Dormancy and barriers to germination

Proceedings of an international symposium of IUFRO Project Group P2.04-00 (Seed Problems)

Compiled and edited by

D.G.W. Edwards
Pacific Forestry Centre

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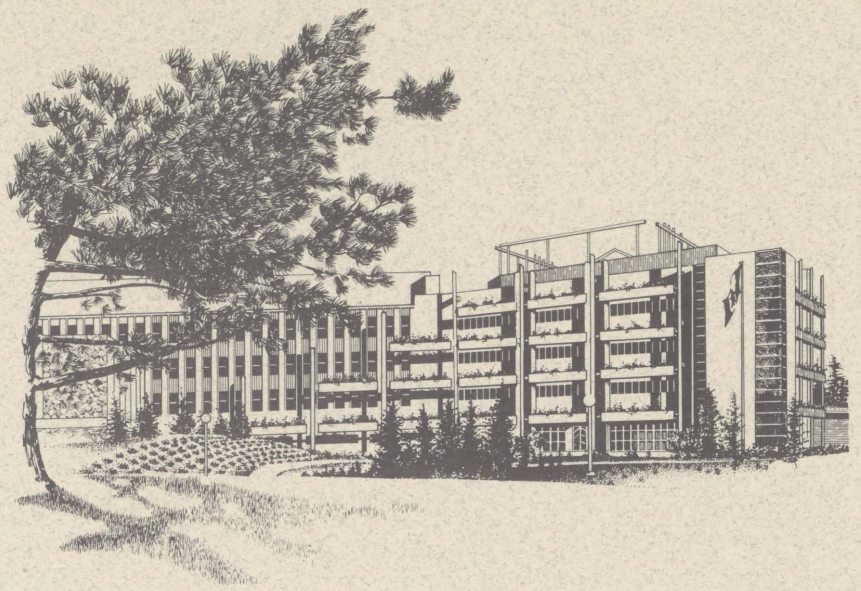

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Cover photo: scanning electron micrograph of a mature seed of Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) showing the three genomes—the diploid embryo surrounded by the haploid megagametophyte and all enveloped by the diploid seedcoat. The micropylar region is in the lower left portion of the view; the remnants of the seedwing are to the right. Scale approx. 40×. Photograph made by L.E. Manning, Pacific Forestry Centre, Victoria, B.C. on a JEOL 35C SEM.

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Victoria, British Columbia, Canada
April 23–26, 1991

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Forestry Canada
Pacific Forestry Centre

1993

Forestry Canada
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Victoria, British Columbia
V8Z 1M5
Phone (604)363-0600

Cat. No. Fo18-25/1991E
ISBN 0-662-20777-7
Printed in Canada

Microfiches of this publication may be purchased from:

MicroMedia Inc.
Place du Portage
165, Hotêl-de-Ville
Hull, Quebec
J3X 3X2

Canadian Cataloguing in publication data

Main entry under title:

Dormancy and barriers to germination: proceedings of an international symposium
of IUFRO Project Group P2.04-00 (Seed Problems)

"Victoria, British Columbia, Canada
April 23–26, 1991"
ISBN 0-662-20777-7
DSS cat. no. Fo18-25/1991E

1. Trees -- Seeds -- Dormancy -- Congresses.
2. Seeds -- Dormancy -- Congresses.
3. Germination -- Congresses.
I. Edwards, D.G.W. II. Pacific Forestry Centre.
III. International Union of Forestry Research Organizations. Project Group P2.04-00 (Seed Problems)

SD403.D67 1993 634.9'562 C93-099642-9

Acknowledgements

The Project Group is grateful to Ms. H. Matson of the Pacific Forestry Centre (PFC) for typing those manuscripts that were not on diskette, and to Mr. S. Glover (Editor, PFC) who ensured that publication of these proceedings meet Canadian Government standards. Typesetting and graphic design was done by Mr. D. Dunaway (PFC).

Grateful acknowledgements are also made to the local organizing committee: Dr. C.L. Leadem (BCMOF), Dr. Y.A. El-Kassaby (CPFP), Dr. M. Meagher and Messrs. D.W. Taylor and F.T. Portlock (PFC). Ms. B. Newbery (CPFP) and Ms. J. Clark (BCMOF) helped with registration. We are also indebted to the BCMOF for graciously hosting the banquet. The guest speaker was Mr. P. Ackhurst, Director of the Silviculture Branch of the BCMOF, who spoke on "Forest Renewal in British Columbia". Thanks are due also to CPFP and BCMOF for providing lunches on the field trips.

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Preface

Of the 254 working parties and project groups (IUFRO Annual Report 1991), Project Group P2.04-00 (Seed Problems) is one of the more active, judging from the number of symposia that it has held since its inception in 1972; the first symposium was held in Norway in 1973. The publications of these proceedings mark the twenty-first anniversary of the Project Group (formerly Working Party S2.01.06), and it is fitting to recognize the efforts of Prof. M. Simak (Sweden), who chaired the Working Party through 1981, together with Dr. S. Asakawa (Japan) and Dr. K. Kamra (Sweden), Co-Chairpersons, and Dr. F.T. Bonner (U.S.A) who chaired the group though 1990, together with Ms. K. Lind-Mulet (Guatemala) and Dr. K. Kamra (Sweden), Co-Chairpersons, for making the present Project Group such a dynamic entity. Our membership list remains at over 200, and plans are being formulated to create three fledgling working parties that will concern the World Directory of Tree Seed Workers, Tree Seed Radiography, and Tropical Tree Seeds.

The 1991 Symposium in Victoria, British Columbia, was held between April 23 and 26. This marked the second time that the Project Group had met in Canada; the first was in 1980. The 1991 meeting attracted more than 60 participants from six countries. The papers were presented in four technical sessions, the first three of which had invited lead papers. Two field trips were held, one to the Canadian Pacific Forest Products' (CPFP) seed orchard in Saanichton, and a second to the British Columbia Ministry of Forests' (BCMOF) Tree Seed Centre in Surrey. The program ended with a project group business meeting, and a tour of the Pacific Forestry Centre (PFC).

Twenty one papers were submitted by tree seed workers from 11 countries, and all are included in these Proceedings, together with an abstract of one (the only one submitted for publication) of the eight posters that were displayed.

D.G.W. Edwards
Compiler and Editor

After-ripening requirement, dormancy and germination in rowan (*Sorbus aucuparia* L.)

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Abstract

Seeds of *Sorbus aucuparia* L. possess both testa and embryo dormancy at maturity. After-ripening of intact seeds at 1°C or at 4°C resulted in over 70% germination after 20 wk incubation. No germination was observed after 20 wk incubation at 10°C, 15°C or 20°C respectively. Fresh samples of intact seeds or dehulled *Sorbus aucuparia* embryos were cultivated aseptically on solid media containing either NAA, IAA, IBA, BAP or GA_3. For isolated embryos, the rate of cotyledon greening, growth of hypocotyl, roots and shoots and germination percentage were determined after incubation at 20°C in continuous light for one month. No germination was recorded for intact seeds subject to any treatment. Gibberellic acid had no observable effects on isolated embryos. All auxin treatments stimulated the greening on cotyledons. Treatments with IAA at 5 mg/L enhanced root growth as did NAA at 2-3 mg/L. Indole butyric acid (IBA) had little effect on root growth. Treatment with BAP resulted in 76% of embryos producing both shoots and roots. Root growth was most significantly enhanced with a lower concentration of BAP (0.1 or 1 mg/L), as was the production of single shoots (0.1-2 mg/L). Higher concentrations stimulated multiple shoot growth and inhibited root growth.

Résumé

L'embryon ainsi que le tégument à maturité des graines de *Sorbus aucuparia* L. ont une dormance. La postmaturation des graines intactes à 1°C ou à 4°C a donné 70 % de germination après 20 semaines d'incubation. Aucune germination n'a été observée passé ces 20 semaines, que ce soit à 10°C, 15°C ou 20°C. Des échantillons frais de graines intactes ou d'embryons décortiqués de *Sorbus aucuparia* ont été cultivés en milieu aseptique sur un milieu solide contenant de l'ANA, de l'AIA, de l'AIB, du BAP ou de la GA3. Dans le cas d'embryons isolés, le taux de verdissement du cotylédon, la croissance de l'hypocotyle, des racines et des pousses ainsi que le pourcentage de germination ont été déterminés après incubation à 20°C sous un éclairement continu pendant tout un mois. Il n'y a pas eu de germination des graines intactes, quel que soit le traitement. L'acide gibberellique n'a pas eu d'effet observable sur les embryons isolés. Tous les traitements aux auxines ont stimulé le verdissement des cotylédons. Les traitements à l'AIA en concentration de 5 mg/L ont stimulé la croissance des racines, tout comme l'ANA en concentration de 2 à 3 mg/L. L'acide indole butyrique (AIB) a eu peu d'effet sur la croissance des racines. Le traitement au BAP a conduit à la production de pousses et de racines chez 76 % des embryons. La croissance des racines a été le plus fortement stimulée à une moindre concentration de BAP (0,1 ou 1 mg/L), tout comme la production de pousses uniques (0,1 à 2 mg/L). Le traitement à des concentrations supérieures a stimulé la croissance de pousses multiples et inhibé la croissance des racines.

Introduction

The seeds of *Sorbus aucuparia* L. have long been known to be dormant. They require after-ripening at a low temperature (1-4°C) for germination to take place. It is also known that the seed coat plays a role in dormancy (Flemion 1931). However, very little work has been performed on the nature of embryo dormancy in this species, and little is known about the possible role of plant growth substances in the regulation of dormancy.

Materials and methods

Rowan seeds were collected from mature trees growing within the campus of the University of Edinburgh during October 1986. Fruits were pulped, seeds extracted, dried, cleaned and stored at 4°C until required.

A. After-ripening

For each treatment at 1°C, 4°C, 10°C, 15°C and 20°C, seeds were surface sterilized for 15 minutes in 1% sodium hypochlorite solution, washed once and imbibed in sterile distilled water for one day. Thirty seeds were then placed aseptically on a 9 cm petri dish containing two layers of Whatman No. 1 filter paper and 5 mL sterile distilled water. Each temperature treatment consisted of 40 such petri dishes sealed with Parafilm and placed randomly into incubators at the various temperatures for up to 20 wk.

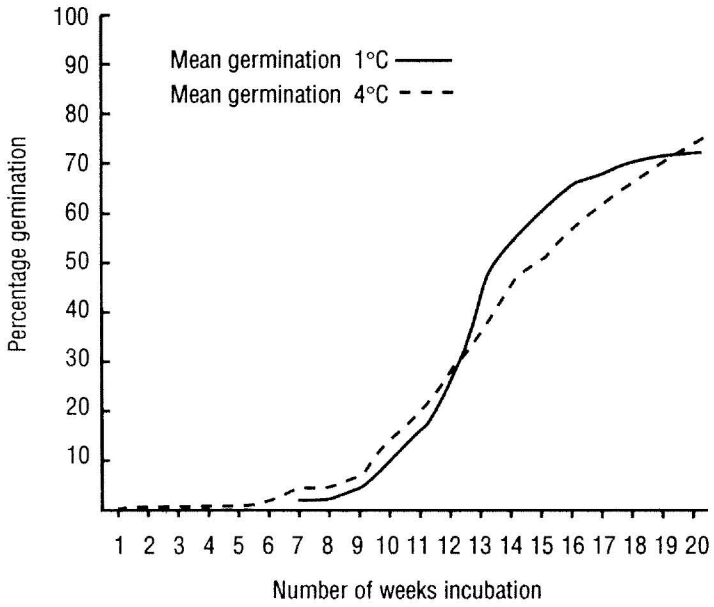

Figure 1. Mean cumulative germination of *S. aucuparia* at two storage temperatures.

***B. Cultivation* in vitro**

Seeds were surface sterilized as above, but imbibed for two days in sterile distilled water that was changed daily. Seed coats were removed aseptically. Embryos or whole seeds were placed into 60 mL specimen jars containing 20 mL half strength Murashige and Skoog (M & S) medium plus 0.8% agar and various concentrations of either benzyl amino purine (BAP), indole acetic acid (IAA), indole butyric acid (IBA), napthalene acetic acid (NAA), or gibberellic acid (GA_3) as follows: 0, 0.1, 1, 2, 3, 4, and 5 mg/L. Each jar contained five seeds or embryos and each treatment was replicated five times. Jars were transferred to a Gallenkamp growth cabinet at 20°C and continuous white fluorescent light. Observations were performed weekly on cotyledon greening, hypocotyl extension, and root and shoot growth. Embryos were considered to have germinated once both a shoot and root were visible.

Results

A. After-ripening

Seeds not only after-ripened, but also germinated at 1°C and 4°C. Visible germination began after 7 wk incubation and, after 20 wk, 74% (1°C) or 75% (4°C) had germinated, there being no significant difference (Fig. 1). No germination was recorded for seeds incubated at 10°C, 15°C or 20°C.

***B. Cultivation* in vitro**

(i) Germination of Intact Seeds

No germination was observed after incubation for 1 month in any treatment.

(ii) Cotyledon Greening

Cotyledons of embryos from dehulled seeds placed on media with no added plant growth substances (PGS) showed complete greening in 70% of cases, the remaining 30% of seeds possessed only one green cotyledon. Treatment with IAA or BAP resulted in complete greening in all cases, whereas treatment with NAA or IBA resulted in complete cotyledon greening in 80% of the embryos. Seeds placed on GA_3 did not develop and senesced within 7 days.

(iii) Embryo Development

Treatment with auxins generally resulted in a decline ($P < 0.01$) in hypocotyl extension and shoot growth ($P < 0.001$ for NAA and IAA, N.S. for IBA) compared with controls (Fig. 2A, 2C). Only 12% of embryos had expanded hypocotyls, roots and shoots in NAA (at 0.1 and 1 mg/L), compared to 16% with IAA (at 1 mg/L), and 8% with IBA (at 2 mg/L).

Treatment with IAA (5 mg/L) significantly ($P < 0.1$) enhanced root growth (average length 3.3 mm); this was the result of a large increase in root growth in only 20% of the seeds (Fig. 2B). Increases in root growth due to NAA (2 mg/L, mean root length 2.8 mm; 3 mg/L, mean root length 1.9 mm) were less dramatic, but occurred in 60% and 40% of embryos respectively.

