EFECTS OF STRATIFICATION AND TEMPERATURE ON THE GERMINATION OF *DALBERGIA COCHINCHINENSIS, PINUS KESIYA* AND *PINUS MERKUSII*

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LEADEM, C.L., BHODTHIPUKS, J. & CLARK, J.M. 1995. Effects of stratification and temperature on the germination of *Dalbergia cochinchinensis, Pinus kesiya* and *Pinus merkusii*. The effects of stratification and incubation temperature on seed germination were investigated for *Dalbergia cochinchinensis, Pinus kesiya* and *Pinus merkusii*. Seeds were either soaked for 24 h, or stratified (soaked in water for 24 h and stored at 2 - 5 °C) for 2 weeks, then incubated for 21 days at 30/20 °C, 30 °C, or 20 °C. Germination test results were compared to results of two rapid viability tests, X-ray and tetrazolium chloride (TZ). This information will assist in determining the true potential of seeds used for reforestation and facilitate nursery operations in tropical countries where *D. cochinchinensis, P. kesiya,* and *P. merkusii* are grown. Total germination of *D. cochinchinensis* was greater when seeds received no stratification treatment. Of the three incubation temperatures, germination was most rapid when seeds were incubated at 30 °C, but highest when seeds were incubated at 30/20°C. Stratification enhanced germination rates of *P. kesiya* and *P. merkusii* under all incubation temperatures. Temperature also enhanced the speed of germination; germination was most rapid when the seeds were incubated at 30 °C, and slowest when incubated at 20 °C. For *D. cochinchinensis,* germination test results agreed well with results of X-ray tests, but poorly with TZ tests. Both X-ray and TZ tests corresponded well with germination tests of *P. kesiya,* but neither quick test correlated well with germination test results of *P. merkusii.* The poor correspondence may have been due to the relatively low quality of the *P. merkusii* seed source used in this study, and further refinement is needed for this species. Nevertheless, the use of X-ray and TZ quick tests appears feasible in those instances when there is insufficient time available for a standard test.

Key words: Seed germination - stratification - temperature - quick tests

LEADEM,C. L., BHODTHIPUKS, J. & CLARK, J. M. 1994. Kesan-kesan penstrataan dan suhu pada percambahan *Dalbergia cochinchinensis, Pinus kesiya* dan *Pinus merkusii*. Kesan-kesan penstrataan dan suhu pengeraman pada percambahan biji benih *Dalbergia cochinchinensis, Pinus kesiya* dan *Pinus merkusii* dikaji. Biji benih direndam selama 24 jam atau distrata (rendam di dalam air selama 24 jam dan disimpan pada suhu 2-5 °C) selama 2 minggu, kemudian dieramkan selama 21 hari pada suhu 30 °C /20 °C, 30 °C atau 20 °C. Hasil-hasil ujian percambahan dibandingkan dengan hasil dua ujian kebolehhidupan yang pantas iaitu Sinar X dan tetrazolium klorida (TZ). Maklumat ini akan membantu dalam menentukan potensi sebenar biji benih yang digunakan untuk penghutanan semula dan memudahkan pengendalian tapak semaian di negara-negara tropika di mana *D. cochinchinensis, P. kesiya* dan *P. merkusii* ditanam. Percambahan keseluruhan *D. cochinchinensis* bertambah apabila biji benih tidak menerima rawatan penstrataan. Percambahan yang paling cepat berlaku apabila biji benih dieramkan pada suhu 30 °C tetapi pertambahan yang paling tinggi berlaku apabila biji benih dieramkan pada suhu 30 °C/20 °C. Penstrataan menambah kadar percambahan *P. kesiya* dan *P. merkusii* untuk kesemua suhu pengeraman. Suhu juga menambah kadar percambahan; percambahan yang paling pantas berlaku apabila biji benih dieramkan pada suhu 30 °C, dan percambahan yang paling lambat berlaku apabila biji benih dieramkan pada suhu 20 °C. Hasil ujian percambahan *D. cochinchinensis* serupa dengan hasil ujian Sinar X tetapi tidak sama dengan ujian TZ. Hasil kedua-dua ujian Sinar X dan TZ sama dengan hasil percambahan *P. kesiya* tetapi tidak sama pula dengan hasil percambahan *P. merkusii*. Ketidaksamaan ini mungkin disebabkan oleh mutu biji benih *P. merkusii* yang agak rendah dan spesies ini perlu diperbaiki selanjutnya. Walau bagaimanapun, penggunaan ujian yang cepat seperti Sinar X dan TZ nampaknya sesuai dalam keadaan kesuntukan masa untuk sesuatu ujian standard.

Introduction

Viable seeds are considered dormant when they are placed under conditions favourable for growth, yet fail to germinate. Dormancy, found in many tree seeds, is an important mechanism to enhance survival by delaying germination until conditions in the external environment are conducive to active growth (Osborne 1981). The expression of dormancy is under genetic control (Naylor 1983), but it is also strongly influenced by environmental factors (Steinhoff *et al.* 1983, Rehfeldt 1983, 1985).

