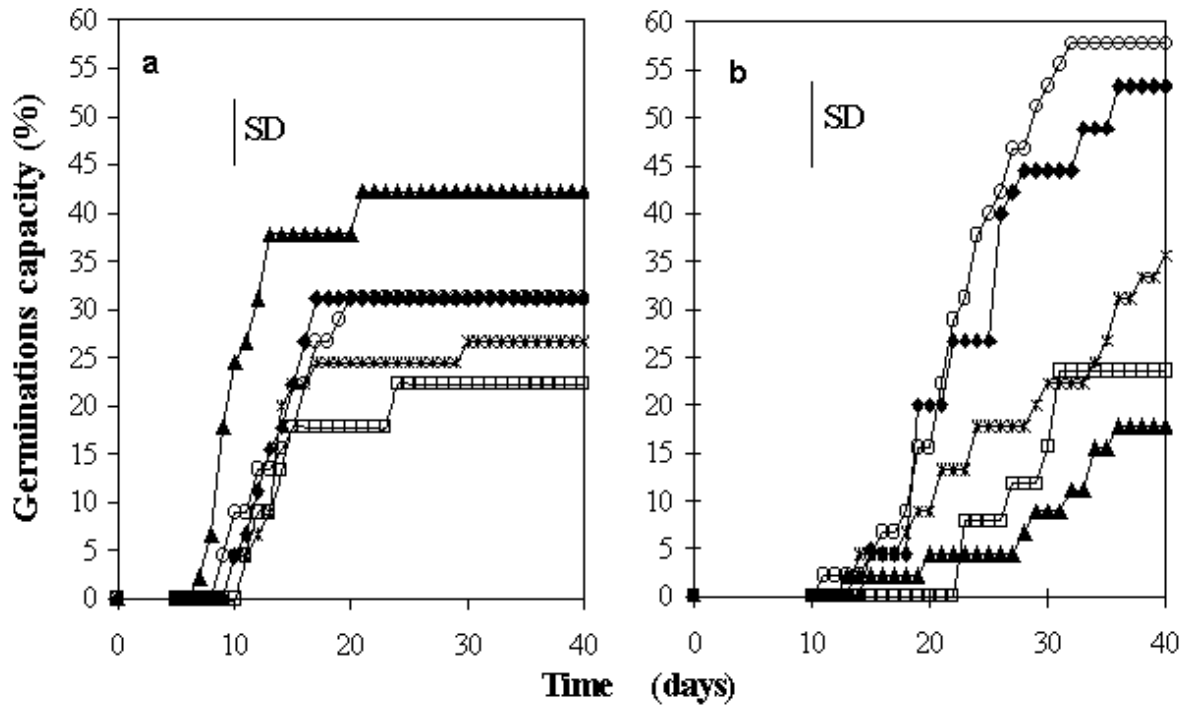
**Figure 5.** Effects of disinfection with sodium hypochlorite at 4% (40°chlorinated water) on the germination of *G. pruinosa* and *C. macrophylla*. Time course of germination with disinfection for *G. pruinosa* (□) and *C. macrophylla* (■) or without disinfection for *G. pruinosa* (◇) and *C. macrophylla* (◆) at 25°C. Means of 4 replicates. Vertical bar denotes the largest SD.



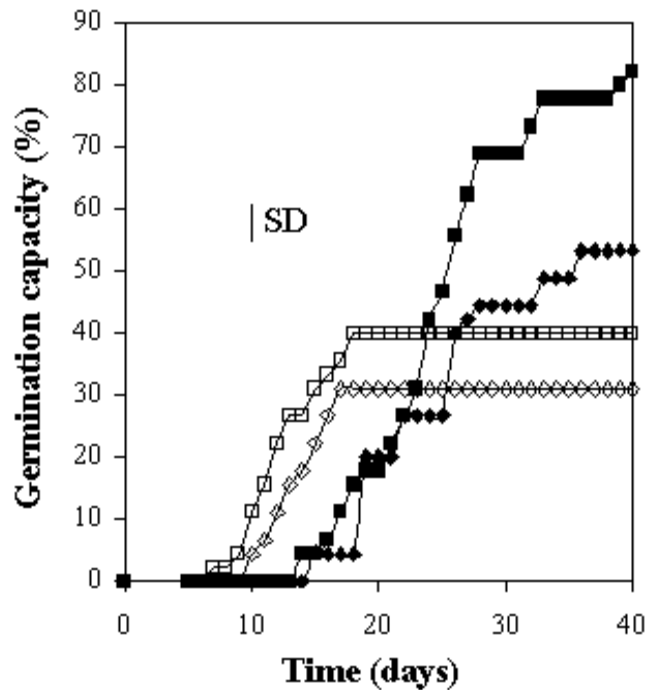
**Figure 6.** Effects of hot water pre-treatment on the germination of *G. pruinosa* (a) and *C. macrophylla* (b). Time course of germination after treatment with hot sterilised distilled water heated at 30°C (□), 40°C (▲), 50°C (○), 60°C (+), 70°C (x) or without treatment (◆). Means of 4 replicates. Vertical bar denotes the largest SD.