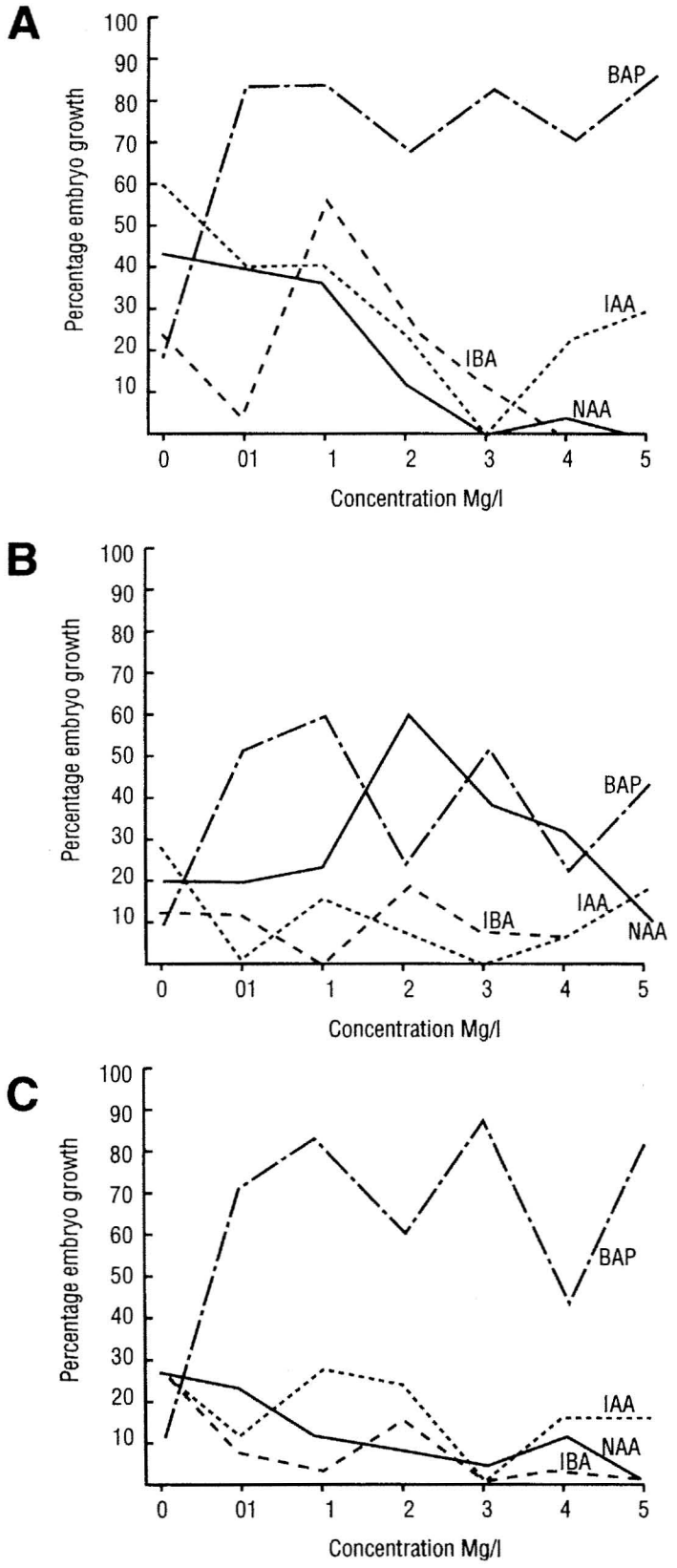

Figure 2. Effect of plant substances on the percentage of dehulled *S. aucuparia* seeds showing growth of (A) hypocotyl, (B) roots and (C) shoots after one month incubation *in vitro* at 20°C in continuous light.

The majority of embryos placed on M & S medium with no PGS failed to develop and were dead in 1 month (Fig. 3B).

Incorporation of BAP into the media resulted in a significant increase in hypocotyl growth ($P < 0.001$) at all concentrations tested (Fig. 4), with an average of 80% of embryos in every treatment showing some hypocotyl extension, compared to 16% for control seeds (Fig. 2A). Root growth was significantly ($P < 0.001$) promoted only at BAP concentrations of 0.1 or 1 mg/L (Fig. 4), with between 50% and 60% of embryos showing some root growth compared to only 8% for controls (Fig. 2B). The number of shoots was significantly increased ($P < 0.001$) by BAP treatment (Fig. 4), an average of 76% of all embryos subjected to treatment with BAP showing some shoot growth (Fig. 2C). The treatment, after 4 weeks incubation, that produced seedlings most closely resembling those of seedlings after-ripened for 4 months, was BAP at 0.1 mg/L (Fig. 3A,C).

Discussion

The period and temperature (1°C or 4°C) required for after-ripening broadly agrees with the work of Flemion (1931).

Low concentrations of the cytokinin BAP can closely reproduce the effects of chilling in dehulled *Sorbus aucuparia* seeds whereas gibberellin (GA_3) treatment had no effect on embryo development. This is in contrast to work performed on apple embryo-dormancy, where GA_3 stimulated greening and germination of embryos. In addition, in apple, there was some relationship between gibberellin concentration and germination which was not apparent with kinetin treatment (Bulard 1985). This contrasts with the present result with BAP at least at the lower rates of application to rowan embryos.

Auxin treatment was generally ineffective at inducing growth in embryos, although NAA and BAP treatments could promote root growth. It would appear, therefore, that auxins have little role in breaking dormancy in rowan seeds.

Incubation of whole seeds *in vitro* did not result in germination. Further work is therefore required to elucidate the mechanism of coat-imposed dormancy. Our

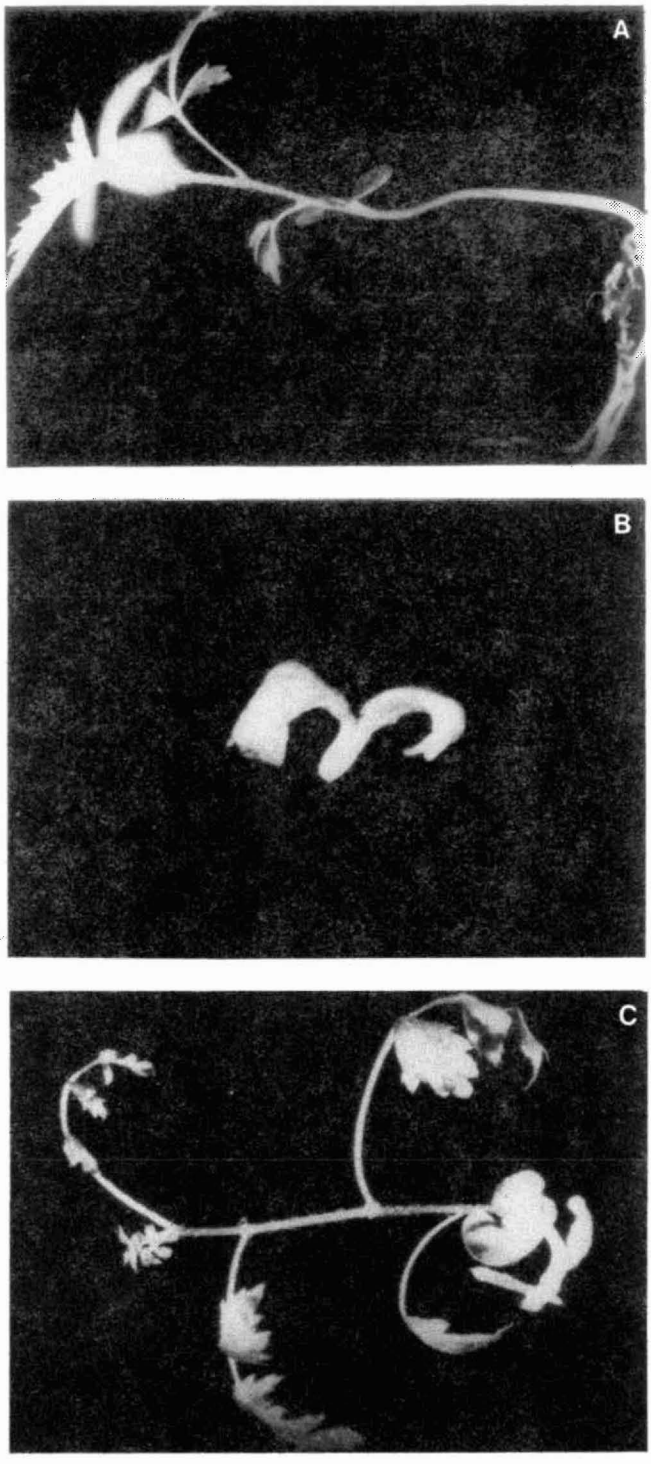

Figure 3. Growth of *S. aucuparia* seedlings: (A) after-ripening seedling, 7 d after germination in soil at 15°C; (B) fresh-excised embryos placed aseptically on half-strength M & S medium for 1 month; (C) as B except on half-strength M & S medium plus 0.1 mg/BAP.

results indicate a role for cytokinins in dormancy breaking in rowan, although more work is required with respect to further cytokinin treatments on intact seeds and other gibberellin application to the intact and dehulled seeds. In providing contrasting results to that recorded for another rosaceous species, they illustrate the diversity of dormancy breaking mechanisms found in seeds.

Acknowledgement

The senior author is grateful to Universiti Pertanian Malaysia for financial support.

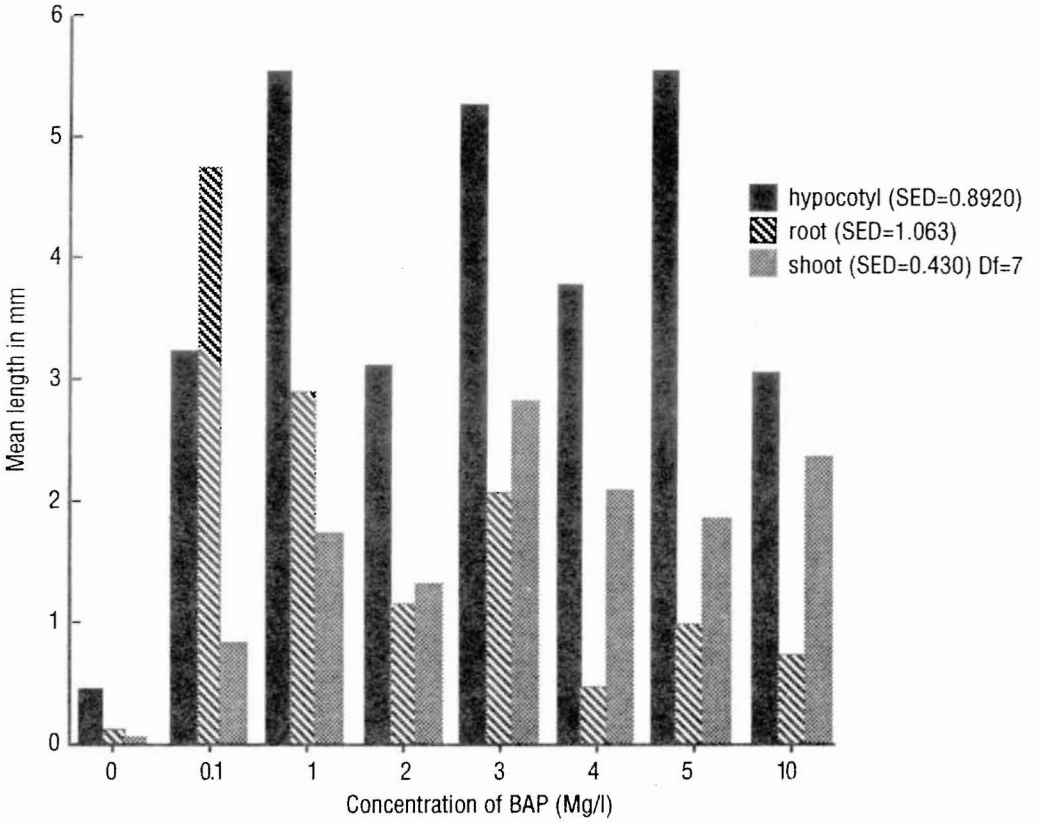

Figure 4. Effect of BAP treatment on dormant embryos of *S. aucuparia* after incubation at 20°C and continuous light for one month.

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Bulard, C. 1985. Intervention by gibberellin and cytokinin in the release of apple embryos from dormancy: a reappraisal. New. Phytol. 101: 241-249.

Removal of dead-filled seeds and invigoration of viable seeds — a review of a seed conditioning concept used on conifers in Sweden

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Abstract

The significant demand in Sweden for using high-quality seeds in forest seedling production is met with a concept for seed conditioning. The basis of the concept is to utilize a battery of analyses so that a proper diagnosis can be made that makes it possible to develop/select the most suitable measure(s) of quality improvement for each individual seed lot. The measures involve methods for elimination of non-productive seeds such as dead-filled seeds and for invigoration of viable seeds. A state-of-the-art review is given about proper conditions for using the IDS method for removal of dead-filled seeds with or without integration of an invigoration method. In the invigoration method seeds are incubated for a specific time at controlled moisture content using a special technique that gives continuous aeration. This method improves the germination rate and percentage at both sub- and supraoptimal temperatures. Different conditioning regimes are suggested for Scots pine (*Pinus sylvestris* L.), Norway spruce (*Picea abies* (L.) Karst.) and lodgepole pine (*Pinus contorta* Dougl.). These regimes can probably be applied for other species as well.

Résumé

La forte demande en Suède de semences de haute qualité pour la production de semis forestiers a trouvé réponse dans un concept de conditionnement des semences. Il s'agit essentiellement d'appliquer une série d'analyses qui mènent au diagnostic permettant de développer et de sélectionner les mesures les plus appropriées d'amélioration de la qualité de chacun des lots de semences. Les mesures comprennent notamment les méthodes d'élimination des semences non productives telles que celles qui sont remplies mais mortes, ainsi que les méthodes de revigoration des semences viables. On fait un examen complet des conditions qui doivent être réunies en vue de l'application de la méthode d'incubation, séchage et séparation pour l'extraction des semences remplies mais mortes, avec ou sans mesure de revigoration. Dans le cas de l'application de cette dernière, les semences sont incubées pendant une période donnée à une teneur ajustée en humidité au moyen d'une technique spéciale qui procure une aération constante. Cette méthode améliore le taux et le pourcentage de germination aux températures supérieures et inférieures à la température optimale. Différents régimes de conditionnement sont proposés dans le cas du pin sylvestre (*Pinus sylvestris* L.), de l'épinette de Norvège (Picea abies Karst.) et du pin tordu (*Pinus contorta* Dougl.). Ces régimes peuvent probablement être appliqués à d'autres essences.

Introduction

The annual production of forest seedlings in Sweden is about 610 million, and more than 80% are raised in containers (Nyström 1989). Each container represents the same costs during cultivation whether it has a seedling or not and it is therefore desirable to reach a seedling emergence as close to 100% as possible. Consequently, the demand for high seed quality is significant, especially if single-seed sowing is to be used. Sowing one seed per container is beneficial not only for eliminating culling costs, but also for minimizing seed consumption, which is especially important in northern Sweden. In this region there is often a seed shortage due to unfavorable climatic conditions for cone production and seed ripening (Sahlen and Bergsten 1992).

The demand for viable, vigorous seeds has been met with a concept for seed conditioning worked out at our laboratory. The basis of the concept is to utilize a battery of analyses so that a proper diagnosis can be made that makes it possible to develop/select the most suitable measure(s) of quality improvement for each individual seed lot. The analyses include both standard tests made according to ISTA rules and tests giving complementary or additional information (cf. Fig. 6; Sahlén and Henriksson 1985).