Many important tropical forest trees are legumes, of which *Dalbergia cochinchinesis* is representative. For most leguminous seeds, the inability to germinate is due to the impermeability of the seed coat (Barton 1947, Brant *et al.* 1971, Ballard 1973, Rolston 1978, Werker 1980). Although hand or mechanical scarification, acid treatments, and water treatments are commonly used to break dormancy of hard-coated leguminous seeds (Willan 1985), scarification was not deemed necessary for *D. cochinchinensis* because its seeds absorb water readily (Kobmoo *et al.* 1990a). Seeds of conifers, however, often require stratification, i.e. chilling of moist seeds at 2 - 5 °C for several weeks to several months to break dormancy and ensure complete germination. Since stratification increases germination rates as well as total germination, seedling emergence is completed within a shorter period (Allen 1960, 1962, Edwards 1973). Another advantage of stratification is that it enhances the ability of seeds to germinate under a wide range of temperatures (Leadem 1989). Nursery managers find stratification especially attractive because

it enhances their ability to manage for the variety of different environmental conditions which may exist in seedling nurseries (Leadem 1989).

Tree seed quality is usually assessed by means of a standard germination test requiring a minimum of three weeks; with stratification, the test can take six weeks or longer. Sometimes the standard test cannot be performed because of time constraints or mechanical or physiological dormancy, and in such instances, "quick tests" of seed viability can provide reasonably good estimates of seed quality (Leadem 1984). Quick tests such as the X-ray and tetrazolium chloride (TZ) tests provide an estimate of viability within several hours. Since quick tests are performed independently of environmental factors, they have an advantage over the usual germination tests because seed quality is assessed irrespective of dormancy status.

The X-ray test is based upon the physical examination of the embryo and endosperm. Although X-radiography can rapidly distinguish good from defective seeds (Kamra 1976), the technique has had relatively limited application in tropical forest seeds, especially in southeast Asia (Kobmoo *et al.* 1990b). X-radiography can be used to detect disease-infested seeds (Kamra 1974), mechanically-damaged seeds (Singh & Banerjee 1968, Kamra 1973), and internal insect infestation (Freeman 1965, Yates 1972, 1974). The X-ray test has been demonstrated to be a reliable indicator of seed viability, showing good correlations with germination test results (Leadem 1981). The use of "soft" (low energy) X-radiation does not affect seed germination or result in any apparent chromosome damage (Kamra & Simak 1965), and is also safer for operators working in seed testing laboratories.

The TZ technique defines live and dead areas of the embryo and endosperm by differential, topographic staining. When seeds are incubated for several hours in tetrazolium chloride solution, dehydrogenase enzymes in actively respiring areas of the seed react with the colourless TZ to form the red-coloured formazan, which precipitates in and stains the tissues. By evaluating the location and intensity of the formazan stain, one can readily differentiate between healthy and damaged portions of the seed.

The TZ evaluation focuses directly upon the physical and physiological condition of the embryo and endosperm (or, in conifers, the megagametophyte), but relies heavily upon the expertise and intuition of the experienced analyst (Leadem 1984). Since the TZ rating is independent of inhibitions imposed by dormancy, results may not relate well to actual performance. TZ viability ratings are often higher than actual germination in standard incubator tests (Kaul & Zentsch 1969, Simak 1970, Leadem 1984). However, incubator tests are usually more relevant to nurseries because results correlate directly to nursery performance. The utility of the test depends upon whether the analyst is interested in ultimate seed potential, or in assessing seed performance under field conditions.

The purpose of this study was to investigate the effects of different incubation temperature regimes (30/20 °C, 30 °C, and 20 °C) on seed germination of *Dalbergia cochinchinensis, Pinus kesiya* and *Pinus merkusii,* and to determine how stratification affected the germination response. Seed viability predicted by the X-ray and tetrazolium chloride (TZ) quick tests was compared to germination test results to determine their suitability for testing these tropical seeds. It is hoped that the

information from this study will facilitate nursery operations in tropical countries where *Dalbergia cochinchinensis, Pinus kesiya* and *Pinus merkusii* are grown.

Materials and methods

Seed sources

Seeds were obtained from the ASEAN-Canada Forest Tree Seed Centre, Muak Lek, Saraburi province, Thailand, and the Silviculture Division, Royal Forest Department, in Bangkok. Species and seed sources are listed in Table 1.

Table 1. Species, seed sources and collection dates

Species	Seed source	Collection date
Dalbergia cochinchinensis	Kang Koi, Saraburi	7 September 1990
Pinus kesiya	Samreng, Chiangmai	January 1987
Pinus merkusii	Tung Phaya, Phitsanulok	23 April 1986

Treatments and germination tests

Seeds of each species were divided into two equal groups which were either (1) soaked for 24 h, or (2) soaked for 24 h, drained and then stratified for 2 weeks at 2 - 5 °C. After soaking or stratification, seeds were incubated for 3 weeks in controlled environment chambers at (1) 30 °C for 8 h and 20 °C for 16 h, (2) 30 °C for 24 h, and (3) 20 °C for 24 h. Light was provided for 8 h daily at a light intensity of about 50 microeinsteins m^{-2} sec^{-1}. For the alternating temperature regime, light was given during the high temperature period.

For laboratory germination tests, four replications of 50 seeds were used for each treatment. Seeds were placed in covered plastic boxes (12 × 12 × 3 cm) on a substrate of one layer of Kimpak covered with two layers of Whatman No. 1 filter paper. The media were moistened with 50 ml de-ionized water, and placed in random order in controlled environment incubators. Germination counts were made daily and terminated after 3 weeks. Seeds were considered germinated once the radicle and hypocotyl had grown to four times the length of the seed coat.