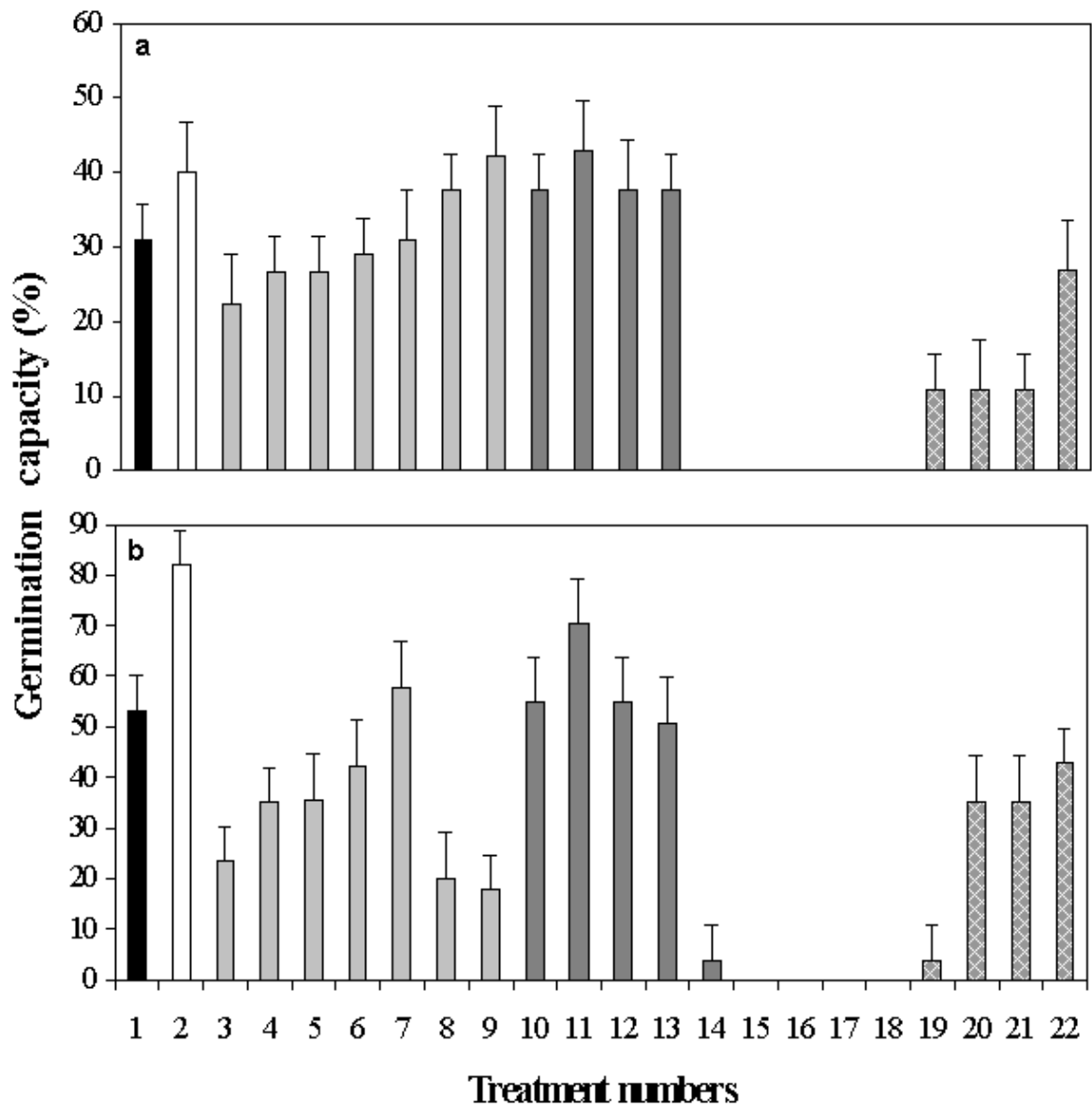
**Figure 7.** Effects of sulphuric acid pre-treatment on the germination of *G. pruinosa* (a) and *C. macrophylla* (b). Time course of germination after pre-treatment with concentrated sulphuric acid 95-97% (□), diluted to 1/2 (x), diluted to 1/4 (▲), diluted to 1/8 (+), diluted to 1/16 (○) or without pre-treatment (◆). Means of 4 replicates. Vertical bar denotes the largest SD.



**Figure 8.** Effects of cold pre-treatment on the germination of *G. pruinosa* (a) and *C. macrophylla* (b). Time course of germination after cold pre-treatment at 4°C for 2 days (□), 7 days (x), 14 days (○), 28 days (▲) or without pre-treatment (◆). Means of 4 replicates. Vertical bar denotes the largest SD.



**Figure 9.** Effects of mechanical scarification on the germination of *G. pruinosa* and *C. macrophylla*. Time course of germination after mechanical scarification for *G. pruinosa* (□) and *C. macrophylla* (■) or without scarification for *G. pruinosa* (◇) and *C. macrophylla* (◆). Means of 4 replicates. Vertical bar denotes the largest SD.



**Figure 10.** Maximal germination capacity of seeds of *Geissois pruinosa* (a) and *Cunonia macrophylla* (b) at 25°C in response to different pre-treatments (means  $\pm$ SD). (1) : control ; for signification of the treatment numbers 2 to 22 refer to material and methods.