The two main measures for improving seedling emergence involve methods for:

I Elimination of non-productive seeds (cf. Simak *et al.* 1985), such as empty or insect-damaged seeds, filled but mechanically-damaged seeds, filled but dead seeds, and germinable seeds not producing

viable seedlings (poorly developed seeds, seeds giving abnormal seedlings, etc.).

II Invigoration of viable seeds

Empty or insect-damaged seeds are easily removed using routine procedures such as pneumatic separation, gravity separation, etc. (Berlage and Brandenburg 1984). Mechanically damaged seeds can be eliminated using the PREVAC method (Lestander and Bergsten 1985). If low absolute pressure is first applied to, and then released, from seeds lying in water, the seeds with cracked coats quickly lose their buoyancy and sink and thus can be separated from the floating undamaged seeds. It is often necessary to use PREVAC in combination with other conditioning methods and it can also be used for removing inert matter. As the physical properties for using PREVAC are described in Bergsten and Wiklund (1987), the method is not considered further in this paper.

Filled dead seeds can be separated using the IDS method (Simak 1981; 1984). This method is based on the principle that viable seeds after water uptake (incubation, I) and during drying (D) initially lose absorbed water at a much slower rate than dead seeds. The two types of seeds can then be separated (S) by, for instance, flotation in water, owing to the induced difference in density. Germinable seeds not producing viable seedlings such as poorly developed seeds, however, cannot be totally eliminated by any method.

Another way of increasing seedling emergence is to improve the vigour (as defined by the Association of Official Seed Analysts 1983) of viable seeds. Various methods that principally result in increased germination rate, and which could be called invigoration (Heydecker 1972) methods, are used for agricultural and horticultural seeds (Heydecker and Coolbear 1977). Osmotic priming using aqueous salts or polyethylene glycol (PEG) solutions is the most widely used treatment for invigoration. This method is also successfully applied to conifer seeds such as *Pinus* and *Picea* species in laboratory or small-scale nursery experiments (Simak 1976; Fleming and Lister 1984).

To fulfill the demands for large-scale treatment and to achieve consistent integration with the IDS method, a new invigoration method was developed in which water is the only treatment agent. Using a special technique, the seeds are incubated for a specific time at controlled moisture content with continuous aeration. The invigoration can be integrated into the IDS method, i.e. it is possible to achieve both removal of dead seeds and invigoration of the viable seeds in the same regime of conditioning (Bergsten 1987). The method improves the germination rate and percentage at both sub- and supra-optimal temperatures (Bergsten 1989).

The concept, including the conditioning methods mentioned, is used operationally in Sweden on Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abies*) and lodgepole pine (*Pinus contorta* Dougl.), which serve the demand for high seed efficiency in nurseries. Even poor-quality seed lots may, after treatment, be used for single-seed sowing. In 1990 about 3 tonnes of seeds were conditioned for the Swedish nurseries at the Institute for Forest Tree Improvement in Sävar.

The precision and accuracy of the methods described are continuously being improved through testing and optimization of the physical properties influencing the results of conditioning. Earlier investigations on conditioning are therefore reviewed and discussed in this paper together with some new findings. The aim is to give a state-of-the-art review about different regimes of the IDS technique with or without integration of the invigoration method. Some experimental results are presented for the purpose of giving guidelines for application of the concept on other species.

Incubation (I)

Two main incubation methods are used at our laboratory:

Incubation $_{\text{standard}}$ ($I_{st.}$)

This method is used for seed lots containing both filled-dead seeds and vigorously viable seeds, i.e. invigoration is not needed. The proportion of dead seeds is determined at the end of the previous germination test using the cutting test according to ISTA rules or the X-test (Simak *et al.* 1989).

The seeds are soaked and then placed in an incubator at the desired temperature. The incubator is an illuminated cabinet with relative humidity close to 100%. The high humidity is achieved from water continuously running down the walls and from a humidifier placed at the bottom of the cabinet. There is no regulation of initial moisture content (IMC) and, consequently, the water supply is generally superfluous. In small laboratory experiments the seeds are kept between moist blotting paper and for large-scale purposes the seeds are treated in net boxes made of stainless steel.

Conditions

Three days of incubation at 15°C and superfluous water supply (Simak 1981; Simak *et al.* 1985) is a suitable treatment for increasing the moisture content to a

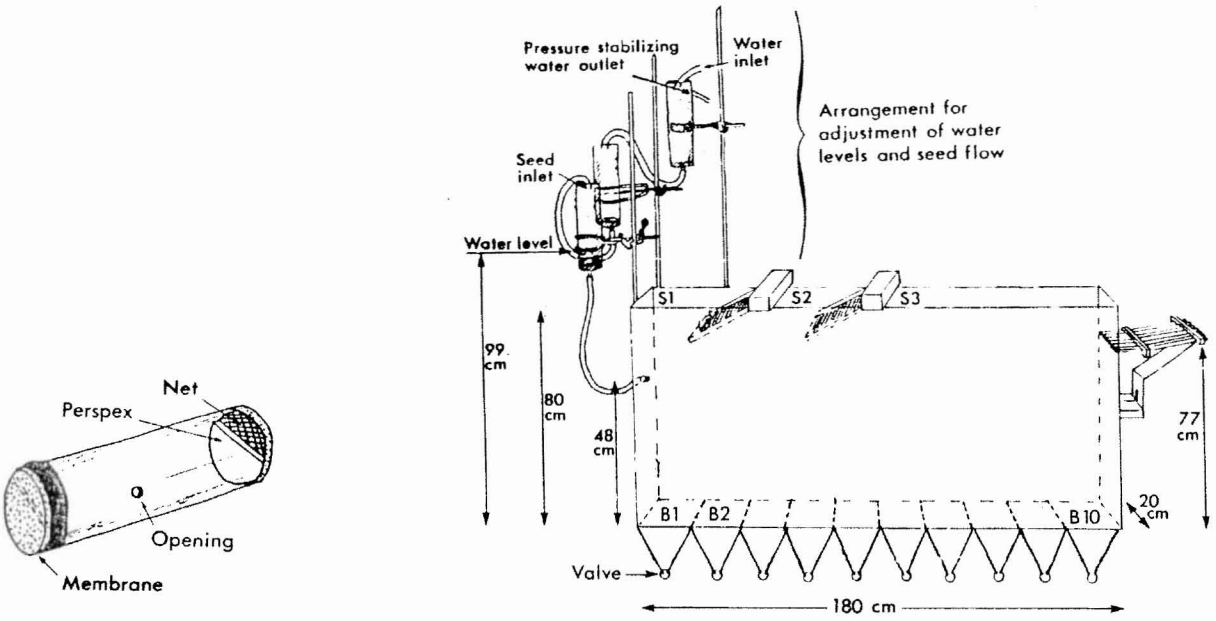

Figure 1. Diagram of membrane tube and sedimentation flume.

maximum level without affecting the germination percentage and rate negatively, thus creating a favourable starting point for the D and S steps. A shorter time than 3 d does not allow the viable seeds to reach a high water holding capacity and a longer time is hazardous since seeds will germinate during the treatment. A temperature higher than 15°C, e.g. 20-25°C, will increase respiration and the risk of negative effects on germination capacity due to superfluous water (Bergsten 1987). For large-scale treatment, a regime of IMC 30% (IMC 30) for 3 d followed by $I_{st.}$ for 16 h (overnight) might be preferable in order to minimize the risk of negative effects.

Incubation invigoration ($I_{inv.}$)

This method is used for seed lots containing seeds with low vigour. If dead-filled seeds are to be eliminated, the $I_{st.}$ is performed subsequent to this method to increase the moisture content and thus enhance results of the D and S steps.

The seeds are placed in a tube with the ends covered by a membrane made from polytetrafluorethylene, trade mark GORE-TEX, which allows gas exchange (Fig. 1). A suitable moisture content for invigoration is chosen and the amount of water necessary to increase the seed moisture content to this level is calculated and supplied (Bergsten 1987). Subsequent incubation is performed at the desired temperature for a certain time.

The membrane tube is placed in the incubator and the moisture content of the seeds is regulated by the interaction between the IMC level and the humidity of the environment. The pores of the membrane (1.4 x 10^9 pores cm^{-2}) ensure that a continuous air supply is obtained without any disturbance from surrounding water drops, i.e. the membrane allows vapour, but not drops to pass through.

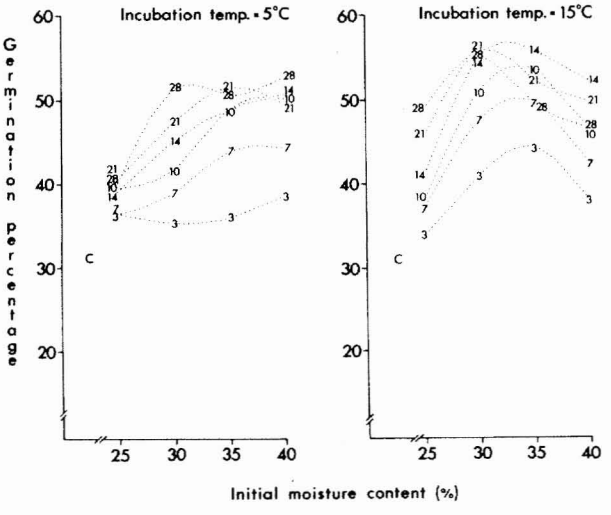

Figure 2. Germination percentage (21 d at 20°C) for re-dried seeds of a Scots pine seed lot after incubation at IMC 25, 30, 35, or 40 for 3-28 d at 5 or 15°C. C is control, figures are time of treatment. Anatomical potential (Simak 1980) = 69 %. Each value represents 4 tests of 4 x 100 seeds. Data from the two photoperiods (24 h-200 µmol m^{-2} s^{-1}; 0h) are pooled. Germination test was performed at 24 h light (cool-white; 25 µmol m^{-2} s^{-1}).

Conditions

The invigoration effect is based mainly on the control of three factors, i.e. temperature, moisture content of the seeds during treatment, and time of treatment. For some species light may also be important.

An incubation temperature of 15°C is the most suitable (Fig. 2,3; Bergsten 1987). This temperature is also used for successful osmotic priming of *Pinus* and *Picea* species (Simak 1976; Fleming and Lister 1984; Simak *et al.* 1984) and is chosen for osmotic priming of many agricultural and horticultural seeds (Heydecker *et al.* 1974; Heydecker 1975). It is also within the range of 10-20°C which is suggested by Bewley and Black (1985) for osmotic priming of most seeds. A higher temperature than 15°C would be more hazardous if the IMC level accidentally exceeds the optimal level, as negative effects on germination capacity would occur earlier during incubation at these temperatures.

The most suitable IMC level should probably be related not only to species (cf. Tesche 1975: different osmotic potential of conifer species), but also to the vigour of the individual seed lot. For Scots pine and Norway spruce the optimum IMC is about 30% for both re-dried seeds and seeds which are germinated directly after incubation. There is a tendency, however, that seeds with high anatomical development respond similarly to treatment at IMC 25 and IMC 30. A consideration of operational importance is that seeds incubated at IMC up to 30% are surface dry, i.e. can be sown without re-drying using standard sowing equipment (Bergsten 1987).

The time needed to achieve a clear invigoration effect by incubation at IMC 30 is about 8 d, and the effect is improved up to at least 12 d for Scots pine and Norway spruce (Bergsten 1987). This time corresponds to the 11 d treatment which is found optimal for osmotic priming of Scots pine seeds (Simak 1976; Simak *et al.* 1984). The activation of the metabolic processes will reduce the nutrient reserves of the seeds after a certain period of incubation and there will be negative effects of increased treatment time. Such effects, however, seem not to appear until several

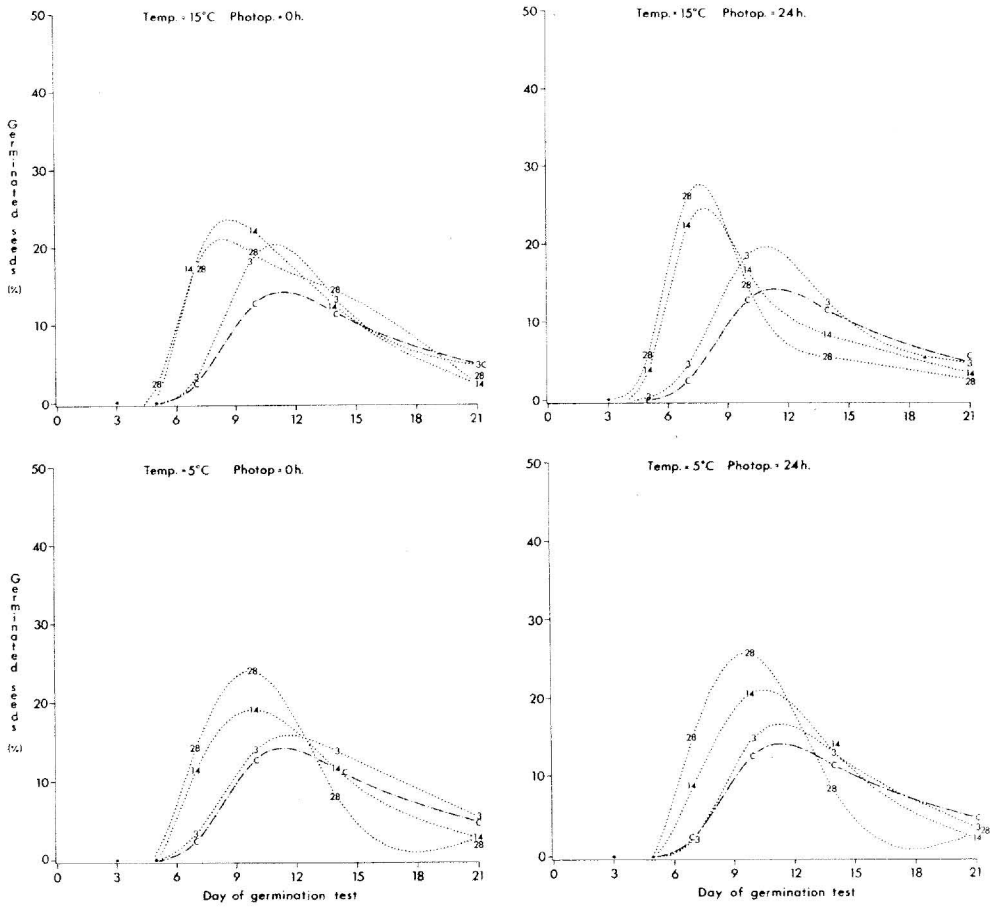

Figure 3. Percentage of germinated seeds at different days of germination test after incubation at IMC 30 for 3-28 d at 5 or 15°C and with 0 or 24 h photoperiod. (For definitions, see Fig. 2).

weeks beyond the recommended time of treatment (Fig. 4).

For Scots pine light quality can affect the germination capacity through the phytochrome pigment (Nyman 1963). The light quality and/or photoperiod during invigoration may also affect the germination rate of poorly developed Scots pine seeds (Fig. 3). However, the effect seems to be small and in the experiment presented no difference was established in germination percentage whether the seeds were incubated in light or not.