X-ray tests

Each replicate of 50 seeds used for the germination tests was X-rayed to determine whether seeds were empty or filled. Settings for the Faxitron X-ray machine were 3 mA and 15 kV, and exposure time was 2 min. Exposures were processed in a Kodak Industrex Instant Processor using light sensitive paper, 8 × 10 inches (20.3 × 25.4 cm) in size, to make the radiographic images. Seed development and viability were determined by evaluating the density, shape and location of opaque matter (bright areas) seen in the X-rays.

Tetrazolium chloride tests

The TZ solution (pH 6.5 - 7.0) was prepared from 2,3,5-triphenyl tetrazolium chloride, diluted to 1% (W/V) in distilled water, and stored at 5 °C in the dark. To determine the most suitable length of time to stain seeds in the TZ solutions, seeds of *D. cochinchinensis, P. kesiya* and *P. merkusii* were incubated in the dark at 37 °C for various periods from 1 to 24 h. Because of the limited amount of seed material, only two replications of 5 seeds were used for each treatment in these preliminary trials. When staining was complete, seeds were immediately rinsed two to three times with distilled water. The period required for adequate staining varied with each species, but light pink to red staining of the embryo was favoured because seeds are easier to evaluate when tissues are less heavily stained (Leadem 1984).

To prepare the seeds for TZ tests, *Pinus* seeds were soaked intact for 24 h in de-ionized tap water, and the seeds were cut longitudinally alongside the embryo to allow for better penetration of the TZ solution. Intact seeds of *D. cochinchinensis* exhibited poor water uptake, so it was necessary to cut off about 1/6 of the seed at the end opposite the embryo to imbibe the seeds. No additional cuts were made prior to incubation in the TZ solution. The seeds of all three species were incubated in 1% TZ solutions at 37 °C in the dark.

D. cochinchinensis seeds were incubated for 24 h, and *P. kesiya* and *P. merkusii* seeds were incubated for 4 h, the periods found to be most effective in preliminary tests (Figure 1). Four replications of 20 seeds each were used for each TZ viability test.

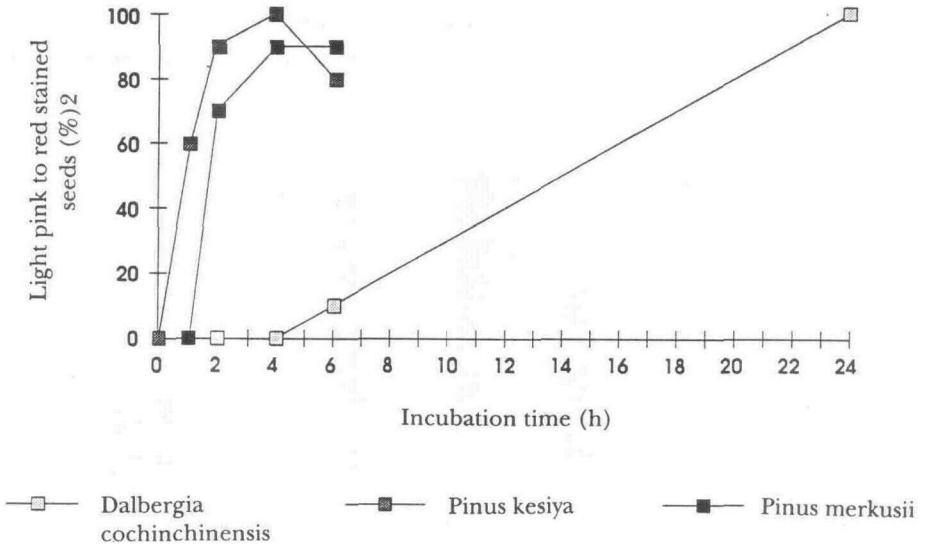

Figure 1. Light pink to red-stained seeds of *Dalbergia cochinchinensis, Pinus kesiya* and *Pinus merkusii* as a percentage of the total

Staining of *Pinus* seeds was evaluated after excising the embryos from the megagametophyte. *D. cochinchinensis* seeds were split along the halves of the cotyledons so that the embryo axis was visible. Seed viability was evaluated by observing the intensity and location of staining, and categorizing each seed according to the four quality classes defined in Table 2. Only those seeds in classes I and II were considered viable for purposes of comparison with results of germination tests.

Table 2. Quality classes for evaluating the tetrazolium test

Class	Description	Viability
I	Embryo completely stained	Viable
II	Very pale staining, or >75% embryo stained	Viable
III	Radicle and/or cotyledon unstained, or >25% of embryo unstained	Non-viable
IV	Embryo unstained	Non-viable

Data analysis

Treatments and replications were completely randomized for each species in the germination test. Germination rates were plotted as the percentage of total seeds sown that germinated each day of the test period. Total germination after 21 days, and the viable seeds as estimated by X-ray and TZ tests were calculated as percentages based on the number of seeds used. Germination data were analyzed by means of ANOVA and statistical differences were determined at 5% probability level. Means were compared using Duncan's multiple range test (Duncan 1965). The t-test at $p = 0.05$ was performed to test the differences between total germination and the viability of seeds as determined from the results of X-ray and TZ tests.