Drying (D)

At least three different types of equipment for drying are used:

a. Net shelf

The seeds are dried in a single layer on a net shelf in dehumidified air in a ventilated drying cabinet. A dehumidifier connected to a cooler is used for the air conditioning. The seeds are occasionally stirred manually. This method is used for small laboratory experiments. After about 16-24 h in this cabinet, the moisture content of incubated seeds is lowered to 4-7%, i.e. the seeds are storage dry.

b. Drum drier

The seeds are dried in a rotating (0.5 r min^{-1}) net drum within a closed drying system with conditioned air (as above). The drum is placed in a sealed cabinet with the air inlet underneath and the outlet above the drum. A perforated metal sheet with adjustable cover is used to spread the air evenly.

c. Fluid-bed drier

For large-scale treatments fluid-bed driers (Nilsson 1986) are used at the Institute for Forest Tree Improvement. This technique consists of a cylindrical vertical tube with a perforated bottom. Conditioned air is blown by fans through the holes of the bottom and the seed mass, thus establishing a rapid and uniform drying.

Conditions

The purpose of the D step is to create a close-to-maximum difference in moisture content and density between viable and dead-filled seeds and thus enhance a successful separation. This difference is related to the drying conditions, and the level of seed moisture content during incubation and the incubation time.

The drying conditions should give a rapid and uniform drying without being hazardous to the seeds. To ensure these criteria we have used a dehumidifier and an air cooler which, in combination, blow air with a temperature of 20-25°C and a humidity of about 5-15% into the drying cabinet. Rapid drying is especially important when using drum- or fluid-bed driers since, at long drying times, e.g. when treating large quantities, there is a risk of mechanical wearing which may cause mechanical damage to the seeds (Huss 1956). A close-to-maximum difference in moisture content between viable and dead-filled seeds is reached after using $I_{st.}$ for 3 d and subsequent drying (net shelf) for at least 2-4 h (Bergsten 1987). The suitable drying time for each drier, volume of seeds, etc., must be determined empirically. An easy way of doing so is to kill a seed sample (e.g. IMC 60 at 60°C for 24 h in a sealed container) and compare the moisture content during drying between killed and not-killed seeds (Fig. 5).

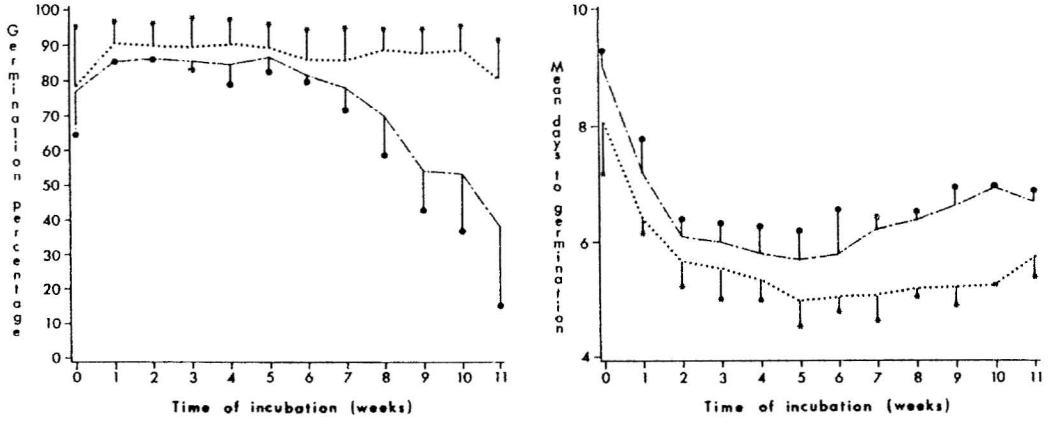

Figure 4. Germination percentage and mean days to germination after incubation at IMC 30 for 1-11 wk at 15°C. Vertical bar is half the range for the mean of two seed lots of Scots pine (···) and Norway spruce (– –). Actual range of moisture content: 29-31% up to 5 wk; 29-32% from 5-8 wk; 29-35% from 8-11 wk.

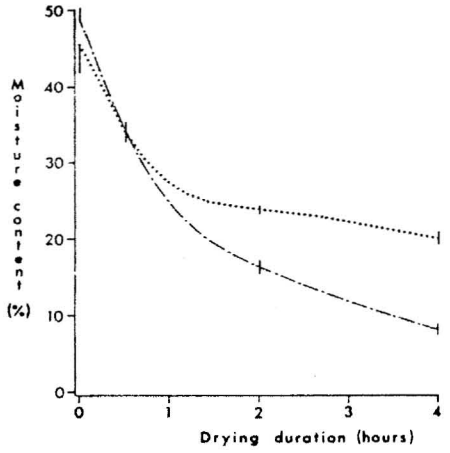

Figure 5a

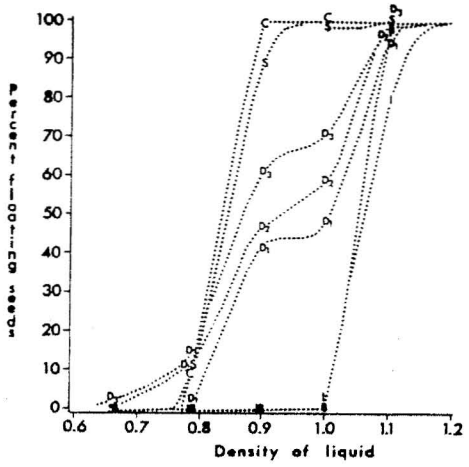

Figure 5b

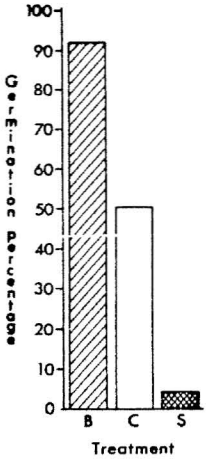

Figure 5c

Figure 5a. Moisture content during drying, for killed ( ) and not killed (····) seeds of a Scots pine seed lot after incubation using I_{st} for 3 d at 15°C. Vertical bar is range for the mean of two tests of 2 x 100 seeds.

Figure 5b. Percent floating seeds at different liquid densities during IDS.

C = Control
I = $I_{st.}$
D_1; D_2; D_3 = D step after 1, 2 and 3 h
B = Bottom fraction at separation
S = Surface fraction at separation
(two tests of 2 x 50 seeds).

Figure 5c. Germination percentage for the control (C) and the bottom (B) and surface (S) fractions after IDS on the Scots pine seed lot (two tests of 2 x 100 seeds).

The most distinct difference in moisture content between dead and viable seeds during drying is reached after incubation at IMC levels that are too high for invigoration. Therefore, for integration purposes additional water must be supplied after the initial invigoration step in order to prepare for a successful IDS separation.

In most seed lots the additional water supply could be provided using $I_{st.}$ at 15°C for 16 h without any significant decrease in germination percentage or rate, even if the invigoration step is fairly long. Treatment for 40 h will cause radicle protrusion and subsequent damage during drying for most seed lots. For practical purposes, a temperature of about 5°C allows a more flexible working schedule, since at this temperature the additional water uptake can occur for at least 40 h without causing any significant negative effects on germination (Bergsten 1987).

Separation

Separation in liquids has attracted the most attention, and the following two methods are used:

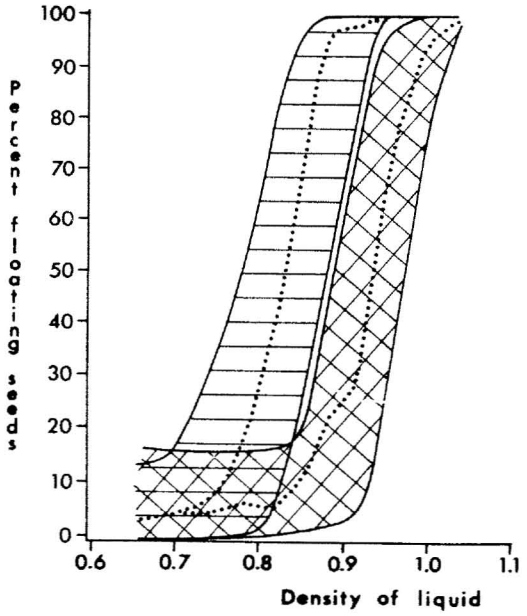

Figure 6. Percent floating seeds of 13 Scots pine (left) and 4 Norway spruce seed lots in liquids with different densities. Pattern shows range. The test was made on 300 storage-dry seeds per density level and seed lot. The liquids used are water + glycerol (> 1) and water + ethylalcohol (< 1).

a. Vertical flotation

The D(dried) seeds are placed in the liquid in a tube-vessel which has a valve at the bottom that facilitates removal of the bottom and surface fractions. The liquid used is water or solutions of water and glycerol (density > 1.0) or hexane and carbon tetrachloride (density < 1.0).

b. Vertical and horizontal sedimentation

This separation is performed using a flume enabling vertical and horizontal separation into a number of compartments for both sunken and floating seeds (Fig. 1). The seeds of each compartment are collected separately. The flume makes large-scale separation of seed lots with very low density possible without using the above-mentioned hazardous organic solvents and also facilitates separation of seed lots with high and/or non-uniform density.

Conditions

Separation in liquids has been used mainly because, in a liquid, the properties of the individual seed seem to be decisive for the efficiency of the separation. Other equipment used for separating seeds or particles with different properties may also be used. However, using gravity tables or air-stream separators seems to be complicated as the separation effiency depends on the interaction within the seed sample and not on individual seed properties.

For seed lots with a uniform density of about 0.10-0.15 units lower than that of water, the S step is successfully accomplished by applying the simple water flotation technique. Seed lots with very low density (e.g. the low values of the Scots pine range - Fig. 6) may be difficult to separate using vertical flotation in water, as the desired difference in moisture content and density is not reached until most seeds are floating. Solutions of hexane and carbon tetrachloride may be used instead if measures of precaution are taken. For seed lots with high and/or non-uniform density (i.e. many Norway spruce seed lots), liquids with higher density than water must be used. The sedimentation flume is a useful "multipurpose" separation apparatus, as both high and low density species may be treated and even a further separation in vigour classes is possible (Bergsten 1988).

However, for successful IDS separation all three steps (I, D and S) must be considered in sequence, i.e. the incubation regime and the drying time must be set bearing the separation medium in mind. If a liquid is used for separation, the drying time must be set so that during drying the density of only the dead-seed fraction will become lower than the density of the liquid. Small seed samples subjected to a floating test are used as a necessary tool in this context. For the example shown in Figure 5, water was chosen as a separation medium as most storage dry seeds had a density of about 0.8-0.9 (Control). After incubation the density is at its highest level and during drying it changes as illustrated as an S-shaped curve in the figure. The S-shape is related to the mixture of dead and viable seeds with different moisture content and density. For this seed lot with 50% dead seeds, the sink test results approached the target value of S percentage = 50 after about 2 h of drying (D step), and separation was performed giving B and S fractions with 94% and 4% germination, respectively.

Application

If the seed lots are free of (or have been cleaned of) empty, insect-damaged and mechanically-damaged seeds, the following treatment regimes are suggested for Scots pine and Norway spruce seeds (cf. also Bergsten 1988 as an example showing a typical regime):

I For seed lots with low germination percentage (a high proportion of dead-filled seeds) and high germination rate:
-incubation using $I_{st.}$ for 3 d at 15°C followed by DS-separation or, especially for large-scale treatment, incubation using $I_{inv.}$ at IMC 30 for 2-3 d at 15°C followed by $I_{st.}$ with additional water supply at the same or at lower temperature, e.g. 5°C, for about 16 h and DS-separation.

II For seed lots with high germination percentage and low germination rate:
-incubation using $I_{inv.}$ at IMC 30 for 1-3 wk at 15°C without DS-separation.

III For seed lots with low germination percentage and low germination rate:
-incubation using $I_{inv.}$ at IMC 30 for 1-3 wk at 15°C followed by $I_{st.}$ with additional water supply at 5°C for at least 16 h and DS-separation.

IV For seed lots with very low germination percentage and/or very low germination rate:
-incubation regimes given should be tested on small samples of the seed lots and the whole seed lot should then be treated only if the effects were positive on the samples.

Low germination percentage may be due not only to the occurrence of dead-filled seeds but also to the existence of slow-germinating seeds not germinating within the test period, i.e. the seeds determined as fresh

at the end of germination test. These low-vigour seeds are difficult to eliminate using DS-separation. However, they often benefit from an invigoration treatment and thus the germination percentage may be improved. The basis for selecting a proper variant of S step should be the density test (Fig. 6).

These regimes can probably be used for other species as well. However, for species needing a dormancy-breaking treatment, such as pre-chilling, the first incubation step should be performed at low temperature (2-5°C) at the prescribed duration (Simak 1983). In this context, it can be suggested that regulation of the moisture content to 30% at the beginning of the treatment followed by a change in temperature to 15°C after the prescribed chilling period may be advantageous as both dormancy release and invigoration can be achieved in the same regime of treatment (Downie and Bergsten, 1991). The appropriate levels of IMC for each species must be determined empirically.

The time suitable for incubation of slow-germinating seeds using $I_{inv.}$ must be chosen considering both the quality and the planned use of the seeds, i.e. the definitions of "high" and "low" germination percentage and rate may vary. A proper base for determination of suitable incubation time should be a standard germination test with frequent counts (e.g. mean days to germination or similar parameters). Also for other conifers, e.g. slash pine (*Pinus elliotti* Engelm.) and loblolly pine (*Pinus taeda* L.), standard germination tests and a measure of germination rate are most accurate for evaluating seed lot vigour (Bonner 1986). It should be emphasized that the basis for assessing the suitable treatment regime for a specific seed lot must be a very thorough seed analysis.

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Overcoming dormancy in loblolly pine (*Pinus taeda* L.)

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Abstract

Dormancy patterns in loblolly pine (*Pinus taeda* L.) were examined by comparing various parameters of germination rate. Both linear and polynomial models showed that mean germination time (MGT) was the most sensitive to chilling period. MGT was then used in a test of a new chilling technique that employed 24-h warm interruptions of chilling. This procedure produced faster rates of germination (lower MGT) in one group of 9 lots, but a second test with 15 lots yielded negative responses to any type of stratification.

Résumé

Chez le pin à encens (*Pinus taeda* L.), les caractéristiques de la dormance ont été examinées par comparaison de différents paramètres du taux de germination. Des modèles linéaires comme polynomiaux ont montré que le temps moyen de germination était le plus sensible à la période d'entreposage en chambre froide. Cette durée moyenne a ensuite été mise à profit dans l'essai d'une nouvelle technique d'entreposage en chambre froide qui comporte des interruptions de 24 h de réchauffement. Cette technique a accéléré les taux de germination (temps moyen de germination raccourci) dans un groupe de 9 lots, mais lors d'un second essai sur 15 lots, a conduit à des résultats négatifs, peu importe le type de stratification.

Introduction

Loblolly pine (*Pinus taeda* L.) is arguably the most widely-planted single tree species in the world. Based on past surveys (McDonald and Krugman 1985) and nursery production data from the United States (Lantz 1988), an annual production estimate of 2 billion seedlings is reasonable. Loblolly is also generally considered to be the most dormant of the southern pines (subsection *Australes*). Mechanical constraint by the seed coat appears to be the primary cause of dormancy (Barnett 1972, 1976), but chilling provides a stimulus to the embryo that promotes sufficient growth to overcome the mechanical restraint (Carpita *et al.* 1983).

For many years, the recommended chilling period for loblolly pine was 30 d (Wakeley 1954), but research has demonstrated improved performance of some seed lots in nurseries with chilling periods of up to 90 d (Barnett 1985). Standard laboratory testing procedures call for 28 d of chilling (International Seed Testing Association 1985). However, loblolly pine, like most temperate-zone species, has variable dormancy. Studies have shown differences in degree of dormancy among seed sources (Richter and Switzer 1982) and among half-sib families (Carpita *et al.* 1983), as evinced by different responses to a range of chilling treatments. Effectiveness of chilling is also directly correlated with seed moisture content. Barnett (1985) reported that full imbibition during chilling produced the most vigorous germination, while any reduction below this level resulted in slower germination. This same relationship has been reported when chilled seeds of loblolly pine are re-dried and stored; loss of chilling stimulus (onset of secondary dormancy) is inversely correlated with seed moisture content (Bianchetti 1986).

Other treatments reported to increase rate of germination in loblolly pine include aerated soaking in cool (5°C) (Barnett 1971) and in warm (24°C) water in the presence of light (Boyer *et al.* 1988), double stratification (chilling, interrupted by redrying and storage) (Boyer *et al.* 1985), and the presence of light during chilling (McLemore 1964).

Although effective methods are available to overcome dormancy in loblolly pine, the variable dormancy exhibited by this species often causes inefficient use of seeds in nurseries. Better ways are needed to quantify dormancy and to relate it to a flexible chilling method that will allow custom treatment of individual seed lots. At the Forestry Sciences Laboratory in Starkville, Mississippi, one of the research goals is to improve chilling methods for

Table 1. Seed sources used in these studies.

	Lot identification	Geographic source	Crop year
Experiment 1	09	Mississippi	1977
	44	Louisiana	1982
	84	Mississippi	1985
	850	Georgia	1987
	90	Mississippi	1990
Experiment 2		First Group	
	6-42*	Virginia	1988
	21-6*	Alabama	1988
	8-553*	Alabama	1987
	1158*	Oklahoma	——
	21*	Louisiana	1988
	8-05*	North Carolina	1988
	21-5*	Alabama	——
	6-8*	North Carolina	1988
	8-43*	North Carolina	1984
		Second Group	
	96	Louisiana	1984
	18*	South Carolina	1985
	06*	South Carolina	1985
	37*	South Carolina	1985
	39*	South Carolina	1985
	85	Mississippi	1985
	84	Louisiana	1984
	79	Alabama	1983
	86	Georgia	1985
	76	Louisiana	1983
	52	Alabama	1983
	69	Mississippi	1984
	44	Louisiana	1982
	48	Southeast U.S. mix	1983
	65	Southeast U.S. mix	1982

*Half-sib lots

loblolly pine through a better understanding of dormancy and how it is measured. In this paper, the research is reported in two parts. First, various parameters of germination rate were examined to determine the one most highly correlated with response to chilling. Second, the best parameter was used to evaluate a new chilling treatment in which single, short interruptions of chilling periods were imposed. This technique was inspired by the success of a similar treatment to reduce time to bud break in dormant loblolly pine seedlings (data on file, Forestry Sciences Laboratory, Olympia, WA).

Materials and methods

Experiment 1

Samples from five loblolly seed lots (Table 1) were subjected to moist chilling at 2°C for weekly increments of 7-56 d. Four samples of 50 seeds each from each lot were soaked overnight in tap water at room temperature (27 ± 2°C) and then sealed in plastic

Table 2. Chilling treatments used in Experiment 2.

Treatment	Chilling treatments*	
	First group	Second group
1	None (control)	None (control)
2	28	28
3	7/21	7/21
4	21/7	14/14
5	7/14/7	21/7
6	56	56
7	14/42	14/42
8	28/28	28/28
9	42/14	42/14
10	14/14/14/14	—

*The 24-h warm breaks are designated by /; numbers designate days of chilling at 2°C.

bags. Control samples were also soaked overnight but did not receive chilling.

Germination testing was performed on white blotters at an alternating 20°/30°C regime according to International Seed Testing Association prescriptions (1985). Germination was counted three times per week, using ISTA standards to determine when seeds were scored as germinated. Dormant, ungerminated seeds were determined by cutting at the end of the 28-d germination test period. Raw data were analyzed with a computer program that reconstructs germination on a daily basis and calculates all germination statistics accordingly.

Germination rate parameters were regressed on length of chilling period to find a model that could best describe the effects of chilling for the five lots. Parameters tested were peak value (PV) and germination value (GV) (Czabator 1962), mean germination time (MGT), variance in MGT, and percentage of dormant seeds at the end of the test. Both linear and nonlinear models were examined to determine which parameter was most sensitive to chilling treatment. The amounts of variation accounted for by the models (R^2 values) were used to determine effectiveness. The best rate parameter was then used with a second data set to evaluate the effects of new chilling treatments.

Experiment 2

Samples from nine half-sib families from an industrial seed orchard (Table 1) received chilling treatments at 2°C for 0, 28, and 56 d, and other treatments in which the 28- or 56-d period was interrupted by a 24-h period of 27°C in the dark (Table 2). Germination was tested, and the raw data were analyzed in the same way as in Experiment 1. Response to chilling was analyzed in a 2-by-2 factorial analysis of variance. This experiment was then repeated with 15 additional seed lots of loblolly from samples stored at the Forestry Sciences Laboratory (Table 1). The two least effective treatments were not repeated, but one new schedule was added. Tests and analyses were the same as in experiment 1.

Results

Experiment 1

Results with simple linear models of chilling periods and germination rates were mixed (Table 3). The best response parameters, PV and MGT, were those most directly related to rate of germination. R^2 values ranged from 0.456 to 0.833 for PV, and from 0.570 to 0.810 for MGT. Of the five lots, four had higher R^2 values for MGT as compared with PV.

When data were transformed, some improvement in the linear fits resulted. From this point, results for PV and MGT only will be discussed (Table 3). Logarithmic transformations increased R^2 values in only 3 of 10 models, but square root transformations improved them in 8 of 10. Again, the higher R^2 values were with MGT compared with PV.

The best models of all, based on R^2 values, were those obtained with second and third order polynomial regressions (Table 4). In 9 of 10 cases, MGT was more highly correlated with chilling period than was PV. R^2 values for MGT ranged from 0.575 to 0.956 in the second-order regression and 0.728 to 0.972 in the third-order regression.

As a result of these analyses, MGT was chosen as the most sensitive parameter to use in comparing chilling treatments in loblolly pine germination.

Experiment 2

In the first group, chilling caused significant reductions in MGT across all seed lots as expected (Table 5). In treatments totaling 28 d of chilling, the 21/7 treatment yielded the lowest MGT (10.9), while the other interrupted treatments were not as effective as a straight 28 d. Among treatments totaling 56 d chilling, the means for all interrupted treatments (11.0-

Table 3. R^2 values of the linear models of the effect of chilling on germination parameters PV, GV, MGT, variance in MGT (VAR), and percent of dormant seeds (DORM).

Dependent variable	Seed lot 09	044	84	850	90
		$Y = a + b\,(X)$			
PV	0.588	0.460	0.833	0.456	0.486
GV	0.467	0.280	0.748	0.229	0.481
MGT	0.794	0.596	0.810	0.570	0.659
VAR	0.008	0.332	0.437	0.351	0.143
DORM	0.518	0.420	0.474	0.073	0.137
		$\log Y = \log a + b\,(\log X)$			
PV	0.622	0.619	0.655	0.068	0.357
MGT	0.739	0.738	0.792	0.269	0.618
		$Y = a + b\,(X)$			
PV	0.668	0.595	0.847	0.291	0.495
MGT	0.895	0.760	0.939	0.489	0.739
		$1/Y = a + b\,(1/X)$			
PV	0.493	0.549	0.425	0.001	0.196
MGT	0.414	0.458	0.442	0.113	0.362

Table 4. R^2 values of the polynomial models of the effect of chilling on rate of germination; PV and MGT are the same as in Table 3.

Dependent variable	Seed lot 09	044	84	850	90
		$Y = a + b_1\,(X) + b_2\,(X^2)$			
PV	0.637	0.598	0.854	0.487	0.506
MGT	0.904	0.855	0.956	0.575	0.761
		$Y = a + b_1\,(X) + b_2\,(X^2) + b_3\,(X^3)$			
PV	0.658	0.606	0.856	0.780	0.519
MGT	0.918	0.878	0.972	0.728	0.763

11.5) were significantly lower than the mean for a straight 56 d of chilling (13.0) (Table 5).

Among individual lots, interrupting chilling at day 21 in a 28-d period was significantly better than a straight 28 d in six of the lots and equal to the straight 28 in two other lots. If the 56-d treatments are compared in a similar way, interrupted chilling was significantly better than a straight 56 d in all but one of the nine lots.

These results strongly suggest, but do not conclusively demonstrate, the potential for an improved rate of germination by short interruptions of chilling. The second group of seed lots was then tested to confirm the first results. However, results were clearly disappointing; all chilling treatments resulted in significantly higher MGT values than the controls across all seed lots (Table 5). Among individual lots, not a single interruption treatment significantly lowered MGT over that of the straight 28- or 56-d treatments.

Discussion

The utility of a good linear model for the effect of chilling on germination rate was emphasized by Sorensen (1980); his analyses of several data sets (some loblolly pine) from the work of others made a good case for the logarithmic model (Sorensen 1983). However, results of the present study did not yield good linear models with the logarithm transformations (Table 3). Much better results were obtained with the polynomial models (Table 4). Donald (1987) also reported good fits with the quadratic function and percentage dormancy for *Pinus pinaster* Ait. In this study, with the exception of lot 850, there was very little improvement of third-order over second-order polynomials.

Nonlinear models are logical because experience shows that germination rates will not respond to increasing chilling periods indefinitely. At some point, response levels off or even declines (germination slows) because excessive chilling may damage seed quality (Allen 1960; Donald 1987; Edwards 1986), especially for low-quality or damaged lots (Leadem 1986; McLemore and Czabator 1961). Responses to chilling are likely to be linear with less than optimum periods, but when supra-optimum periods are used, the polynomial model is appropriate.

The better performance of MGT as compared with PV for a measure of germination rate was mildly surprising. In past research on seed vigor measurement in loblolly pine, PV had usually produced better results than MGT (Bonner 1986). However, the seed vigor studies did not test response to varying lengths of chilling, which may account for the difference.

The failure of interrupted chilling treatments to provide a consistent response was disappointing. This treatment could be a simple technique that would require no additional equipment and little time for nursery workers to use. The failure of the second group of seed lots to respond to any chilling treatment is hard to understand. Procedures and data collection were reviewed thoroughly, however, and no discrepancies were found. Another test of this concept may be justified. The possibility of a correlation between seed dormancy and bud dormancy is intriguing also, because seed coats and bud scales are similar in constraint and embryonic axes and vegetative buds are similar in temperature stimuli.

Table 5. MGT as affected by chilling and interrupted chilling treatments for both groups of seed lots in Experiment 2; means within a column followed by the same letter do not differ significantly at P = 0.05 as determined by Duncan's Multiple Range Test.

Treatment	Group 1 (n = 9)	Group 2 (n = 15)
control	15.3 f	11.3 a
28	11.8 d	11.6 b
7/21	12.9 e	13.1 f
14/14	——	12.8 e f
21/7	10.9 a	12.7 c d e
7/14/7	12.8 e	——
56	13.0 e	12.7 d e
14/42	11.5 c	12.5 c d
28/28	11.2 a b	12.4 c
42/14	11.4 b c	12.7 c d e
14/14/14/14	11.0 a	——

Conclusions

Of all germination rate parameters studied, MGT proved to be the most sensitive to chilling period. Interruption of chilling with 24-h periods of 27°C further reduced MGT for most seed lots in which straight chilling alone reduced MGT, but these treatments had no effect on MGT for seed lots in which straight chilling alone had no effect.

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Variation in germination parameters within and among populations of Pacific silver fir on Vancouver Island

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Abstract

Cones from 42 individuals of Pacific silver fir (*Abies amabilis* [Dougl.] Forbes) collected at six sites on Vancouver Island, British Columbia, provided seeds for a paired germination test which revealed that strong family differences in germination responses exist, irrespective of germination treatment. Seeds subjected to 28-day stratification also exhibited marked differences among populations in both germination capacity and speed of germination. Possible explanations of these differences in germination behaviour and implications for nursery germination are discussed.

Résumé

Des cônes prélevés sur 42 sapins gracieux (*Abies amabilis* [Dougl.] Forbes) à 6 stations sur l'île de Vancouver (Colombie-Britannique), ont fourni les graines qui ont été utilisées dans un test jumelé de germination. Ce test a révélé de fortes différences, sur le plan de la germination, entre les familles, peu importe le traitement de germination appliqué. Les graines soumises à une stratification de 28 jours révélaient aussi l'existence de profondes différences entre les populations sur le plan de la faculté germinative et de la vitesse de la germination. Les raisons possibles de ces différences au niveau de la germination et l'importance sur le plan de la germination en pépinière sont étudiées.

Introduction

Pacific silver fir is one of three true fir species native to British Columbia. Its distribution is restricted to Vancouver Island and along the mainland coast from the Alaska panhandle southward, and seldom extends more than 300 km inland (Fowells 1965; Fig. 1). Pacific silver fir is an important component of the wetter maritime coastal western hemlock biogeoclimatic subzone, attaining its highest productivity in areas where annual precipitation exceeds 2500 mm, at elevations beyond which Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) does not grow well (Krajina *et al.* 1982; Klinka *et al.* 1991).

Over the past 10 years in excess of 10 million seedlings of Pacific silver fir have been planted in the Vancouver Forest Region, which includes all of Vancouver Island and most of the range of Pacific silver fir in B.C. (R.G. Knotts, B.C. Min. of Forests, pers. comm. March 1991). The production of containerized planting stock demands the use of high quality seeds. Edwards (1982) reported that seeds of *Abies* species are frequently of lower quality than other conifers, the range in nursery germination being typically 20-50% (Franklin 1974).

Although improvements in the collection and handling of *Abies* seeds have increased germination, true fir seeds are known to exhibit varying degrees of dormancy which hampers nursery production (Leadem 1986). Stratification (or prechilling) has been shown to improve the germination (in terms of capacity and/or speed) of several *Abies* species, which is also taken as evidence that dormancy exists in these seeds (Edwards 1962). The ameliorating effects of prechilling on the germination of grand, subalpine and Pacific silver fir have been found to vary with both species and seedlot (Edwards 1982; Leadem 1986).