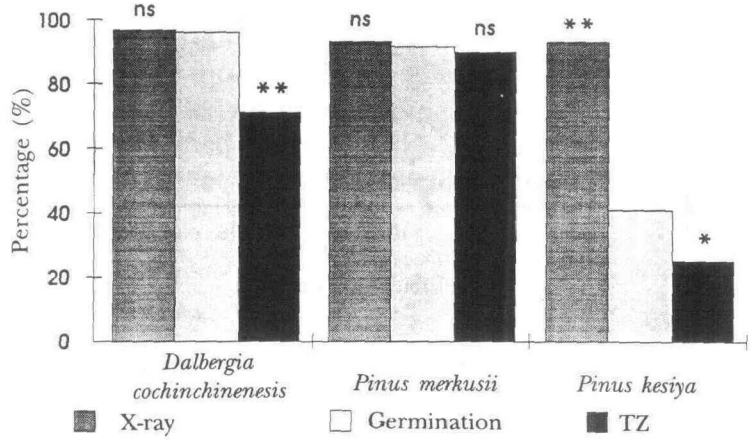

Figure 2. Comparison of total germination percentage using the most suitable treatment for each species with the percentage of viable seeds as evaluated from X-ray and the TZ test results. Using the t-test, ns = not significantly different from the germination test; * significantly different, $p = 0.05$; ** significantly different, $p = 0.01$.

Results and discussion

Dalbergia cochinchinensis

Stratification did not appreciably improve the germination speed of *D. cochinchinensis* seeds in any of the temperature regimes (Figures 3, 4). The fastest rates were obtained with unstratified seeds incubated under 30 °C, and the slowest rates were achieved with stratified seeds under 20 °C temperatures (Figure 3). Under the two warmest regimes, germination generally started 5 to 6 days after sowing. Under 20 °C, unstratified seeds started to germinate on day 15, whereas stratified seeds did not germinate at all.

Table 3. Effects of stratification and incubation temperature on germination capacity of *Dalbergia cochinchinensis, Pinus kesiya* and *Pinus merkusii*

Treatment		Germination capacity (%)		
Strat.	Incubation	*Dalbergia cochinchinensis*	*Pinus kesiya*	*Pinus merkusii*
None	30/20 °C	96.5 a	91.0 b	37.0 ab
None	30 °C	91.0 a	93.5 ab	37.5 ab
None	20 °C	4.5 d	96.0 a	28.0 ab
2 wk	30/20 °C	88.0 bc	94.0 ab	37.5 ab
2 wk	30 °C	83.0 c	92.0 ab	41.0 a
2 wk	20 °C	0.0 d	92.0 ab	24.5 b

Values followed by the same letter in each column are not significantly different at p = 0.05 as determined by Duncan's multiple range test (1965).

Total germination after 21 days differed significantly (ANOVA, p = 0.05) among the various treatments (Table 3). The highest germination percentages were achieved by unstratified seeds incubated under 30/20 °C (96.5%) and 30 °C (91.0%). Total germination of stratified seeds incubated under the same temperatures was significantly lower (Figure 4). Under 20 °C, germination of stratified and unstratified seeds was low or non-existent.

To determine whether quick tests could be used to predict viability of *D. cochinchinensis*, comparisons were made between total germination under the best test conditions (no stratification at 30 °C/20 °C) and the results obtained from X-ray and TZ tests (Figure 2). Significant differences (t-test, p = 0.01) were found between TZ test results (72.5%) and germination tests (96.5%). The relatively low percentage achieved with the TZ test was most likely due to the impermeability of the seed coat. In spite of the care taken to cut the coat and imbibe the seeds prior to incubation in the TZ solution, there appeared to have been inadequate penetration of the TZ solution into the tissues. Of the three species studied, *D. cochinchinensis* required far longer incubation in the TZ solution

to achieve acceptable staining of the seeds (Figure 1). Based on the results of this study, incubation longer than 24 h may be necessary.