Single-tree cone collections, which formed part of a larger study of variation patterns in Pacific silver fir on Vancouver Island (Davidson 1990), permitted analyses of germination responses on a level below that of the seedlot (where seedlot is seen as equivalent to population).

A germination test of seeds of wind-pollinated families from six populations of Pacific silver fir (Fig. 2) was designed to obtain some estimate of the magnitude of family variation in germination response,

relative to that of population and collection region (populations grouped by latitude), and to examine how a given stratification regime might affect variance structure.

Materials and methods

Cones were collected at six locations on Vancouver Island over a four-week period in the fall of 1983 following, where possible, IUFRO guidelines (Lines 1967). Populations were grouped into three broad collection regions representing north, mid and south Vancouver Island. Seven trees from each population were selected and the identity of maternal parents was maintained throughout cone and seed processing. Cones were kept in mesh bags at 4°C until all collections were obtained and then air-dried at 12-20°C for two weeks. Seeds were extracted using a small vibratory separator, dewinged by hand, and empty seeds removed using an aspirator separator (Edwards 1979). Seeds were further air-dried until samples of less than 10% moisture content were obtained, then stored at 4°C for 14 months. Six replications of 50 filled seeds (determined by x-ray) were obtained for each tree (42 in total).

A paired germination test (unstratified seeds together with seeds subjected to 28-day stratification) was carried out, modified from ISTA (International Seed Testing Association 1985) rules by using 50-seed replications and a 42-day test period. Stratification consisted of placing dry seed samples in clear plastic, closed germination boxes (12 x 12 x 3 cm) on three layers of filter paper over cellulose towelling wetted with 43 mL distilled water, and chilling in darkness at 1-4°C for 28 days.

Boxes were randomly distributed in 2 germination cabinets maintained at 30°C for 8 h (day) and 20°C for 16 h (night), with light, at approximately 1000 lux, provided by cool-white fluorescent tubes, during day hours. Germinants were counted eight times during the first 28 days and also at 35 and 42 days, to better reflect the course of germination for slower-germinating trees. A germinant was removed when its radicle reached the length of its seed coat (Edwards 1982).

Germination counts were summarized as two response variables: germination capacity (GC), the number of germinants, expressed as a percentage of filled seeds, at the end of the test; and germination value (GV), computed according to Czabator (1962). The higher the value of GV, the more complete and/or the more rapid the germination process.

Multi-way contingency table analysis (Fienberg 1970; Schoener 1970) revealed that stratification, when considered as one of the hypothesized sources of variation affecting germination response (i.e. by pooling counts of both stratified and unstratified seeds into one model), was the single most important factor affecting germination pattern. More homogeneous data sets were obtained by considering each pregermination treatment separately, and, after appropriate transformation (Davidson 1990), GC and GV were analyzed according to the following ANOVA model:

$$Y_{ijklm} = m + R_i + P_{j(i)} + T_{k(ij)} + C_l + CR_{il} + CP_{jl(i)} + CT_{kl(ij)} + e_{m(ijkl)}$$

where

m	= overall mean germination response
R_i	= climatic region (i = 1,2,3)
$P_{j(i)}$	= population within climatic region (j = 1,2)
$T_{k(ij)}$	= tree within population (k = 1,...,7)
C_l	= cabinet (l = 1,2)
$e_{m(ijkl)}$	= error (m = 1,2,3)

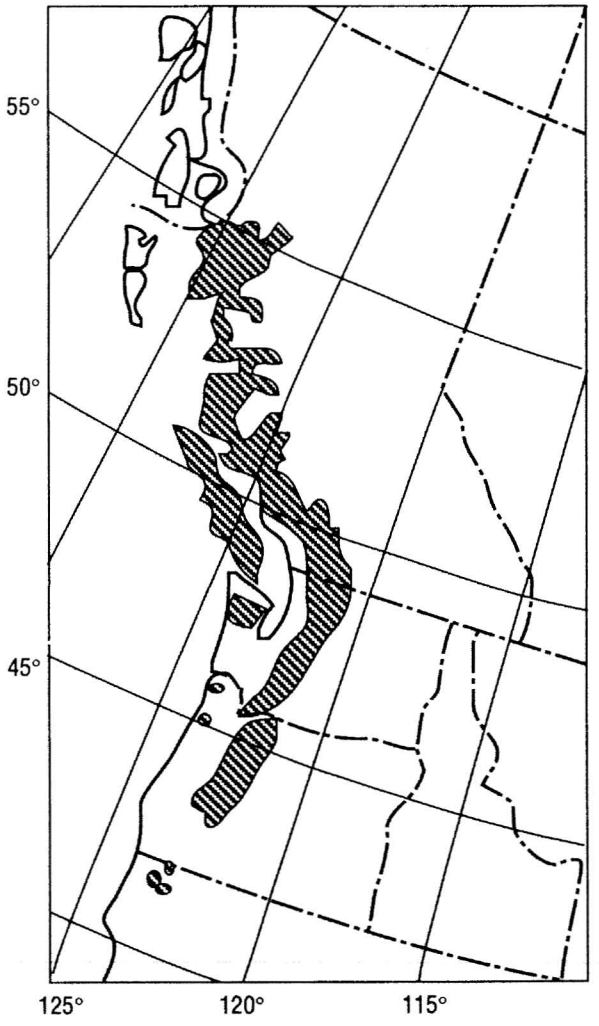

Figure 1. Distribution of Pacific silver fir in Western North America (after Fowells 1965).

All effects were considered random, except R and C, which were deemed to be fixed.

The relative magnitude of variation ascribed to each hypothesized source was estimated as a ratio of the appropriate variance component to the total of all components and is presented graphically. Cabinet effects were negligible in all analyses and these terms were removed from the model, increasing the error degrees of freedom.

Results and discussion

The overall mean GC for unstratified seeds was 79.6±1.8% (95% confidence interval) and 89.9±1.8% for seeds subjected to stratification (calculated from untransformed data). The influence of stratification on both the total amount and rapidity of germination is reflected in the overall increase in germination value, from an average of 4.42±0.22 to 11.37±0.44 (95% confidence intervals), suggesting that this sample of Pacific silver fir seeds possessed some degree of dormancy and that 28-day stratification was effective in overcoming at least some of that dormancy. However, the variances associated with average GC and GV do not follow the pattern of germination usually observed when conifer seeds are stratified, that is, a hastening of germination with a concomitant reduction in variability (Allen and Bientjes 1954; Edwards 1969). In fact, the variance in average GV for stratified seeds is twice that of unstratified seeds.

Nested ANOVA for both unstratified and stratified seeds revealed that the largest source of variation in both GC and GV is associated with differences among trees (Fig. 3). Strong among-tree variation in germination behavior has been detected in other tree species (Barnett and Farmer 1978; Bramlett *et al.* 1983; Farmer and Reinholt 1986; El-Kassaby *et al.* 1992).

Regions were, in most analyses, relatively unimportant except for GV of unstratified seeds, where differences among the three collection regions

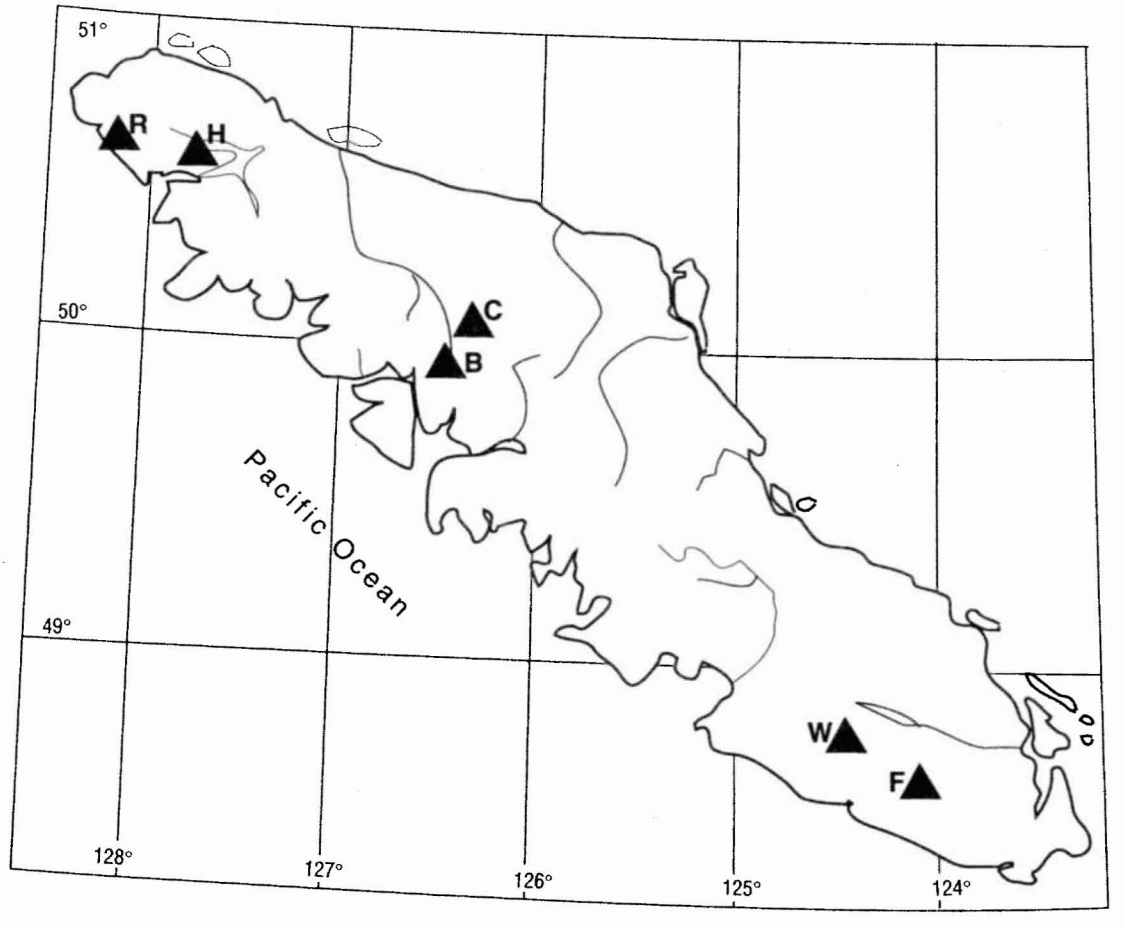

Figure 2. Location of six populations of Pacific silver fir on Vancouver Island.

accounted for nearly 12% of the total variance (in this case, within-tree variation was almost 19%). In contrast, Campbell and Ritland (1982) found that populations of western hemlock at higher latitudes exhibited earlier and more rapid germination, which was viewed as an adaptation to shorter growing seasons.

Population differences in GC and GV of unstratified Pacific silver fir seeds were negligible. When seeds were stratified, however, large differences among populations became evident, accounting for 40-50% for the total variation in the data (GV and GC respectively). Intra-tree variation remained approximately the same. These results suggest that populations within the same latitudinal band responded differently to the particular stratification conditions employed.

Because the among-tree variance component appears so large for germination responses of unstratified seeds and is reduced when seeds are subject to stratification, it is tempting to speculate that such a dramatic shift in source effect from the individual to the population indicates that a high level of adaptive variation in dormancy (Levins 1969) exists in Pacific

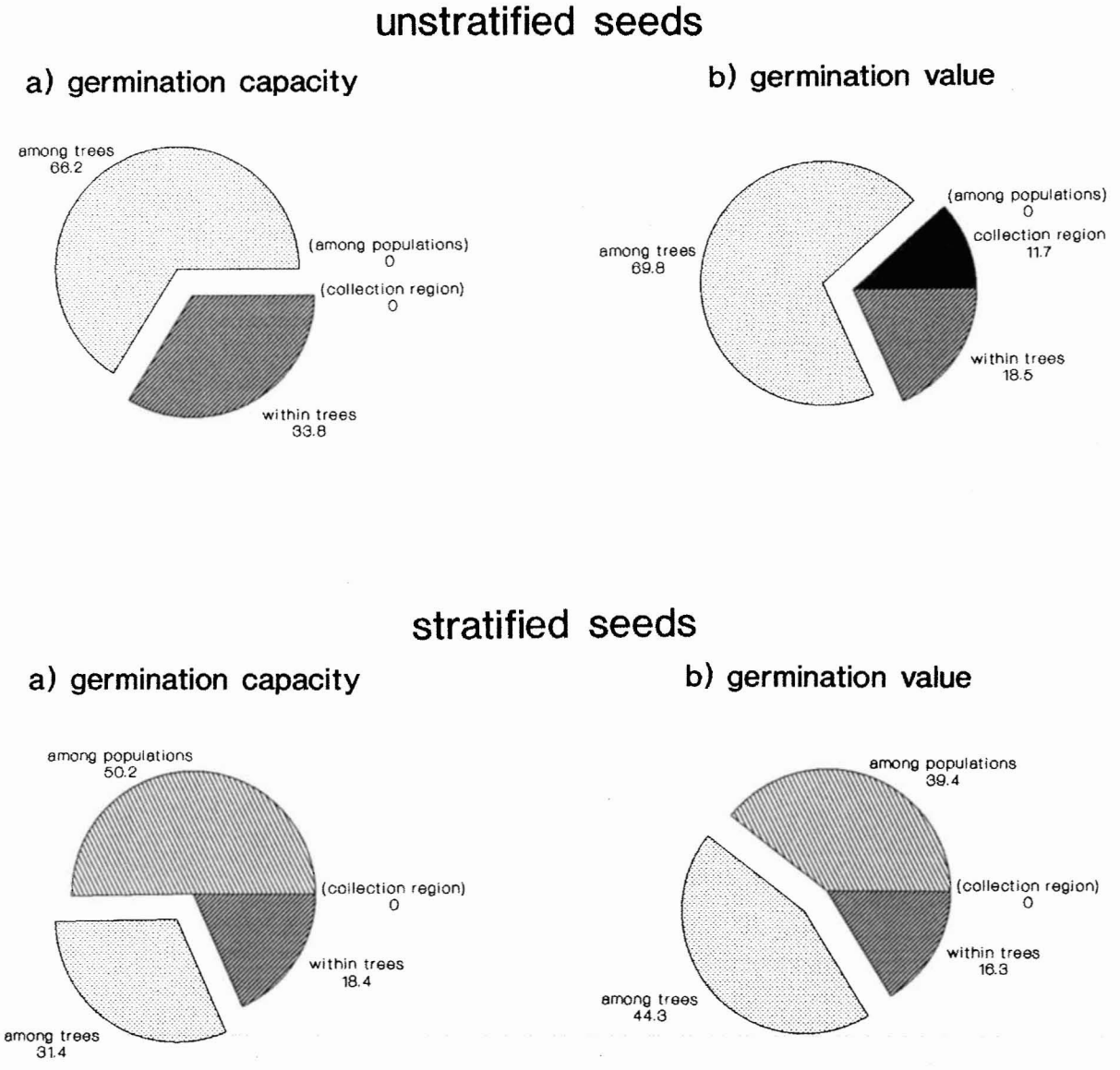

Figure 3. Results of ANOVA of a) germination capacity and b) germination value for unstratified and stratified seeds (top and bottom, respectively). Numbers indicate the percentage of total variation in data associated with a given source (% components of variance).