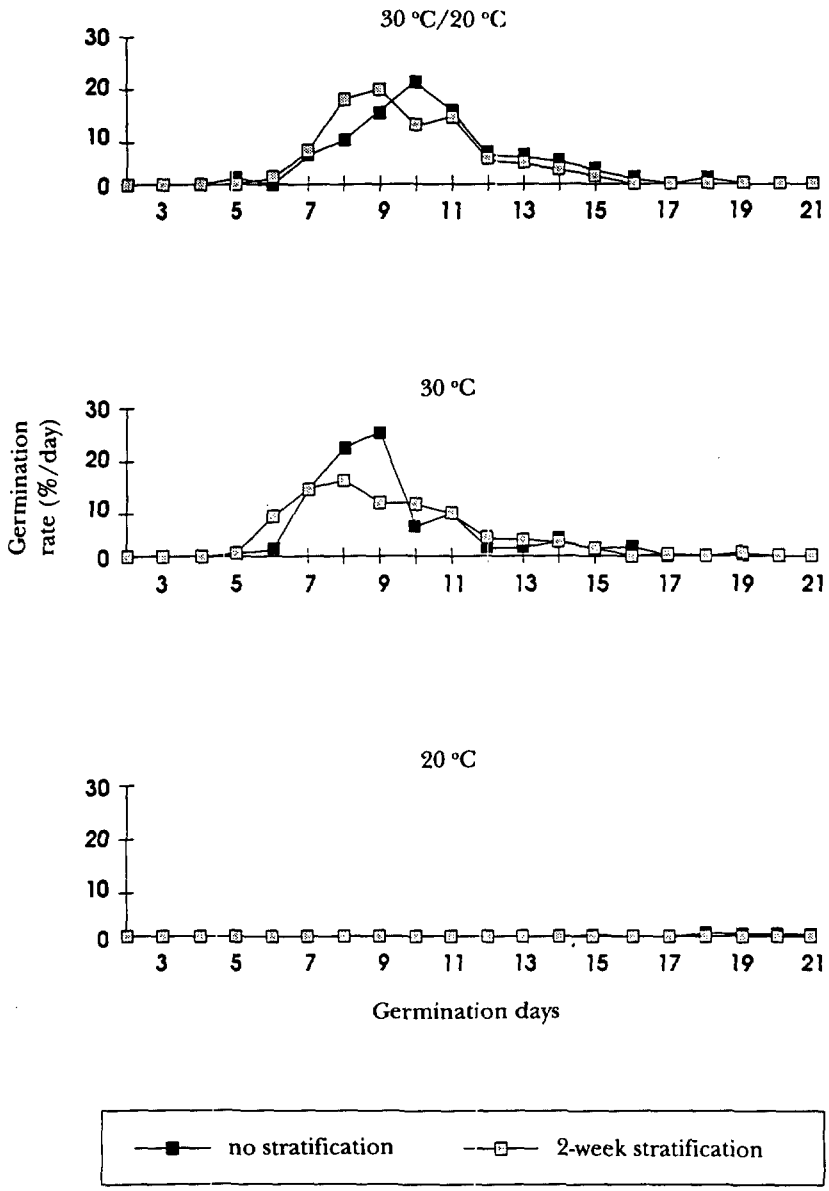

Figure 3. Effects of stratification on the germination rate of *Dalbergia cochinchinensis* seeds at three incubation temperatures

X-ray test results (97%) did not differ significantly from the results of germination tests (96.5%) (Figure 2). For a species such as *D. cochinchinensis*, X-ray tests would probably have good potential for the rapid determination of seed quality because X-rays work best in species which exhibit no dormancy. In dormant seeds, X-ray tests tend to have poor predictive ability because visual

evaluation is made irrespective of the dormancy status of the embryo (Leadem 1984).

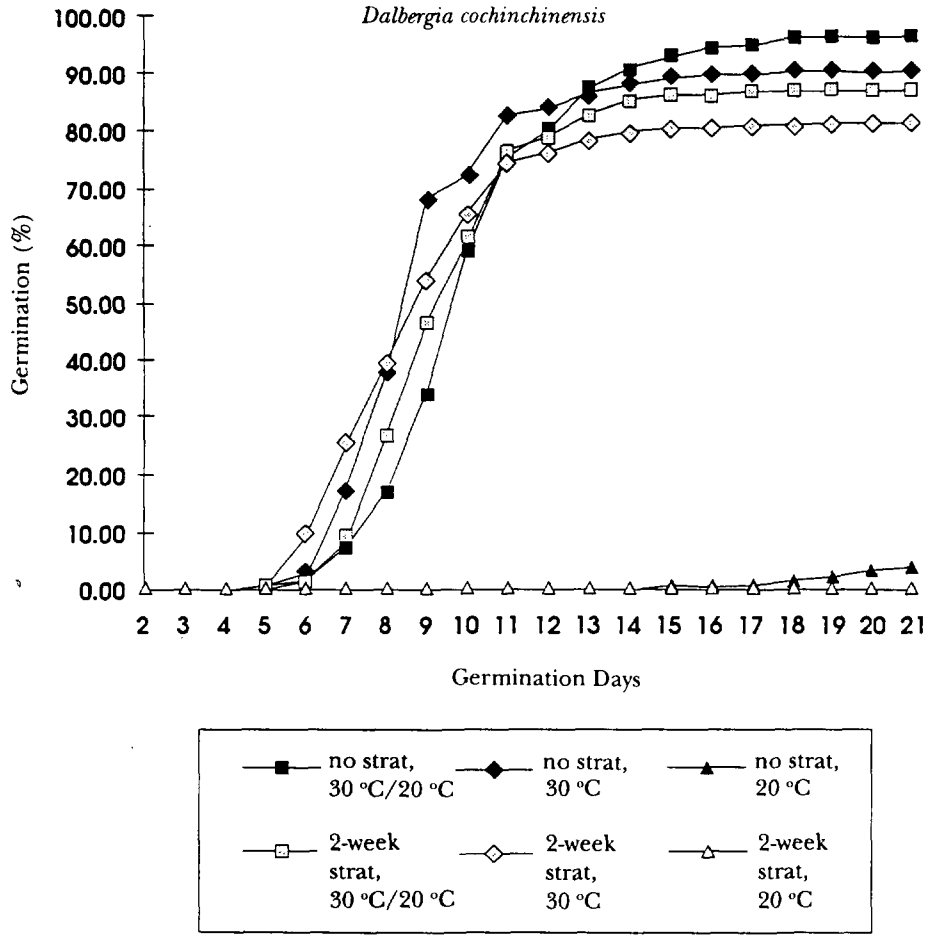

Figure 4. Effects of stratification treatment and incubation temperature on the germination of *Dalbergia cochinchinensis* seeds

Pinus kesiya

Stratification for 2 weeks enhanced the germination rate at all three incubation temperatures (Figure 5). Germination started on days 4, 5 and 8 for seeds which were stratified and incubated at 30 °C, 30/20 °C and 20 °C respectively. Unstratified seeds commenced germination on days 5, 6 and 9 when incubated at 30 °C, 30/20 °C and 20 °C, respectively (Figure 5). The germination rate was highest on day 6 (42%) for stratified seeds under 30 °C (Figure 5), and the slowest was observed for unstratified seeds incubated at 20 °C.