Table 1. Average germination capacity (95% confidence interval) for six populations of Pacific silver fir for both unstratified and stratified seeds.

Region	population[1]	Pregermination treatment	
		No Stratification	28-day Stratification
North	R	82.2 (3.7)	92.5 (2.9)
	H	76.2 (4.9)	91.0 (2.9)
Middle	B	76.2 (5.5)	89.2 (3.5)
	C	79.7 (3.7)	94.7 (2.4)
South	F	86.6 (3.9)	97.8 (1.4)
	W	76.8 (3.7)	68.9 (5.9)

[1]See Figure 2 for locations.

Table 2. Average germination values (Czabator, 1962) followed by 95% confidence interval for six populations of Pacific silver fir, for unstratified and stratified seeds.

Region	population[1]	Pregermination treatment	
		No Stratification	28-day Stratification
North	R	4.55 (0.51)	11.06 (0.94)
	H	3.93 (0.51)	11.63 (0.96)
Middle	B	4.36 (0.63)	11.82 (1.10)
	C	4.16 (0.43)	12.83 (0.69)
South	F	5.48 (0.49)	13.70 (0.59)
	W	5.34 (0.45)	7.15 (0.90)

[1]See Figure 2 for locations.

silver fir. However, germination behavior observed in the laboratory is influenced not only by i) the genetic background of the seeds - maternal, paternal and adaptive, and ii) their developmental environment ("preconditioning" *sensu* Rowe 1964 and "pseudo-preconditioning" *sensu* El-Kassaby *et al.* 1992), but also iii) handling techniques imposed on the seeds, including processing, storage, and stratification.

None of the samples could be considered deeply dormant as average germination capacities for unstratified seeds in all six populations were consistently high (Table 1). In response to the stratification regime applied in this study, total germination (GC) was significantly ($P < 0.05$) increased and variability reduced in all populations except W (Table 1). Fewer germinants were obtained and germination was more variable for stratified seeds than unstratified seeds in this population. The speed of germination (inferred from GV) of unstratified seeds was higher in the two southern populations (Table 2). In stratified seeds, GV was significantly increased in all populations, but improved the least in population W (Table 2). The variance of GV was higher in all populations. This result suggests that the particular stratification conditions were suboptimal for many trees in the test, producing more rapid but less uniform germination as was especially apparent in trees of population W (Table 3).

Average germination percentages of seven trees representing population W ranged from 54 to 92 for unstratified seeds and from 38 to 95 for stratified seeds. The stratification treatment appeared to be detrimental to seeds from four of the seven trees in the sample (mean germination capacity significantly reduced). Germination value was significantly improved only in the three trees in which germination capacity did not diminish in response to stratification (Table 3).

Campbell and Ritland (1982) acknowledged that environmental preconditioning can mimic genetic differences among populations. As well, any genetic component may be masked by the kind of handling seeds receive (Allen 1958). The extent to which environmental preconditioning and handling techniques affect germination performance cannot be discerned in this experiment, but they are probably not negligible. Several studies on tree seed maturity

Table 3. Mean germination capacity (GC) and germination value (GV) for individual trees of population W (each mean based on six 50-seed replications) and their associated 95% confidence intervals, and population mean values for both germination responses.

	Mean germination capacity	
Tree No.	No Stratification	Stratification
1	75.1 (2.6)	58.7 (4.6)*
2	77.7 (3.6)	64.5 (8.9)*
3	85.7 (4.2)	85.2 (4.5)
4	54.5 (8.1)	38.2 (7.1)*
5	77.0 (4.3)	64.7 (7.0)*
6	92.0 (3.2)	95.6 (1.9)
7	75.5 (3.9)	75.7 (9.9)
Mean	76.8 (3.7)	68.9 (5.9)

	Mean germination capacity	
Tree No.	No Stratification	Stratification
1	5.23 (0.25)	5.07 (0.69)
2	5.86 (0.82)	6.99 (1.73)
3	6.95 (0.89)	9.49 (0.54)*
4	2.54 (0.83)	2.36 (0.81)
5	5.54 (0.65)	7.15 (1.62)
6	6.23 (0.39)	10.67 (0.51)*
7	5.03 (0.48)	8.29 (1.52)*
Mean	5.34 (0.45)	7.15 (0.90)*

* $P < 0.05$

reviewed by Edwards (1980) have shown that immature seeds tend to be lighter in weight, germinate slowly if at all, show reduced germination as a result of prechilling, and are more susceptible to disease. Thus, it is presumed that genetic, environmental, and handling differences all affect seed maturation.

If seed weight and/or collection date are used as indices of seed maturity, then it is not likely that immaturity was a major factor contributing to the poor germination performance of stratified seeds from population W. Population W produced the heaviest seeds (53.1±12.5 g is the mean and standard deviation for 1000-seed weight) of all six populations (overall average 33.3±13.1 g) and, along with population F, was the last to be collected (September 30, *vs.* September 8/9 for B and C and September 28 for R and H).

It was observed during the course of the germination test that seeds from population W were the most susceptible to mold growth, particularly the stratified seeds. Every effort was made to avoid damage to the resin vesicles present in the seed coats of Pacific silver fir obtained for this study. The actual function of the resin is unknown in Pacific silver fir but it was found to inhibit germination in seeds of white and red firs (Kitzmiller *et al.* 1975) and to be a good medium for fungal development (Kitzmiller *et al.* 1973). Mold growth is a frequent problem in germination studies of *Abies* species (Edwards 1969, 1982; Kitzmiller *et al.* 1973; Adkins 1983; Blazich and Hinesley 1984) and high temperatures increase susceptibility to fungal attack (Davidson *et al.* 1984; Leadem 1986).

The stratification method employed in the present study, where the soaking step is by-passed and seeds imbibe whatever water is available from the surroundings, is thought to mimic natural conditions more closely than most nursery prechilling methods. This technique was shown to be an effective dormancy-breaking treatment in five of six populations (Table 1), and its effectiveness varied significantly among trees (Fig. 3). However, for most of the trees in population W, the conditions of stratification created unfavorable environments for germination to proceed in presumably non-dormant, as well as dormant, seeds (Tables 1 and 2), while providing favorable environments for the growth of fungi. Individual trees still varied in their propensity to germinate despite the presence of fungi (Table 3).

Conclusions

This study does not reveal any strong geographic trend in dormancy over the sampled range, but the high degree of family variation has important implications for nursery germination in that there may be inadvertent selection for rapid-germinating, less-dormant families within a given seedlot. In addition, stratification is likely to affect seedlots differently and its effectiveness may be a complex function of the environments of developing seeds, handling conditions as well as inter- and intra-tree variation in dormancy and germination responses. The large familial component of variation detected in this study

suggests that seedlings of Pacific silver fir may be more efficiently obtained by single tree cone collection and germination/planting on a family basis.

Acknowledgements

The author would like to thank D.G. Edwards and D. Taylor of Natural Resources Canada, Pacific Forestry Centre for their support and assistance in collecting the data and M. Banerjee and Y. El-Kassaby for their review of this paper.

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Determination of the major cause of seed dormancy of *Pinus bungeana* Zucc.

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Abstract

Experiments were conducted that show i) while the seedcoat is a major factor in the dormancy of *Pinus bungeana*, ii) seedcoat permeability is not the main constraint to germination. The role of inhibitors is discussed.

Résumé

Les auteurs ont effectué des expériences qui montrent i) que même si le tégument joue un rôle important dans la dormance de *Pinus bungeana*, ii) sa perméabilité n'est pas le principal obstacle à la germination. Le rôle des inhibiteurs est examiné.

Introduction

Seeds of *Pinus bungeana* Zucc. have hard coats, and a deep-seated dormancy. Seedcoat permeability, and/or inhibitors, have been suspected as playing important roles (Dong L. *et al.* 1987; Tao 1956; Tan *et al.* 1984; Yu 1983). However, no studies have reported any quantitative relationship between these factors and seed dormancy in this species. The objective of this study was to determine the major cause of seed dormancy of *Pinus bungeana*.

The work was carried out in two parts:

1. Determination of the major cause of dormancy of *Pinus bungeana* by applying the theory of quantification (Dong W. *et al.* 1977). Three treatments, *viz.*, soaking, seedcoat removal, and chilling are often used to promote germination in a wide variety of species (Khan 1977; Yu 1983). These were tested on *P.bungeana* to determine which produced the most significant increase in germination.
2. Determination of the effect of increased permeability of the seed coat on *P. bungeana* germination.

Materials and methods

Seeds of *Pinus bungeana* were obtained from Shaanxi Province, China. All germination tests were conducted on three replications of 50 seeds for each treatment combination. Germination was tested in an illuminated incubator set at 25°C, using sterilized clean river sand as the medium.

Part 1. Determining the major cause of seed dormancy

Three treatments were tested:

A. Soaking in water

Previous work (Dong L. unpublished data) revealed that seeds soaked in water for 10 days germinated well at 15–25°C. Thus, three soaking durations were tested: (i) no soaking (A_0), ii) soaking for 5 days (A_5), and (iii) soaking for 10 days (A_{10}). The tap water used was changed daily.

B. Removing the seedcoat

Seeds were tested (i) with their seedcoats intact (B_1), and (ii) after the entire outer seedcoat and part of the inner seedcoat had been removed (B_2).

C. Chilling

Traditionally, 15 days chilling had been used as the optimal chilling duration. Four different chilling (at 5°C) durations were tested: (i) no chilling (C_0), (ii) chilling for 5 days (C_5), (iii) chilling for 10 days (C_{10}), and (iv) chilling for 15 days (C_{15}).

Treatments were applied in combinations, 24 in all, as shown in Table 1.

Part 2. The effect of improved seedcoat permeability on germination.

A. To determine if the permeability of the seedcoat was a major factor, seeds were soaked for 0, 5 and 10 days as in Part 1 (i.e. treatments A_0, A_5 and A_{10}), then

Table 1. Effect of 24 treatment combinations on the germination of *Pinus bungeana*.

Treatment number	Treatment combination[1]	Germination[2]
1	$A_0B_1C_0$	58.0
2	$A_0B_2C_0$	0.6
3	$A_0B_1C_5$	49.3
4	$A_0B_2C_5$	4.6
5	$A_0B_1C_{10}$	46.0
6	$A_0B_2C_{10}$	13.0
7	$A_0B_1C_{15}$	33.3
8	$A_0B_2C_{15}$	16.0
9	$A_5B_1C_0$	57.6
10	$A_5B_2C_0$	7.3
11	$A_5B_1C_5$	56.0
12	$A_5B_2C_5$	22.0
13	$A_5B_1C_{10}$	54.6
14	$A_5B_2C_{10}$	28.0
15	$A_5B_1C_{15}$	49.3
16	$A_5B_2C_{15}$	38.0
17	$A_{10}B_1C_0$	60.0
18	$A_{10}B_2C_0$	24.0
19	$A_{10}B_1C_5$	66.0
20	$A_{10}B_2C_5$	30.0
21	$A_{10}B_1C_{10}$	70.7
22	$A_{10}B_2C_{10}$	32.0
23	$A_{10}B_1C_{15}$	48.0
24	$A_{10}B_2C_{15}$	36.0

[1]A = Soak duration (0, 5 or 10 days)
B = Seedcoat (outer plus inner) removal (1 = removed, 2 = left intact)
C = Chilling duration (0, 5 or 10 days at 5°C)
[2]Germination percent after 15 days at 25°C

placed in the germinator with either: (i) their seedcoats intact (B_2), or (ii) after cracking the seed in the micropylar (pore) region (by squeezing in a vise) (B_{11}), or (iii) after removing the both outer and inner seedcoats (B_{12}).

B. To determine how different amounts of seedcoat covering the seed tissues affected germination, seeds were placed in the germinator either: (i) after removing the outer coat, all of which was then replaced around the seed tissues (B_{13}), or (ii) after removing the outer coat, half of which was then replaced around the seed tissues (B_{14}), or (iii) after removing the outer coat (B_{15}), none of which was replaced around the seed tissues. In all instances, the inner seedcoat was not removed.

C. In addition, seeds were soaked in water for 4 days (A_5, as in Part 1), then placed in the germinator either: (i) after removing both outer and inner coats (B_{12}), or (ii) after removing the outer seedcoat only (B_{15}).

Results and discussion

Part 1. Determining the major cause of dormancy

From among the 24 treatment-combinations, seeds from which the seedcoats had been removed (B_1) germinated consistently better than seeds with intact seedcoats (B_2) (Table 1). Soaking the seeds for 5 (A_5) or 10 days (A_{10}), and/or chilling them for 5 (C_5), 10 (C_{10}) or 15 (C_{15}) days improved germination to a much lesser degree. The highest germination, 70.7%, was obtained with a treatment combination of 10 days' soaking, removal of the seedcoats, and 10 days' chilling. Longer chilling appeared to reduce germination (Table 1).

The theory of quantification (Dong W. *et al.* 1977) states that:

$$Y_i = \sum_{j=1}^{m} \sum_{k=1}^{r_j} \delta_i (j, k)\, b_{jk} + \varepsilon_i$$

where i=1, · · ·, 24; m=3, r_1=3, r_2=2, r_3=4.

Using this theorem, the scores bjk were calculated and were statistically compared. Removal of the seedcoat (treatments B_1 and B_2) produced the highest score (31.492), followed by soaking (treatments A_0-A_{10}) (18.237), with chilling (treatments C_0-C_{15}) producing the lowest score (7.981) (Table 2), confirming the observations made above. Partial correlation coefficient showed that the effects due to soaking and removal of the seedcoats were statistically significant (Table 2).

Thus, removing the entire outer and inner seedcoats had a significant effect on germination, so we might

Table 2. Statistical analysis of the effects of soaking, seedcoat removal and chilling on the germination of *Pinus bungeana*.

Item	A			B		C			
Category	A_0	A_5	A_{10}	B_1	B_2	C_0	C_5	C_{10}	C_{15}
	b_{11}	b_{12}	b_{13}	b_{21}	b_{22}	b_{31}	b_{32}	b_{33}	b_{34}
Score	41.729	49.292	59.966	0	-31.492	0	-1.848	6.133	2.183
Rate of score			18.237	31.492		7.981			
Partial correlation coefficient			0.616	0.853		0.295			
Partial correlation test			3.496**	7.298**		1.383			

conclude that seedcoat is the major cause to the seed dormancy of *Pinus bungeana*. But the effect of the seedcoat is an integrated result of various factors that include its permeability, and the presence of inhibitors (Khan 1977; Tan *et al.* 1984; Wang *et al.* 1986). Part 2 of this study looked into the role of seedcoat permeability in this effect.