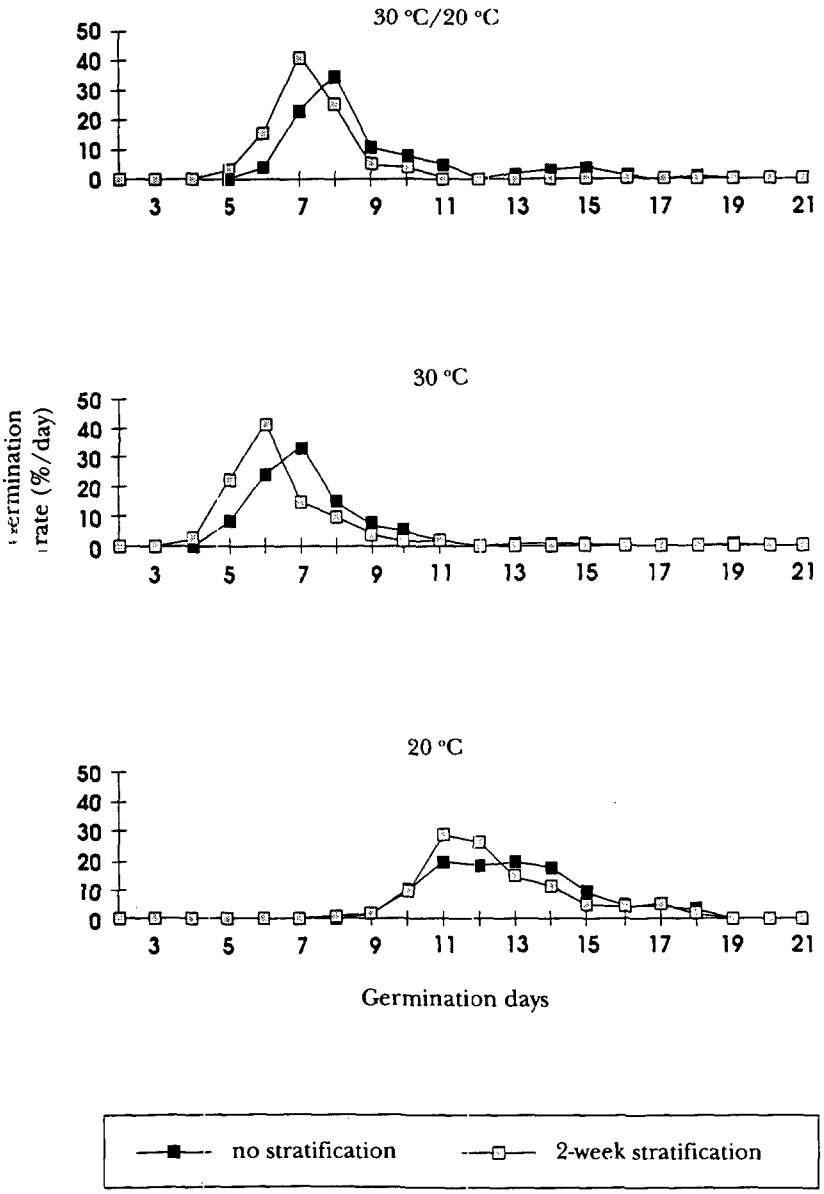

Figure 5. Effects of stratification on the germination rate of *Pinus kesiya* seeds at three incubation temperatures

Germination was nearly complete by day 10 for seeds which were incubated at 30 °C and 30/20 °C (Figure 6). For seeds incubated at 20 °C, germination continued until day 16. By day 21, total germination of all treatments was approximately the same, ranging from 91 to 96% (Table 3). The results of ANOVA and Duncan's multiple range test indicated no significant differences in germination between most of the stratification and temperature treatments.

Only unstratified seeds incubated at 20 °C and unstratified seeds incubated at 30/20°C were significantly different from one another, and even then, the 5% increase in germination was not large enough to be of practical importance.

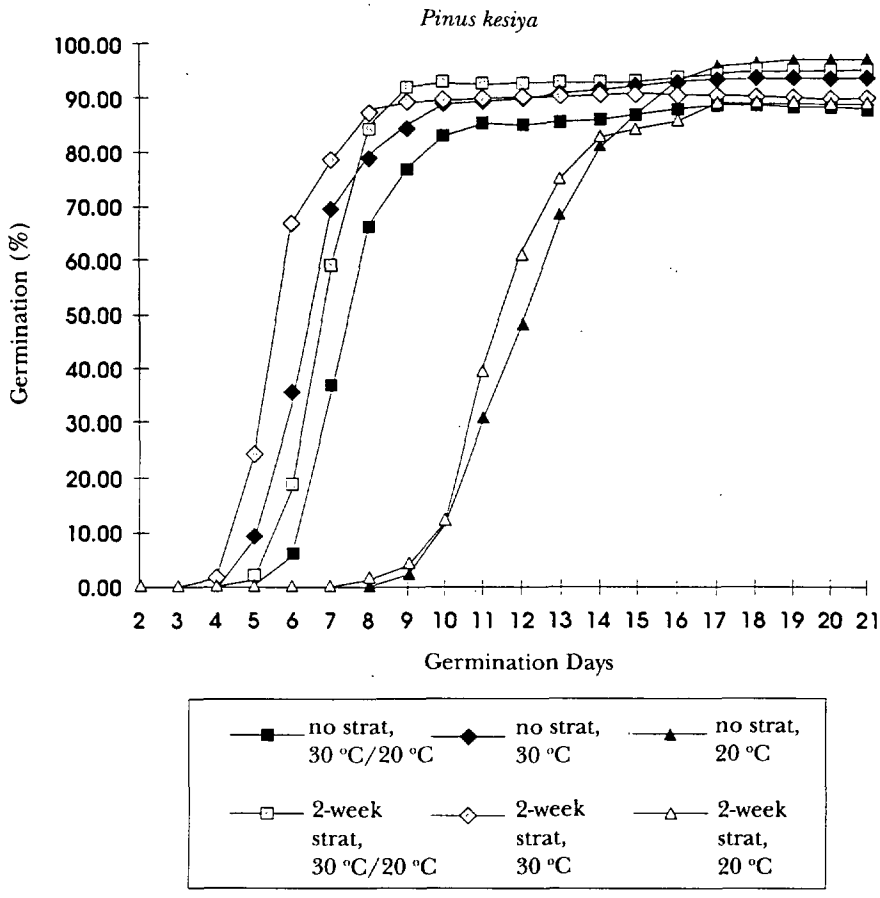

Figure 6. Effects of stratification treatment and incubation temperature on the germination of *Pinus kesiya* seeds

When the t-test (p=0.05) was used to evaluate the efficacy of different viability tests for *P. keysia* seeds, no significant differences were found between any of the three test procedures (Figure 2). Total germination under the best treatment conditions (2-weeks' stratification and 30 °C incubation temperatures) was 96.0%, compared to a viability estimate of 93.5% for the X-ray test, and 90.0% for the TZ test.

Pinus merkusii

When incubated at 30 °C, 30/20 °C and 20 °C, germination of stratified seeds began on days 7, 8 and 14 respectively (Figures 7, 8). Unstratified seeds began

germinating on days 8, 9 and 15 at 30 °C, 30/20 °C and 20 °C, respectively. The germination of stratified and unstratified seeds incubated at 30 °C/20 °C and 30 °C was nearly complete by 21 days, but under 20 °C temperatures, germination did not appear to be complete by the end of the test (Figure 8).

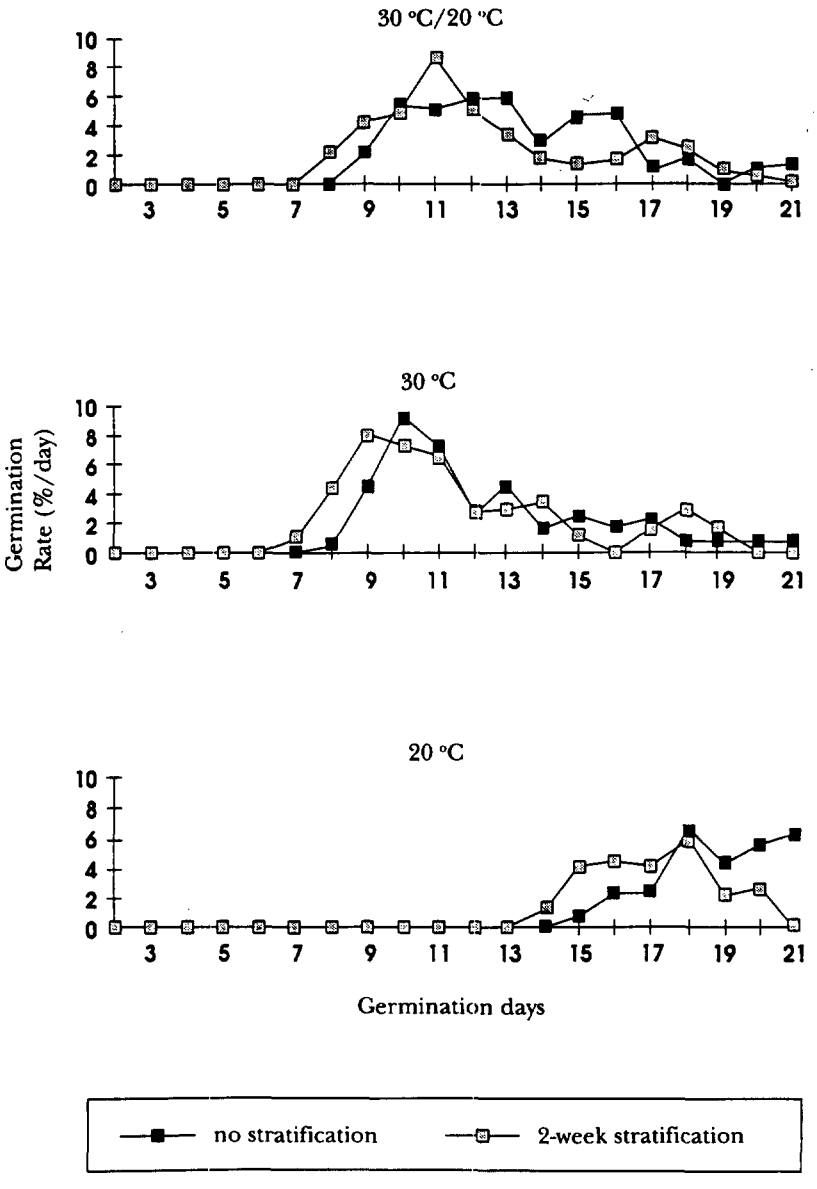

Figure 7. Effects of stratification on the germination rate of *Pinus merkusii* seeds at three incubation temperatures

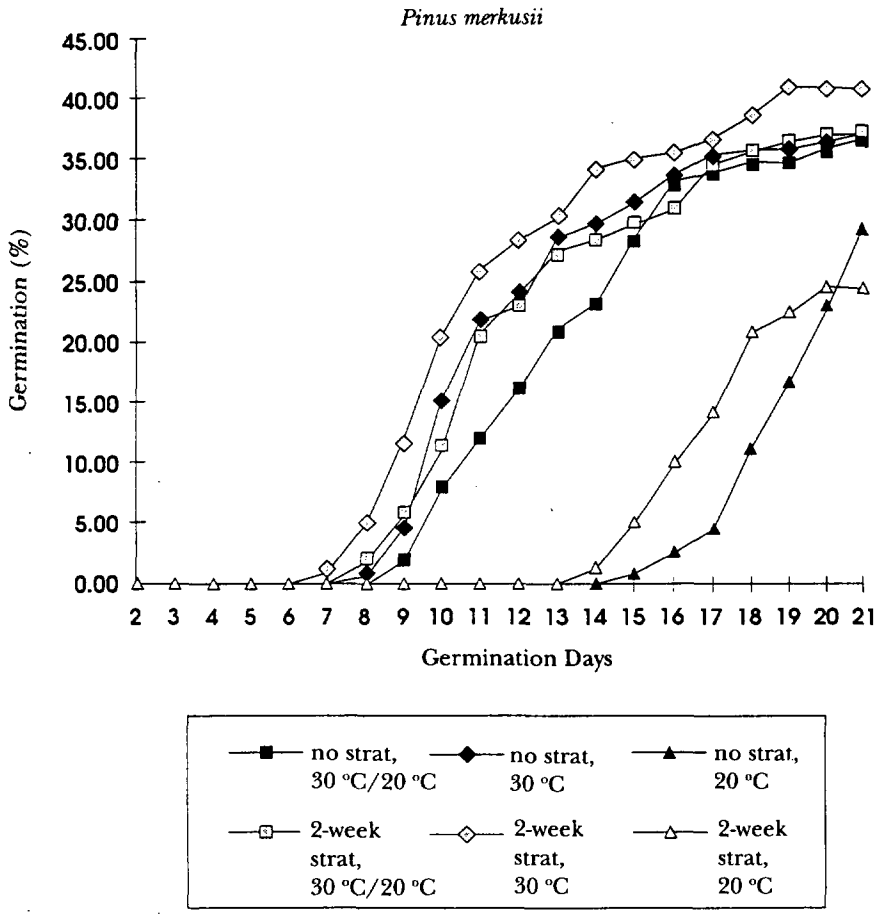

Figure 8. Effects of stratification treatment and incubation temperature on the germination of *Pinus merkusii* seeds

Total germination from all treatments after 21 days ranged from 24.5 to 41.0% (Table 3). Results of a statistical analysis using ANOVA and Duncan's multiple range test showed significant differences between the stratified seeds incubated at 30 °C and 20 °C, but no significant differences between any of the other treatments.

The correspondence between total germination under the best treatment conditions (2-weeks' stratification at 30 °C) and viable seeds determined from the X-ray and TZ tests was not as close for *P. merkusii* as it was for *P. kesiya* (Figure 2). The X-ray test results (94.0%) were significantly higher (t-test, p=0.01), and the TZ test results (26.3%) were significantly lower (t-test, p=0.05) than germination test results (41.0%) (Figure 2). Thus, X-rays substantially overestimated the number of viable seeds, and TZ underestimated viability.

The X-ray test assesses seed viability solely on physical characteristics. Whether or not physically sound seeds germinate, however, depends upon physiological factors. Environmental conditions also play an important role. Any or all of these variables could be responsible for the discrepancies between the tests. The TZ test is based on physiological characteristics of seeds, but it also failed to accurately predict viability. This was probably due to insufficient penetration of the stain. Longer incubation in the TZ solution would have possibly increased the degree of staining of the tissues, and allowed for more accurate interpretation of test results.

Another significant factor was the relatively low germination of the *P. merkusii* seed source used in this study. Poor quality seeds are much more variable in their responses to different treatments and test conditions (Edwards 1980, Leadem 1986, Sorenson 1980). With such material, it is frequently difficult to interpret results, regardless of the method used. The use of improved seed treatments, more suitable incubation conditions and a better quality seed source would undoubtedly improve the correspondence between germination, TZ, and X-ray tests.

Conclusions

Stratification enhanced germination rates of *Pinus kesiya* and *Pinus merkusii* under all incubation temperatures, but did not increase the germination speed of *Dalbergia cochinchinensis* seeds. High temperature (30 °C) enhanced germination speed in all three species, but total germination was not always the greatest under the highest temperature.

With regard to quick tests, X-ray test results correlated well with germination test results obtained for *Dalbergia cochinchinensis* and *Pinus kesiya*. For *Pinus merkusii*, X-rays substantially overestimated (by more than twice) the number of seeds which germinated under standard test conditions. This discrepancy may not represent limitations of the X-ray procedure, but may reflect an inappropriate choice of germination test conditions, or the manner in which seeds were treated prior to testing.

Results of the TZ tests corresponded well with the results of germination tests for *Pinus kesiya*, but not for either *Dalbergia cochinchinensis* or *Pinus merkusii*. TZ tended to underestimate viability, relative to results achieved in the germination test. Penetration of the stain was definitely a problem; this could be rectified by longer incubation periods or by altering test conditions such as temperature. It is evident that further studies are needed to determine the best TZ test procedures for these forest tree seeds.

Since only a single seed source was used per species, these results cannot be extrapolated to the species level. Seeds of most tree species are inherently variable in their developmental and physiological characteristics, and additional seed sources must be examined before establishing standard protocols for the germination of these important tropical forest tree species.

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