Part 2. The effects of improved permeability of the seedcoat on the seed germination

A. (i) Cracking the seedcoat

As might have been expected, cracking the seed in the micropylar (pore) region (B_{11}) improved germination, particularly germination speed and the time at which the seeds first sprouted, by modifying the permeability of the seedcoat, compared to intact seeds (B_2) (Table 3).

The first signs of germination in cracked seeds was 4 days earlier than in intact seeds when the seeds were not soaked (A_0B_{11}), 2 days earlier when seeds were soaked for 5 days (A_5B_{11}), and 1 day earlier when seeds were soaked for 10 days ($A_{10}B_{11}$) (Table 3). Soaking the seeds for 5 days, compared to unsoaked seeds, did not produce earlier germination when seeds were cracked, but it did produce earlier germination in intact seeds. It also increased the overall rate of germination. However, when seeds were soaked for 10 days the first signs of germination in cracked and intact seeds were later than after 5 days soaking, and overall germination speed was also reduced (Table 3).

Although cracking the seeds appeared to speed up the initial germination, statistical analysis showed that none of the early differences were significant. These apparent delays, therefore, can be ignored, and comparisons can be made between the observed germination levels by moving forward the percentages occurring in intact seeds. Thus, for seeds that were not soaked, germination percentages can be moved forward by 4 days; for seeds soaked for 5 days, percentages can be moved forward by 2 days, and for seeds soaked for 10 days percentages can be moved forward 1 day. When statistical comparisons are made on this basis it was found that germination differences later in the test period were significant, with intact seeds performing better than cracked seeds. By the 35th day of the test, for those that were not soaked, intact seeds germinated better than cracked by 15%; when soaked for 5 days, intact seeds germinated better by 20%, and when soaked for 10 days, intact seeds germinated better by 6.5%.

The main conclusion that can be drawn from these data is that the seedcoat of *P. bungeana* is permeable since a) the longer the seeds were soaked, the shorter the interval for the first signs of germination between intact and cracked seeds to be recorded, and b) the overall germination of intact seeds remained superior at the end of the test, irrespective of the duration of soaking. Thus, seedcoat permeability cannot be considered as a major factor in constraining germination in this species.

(ii) Cracking vs entire seedcoat removal

When the outer and inner seedcoats were removed (treatment B_{12}), germination differences with cracked seeds were much more marked. Irrespective of the duration of soaking, seeds from which the entire seedcoat had been removed germinated much more rapidly and more completely than seeds that had been cracked around the micropylar region (treatment B_{11}) (Table 4). Soaking the seeds for 5 days, then removing

Table 3. Effect of soaking, and cracking the seedcoat on the germination of *Pinus bungeana*.

Treatment combination[1]	First sign of germination	Germination % after days in incubator													
		7	8	9	10	11	12	13	15	17	20	25	30	35	40
A_0B_{11}	7th day	5.0	5.0	10.0	12.5	15.0	15.0	20.0	27.5	35.0	35.0	35.0	35.0	35.0	
A_0B_2	11th day	0.0	0.0	0.0	0.0	2.5	2.5	7.5	17.5	20.0	35.0	42.5	47.5	47.5	50.0
A_5B_{11}	7th day	2.5	5.0	20.0	25.0	25.0	27.5	30.0	45.0	50.0	52.5	52.5	52.5	52.5	
A_5B_2	9th day	0.0	0.0	5.0	8.5	16.5	20.0	25.0	35.0	45.0	50.0	61.0	70.0	72.5	74.0
$A_{10}B_{11}$	9th day	0.0	0.0	6.5	10.0	13.5	13.5	16.5	23.3	30.0	33.5	33.5	33.5	33.5	
$A_{10}B_2$	10th day	0.0	0.0	0.0	3.5	6.7	10.0	16.5	26.5	33.5	36.5	40.0	40.0	40.0	

[1]A = Soak duration (0, 5 or 10 days)
B = Cracking treatment (B_{11} seedcoat cracked at the micropyle, B_2 not cracked)

Table 4. Effect of soaking, cracking the seeds at the micropyle and removal of the outer and inner seedcoats on the germination of *Pinus bungeana*.

Treatment combination[1]	First sign of germination	Germination % after days of test												
		5	6	7	8	9	10	11	12	13	15	17	20	22
A_0B_{12}	5th day	15.3	18.7	24.7	30.7	34.0	40.7	48.7	58.0	62.7	64.0	64.0	64.0	64.0
A_0B_{11}	7th day	0.0	0.0	5.3	5.3	10.0	12.7	15.3	15.3	20.0	27.3	34.7	34.7	34.7
A_5B_{12}	5th day	27.3	45.3	47.3	50.0	50.0	52.7	52.7	55.3	55.3	55.3	55.3	55.3	55.3
A_5B_{11}	7th day	0.0	0.0	2.7	5.3	20.0	25.3	25.3	27.3	30.0	45.3	50.7	53.3	53.3
$A_{10}B_{12}$	5th day	7.3	18.7	29.3	32.7	43.3	44.7	50.0	57.3	58.7	60.0	60.0	60.0	60.0
$A_{10}B_{11}$	9th day	0.0	0.0	0.0	0.0	6.7	10.0	13.3	13.3	16.7	23.3	30.0	33.3	33.3

[1]A = Soak duration (0, 5 or 10 days)
B = Seedcoat treatment (B_{11} seedcoat cracked at the micropyle, B_{12} outer and inner seedcoats removed)

Table 5. Effect of removing outer seedcoat, then replacing all, half or none around the seed tissues on the germination of *Pinus bungeana*.

Treatment combination[1]	Germination % after days in incubator										
	7	8	9	10	11	12	13	14	15	16	17
B_{13}	1.0	1.0	2.3	10.4	10.4	14.3	15.6	15.6	17.9	23.1	25.0
B_{14}	0.0	0.0	3.6	8.0	10.1	12.6	20.0	24.5	27.9	29.0	33.8
B_{15}	17.5	24.6	26.3	28.1	29.8	31.6	33.3	33.3	33.3	33.3	33.3

[1]B_{13} = outer seedcoat removed, then replaced around seed tissues
B_{14} = outer seedcoat removed, then half of it replaced around the tissues
B_{15} = outer seedcoat removed (not replaced)

their seedcoats, yielded the fastest, though not the overall best germination. Decorticated (seedcoats removed) seeds showed the first signs of germination 7-9 days earlier than cracked seeds (Table 4) and 8-11 days earlier than intact seeds (Tables 3 and 4). When seeds were soaked for 5 days, no significant difference was noted between the germination at 22 days of decorticated seeds and those that had been cracked. However, when seeds were not soaked, or had been soaked for 10 days, seeds from which the coats had been removed had germinated approximately 1.8 times more than cracked seeds by the 22nd day of the test (Table 4).

While these data appear to contradict the conclusion that permeability of the seedcoat is not a major constraint, it must be pointed out that inhibitors in the seedcoat may have reduced germination of seeds that had been cracked, since the coat was still connected to the seed tissues. Such inhibitors would have been eliminated when the seedcoat was removed.

B. Effects of replacing different amounts of seedcoat

Removing the outer seedcoat, then replacing all (B_{13}) or some (B_{14}) of it around the seed tissues, would have affected permeability very little from seeds from which the coat was simply removed (B_{15}) since there would be many leaks and water would penetrate easily. Thus, these treatments were regarded as offering no permeability differences. In these tests, the seeds were not soaked prior to seedcoat removal.

Seeds from which the seedcoat was removed and not replaced (B_{15}) germinated faster than seeds that had half the seedcoat replaced (B_{14}), and faster still than seeds that had all the seedcoat replaced (B_{15}) (Table 5). For example, seeds with no seedcoats germinated 17.5% after 7 days, whereas seeds with the full seedcoat replaced around their tissues required 15 days to germinate 17.9%. Toward the end of the test, germination differences were disappearing among the treatments (Table 5). The differences in early germination among the three treatments are probably related to the inhibitors in the seedcoat, or to other factors.

C. The effects of the outer and inner seedcoats

No significant differences were found when both outer and inner seedcoats, or only the outer coat, were removed (Table 6). With the inner coat still on the seeds, germination appeared to be 1 day slower than in seeds completely decorticated. The outer and inner seedcoats are connected at the base of the seed, so when removing the outer seedcoat, the inner coat likely was damaged to some degree. Thus, in terms of the level of permeability, the differences between the two treatments probably was minimal. The difference of 1-day earlier germination in seeds with both coats removed could have been related to inhibitors present in the inner seedcoat, nonetheless.

Table 6. Effect of soaking for 5 days then removing the outer and inner seedcoats, or the outer seedcoat alone on the germination of *Pinus bungeana*.

Treatment combination[1]	First sign of germination	Germination % after days in incubator								
		5	6	7	8	9	10	11	12	20
A_5B_{12}	5th day	27.5	45.0	47.5	50.0	50.0	52.5	52.5	55.0	55.0
A_5B_{15}	5th day	2.5	25.0	37.5	47.0	52.5	55.0	60.0	62.5	62.5

[1]A = Soaking duration (5 days)
B = Seedcoat treatment (B_{12} outer and inner coats removed, B_{15} outer coat only removed)

Conclusions

The experiments described demonstrate that the seedcoat is a major factor, and may be the determining cause, in the dormancy of *Pinus bungeana* seeds. Permeability of the seedcoat does not appear to be a major constraint, there is reason to suspect that seedcoat inhibitors may play a role.

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Genetic control of germination parameters of Douglas-fir, Sitka spruce, western redcedar, and yellow-cedar and its impact on container nursery production

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Abstract

The genetic control of germination parameters (germination capacity, peak value, and germination value) in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), Sitka spruce (*Picea sitchensis* (Bong.) Carr.), western redcedar (*Thuja plicata* Donn), and yellow-cedar (*Chamaecyparis nootkatensis* (D. Don) Spach.) was studied using wind-pollinated seeds collected from several seed orchards. The extent of genetic control over these parameters was assessed through the determination of broad-sense heritabilities. The impact of genetic control of these parameters on the expected genetic diversity of container nursery seedling crops is evaluated.

Résumé

Le contrôle génétique des paramètres de la germination (faculté germinative, maximum et valeur germinative) chez le Douglas taxifolié (*Pseudotsuqa menziesii* [Mirb.] Franco), chez l'épinette de Sitka (*Picea sitchensis* [Bong.] Carr.), chez le thuya géant (*Thuja plicata* Donn), ainsi que chez le cyprès jaune (*Chamaecyparis nootkatensis* [D. Don] Spach) a été étudié sur des graines pollinisées par le vent qui provenaient de plusieurs vergers producteurs de graines. L'importance du contrôle génétique sur ces paramètres a été évaluée par détermination d'héritabilité. L'effet du contrôle génétique de ces paramètres sur la diversité génétique des cultures de semis en contenants est évalué.

Introduction

Forest tree seed orchards and nurseries represent the vehicles for packaging and delivering genetic gains, achieved through selection and breeding, to the field forester. Therefore, the genetic evaluation of these delivery systems (i.e., seed orchards and seedling nurseries) is of great importance to ensure that their purpose is fulfilled in an optimal manner. Studies on several coniferous species seed orchards established the fact that the reproductive output (i.e., seed production) is under strong genetic control and that an orchard's genetic entities (i.e., clones or families) contribute to the resultant seed crops with different proportions (see El-Kassaby 1989; El-Kassaby *et al.* 1989 for reviews). To date, nursery practices have not been genetically evaluated.

Seedlots represent the link in the operations between seed orchards and nurseries. In most cases, these two operations are being managed independently with minimum interaction. Seed orchardists and seedling growers strive to achieve their respective programs' goals. Although these operations are linked their goals are at variance, and a paradox has emerged: maximizing the diversity in orchard crops is at odds with the uniformity required for large scale seedling production in the nursery.

Bulk seedlots from a seed orchard represent the reproductive output of several parents, each with different proportions, dormancy requirements, germination rates, and germination capacities. The viability of a bulk seedlot is the weighted average of the viabilities of all seed parents. Since seedlot viability is an important parameter for nursery operations, knowledge of the individual parent's germination parameters is also important. (Note: the germination capacity of a seedlot determines the seed sowing factor that should be implemented by the

Table 1. Species, seed orchard, number of trees, and year of collection of seedlots used in this study.

Species	Seed Orchard[1]	# of trees	Year of Collection
Douglas-fir (Df)	CPFP	19	1988
Sitka spruce (Ss)	CPFP	18	1989
Western redcedar (Cwr)	FC	22	1989
Yellow-cedar (Cy)	CPFP	12	1989[2]

[1]CPFP; Canadian Pacific Forest Products Limited, FC; Fletcher Challenge Canada. These seed orchards are located in Saanichton, B.C. (latitude 48° 35' N, longitude 123° 24' W, elevation 50 m).

[2]Seeds collected from 10-month old seed cones (see El-Kassaby *et al.* 1991).

nursery (Vyse and Rudd 1974)). Despite this, very little research has been done on the inheritance of germination parameters of forest tree seeds.

In this paper, we report on the genetic control of germination parameters for four species and its relevance to container nursery production. Seeds used in the study were obtained from seed orchards, i.e., from seed parents growing in a common and uniform environment. Seed pretreatments applied were similar to those used in operational container nurseries. Germination parameters were compared among the four species and among parents within species. The extent of genetic control over germination parameters and estimates of broad-sense heritabilities were determined following Falconer (1986).

Materials and methods

Orchard-grown seed crops from individual parents (clones and/or families) of four coniferous tree species were used (Table 1). To mimic standard seed pretreatment practiced in container nurseries, seed pretreatment for Douglas-fir and Sitka spruce was a 1-day soak in water at room temperature followed by a 21-day prechilling at +2°C, while for yellow-cedar it was a 1-week soak in water at room temperature followed by 1-month warm (20°C) and 3 months cool (1–3°C) stratification. Western redcedar was germinated without pretreatment. For each species and seed parent, four replications of 100 seeds each were used. All samples, whether prechilled or not, were spread in clear plastic germination boxes lined with moistened cellulose wadding (Kimpak) overlaid with filter paper, then placed in an incubator set at an alternating temperature of 30°C for 8 h followed by 20°C for 16 h. Light, at approximately 1000 lux, was provided during the high-temperature period by means of cool-white fluorescent tubes (International Seed Testing Association 1985). Germinants were counted on alternate days for 21 days for Douglas-fir, Sitka spruce, and western redcedar, and for 34 days for yellow-cedar.

Germination data were expressed in three ways: 1) germination capacity (GC), 2) peak value (PV), and 3) germination value (GV) (see Table 2 for explanation). Germination parameters (GC, PV, and GV) were analyzed using a simple one-way ANOVA after the appropriate data transformation (Table 2).

Results

Significant differences were observed among individual seed parents for all three germination parameters, as well as among the four species (Table 2, Figs. 1–4). Estimates of broad-sense heritabilities were relatively high and ranged between 0.91–0.93 for Douglas-fir, 0.42–0.78 for Sitka spruce, 0.78–0.80 for western redcedar, and 0.28–0.42 for yellow-cedar, indicating the presence of high genetic control. Yellow-cedar showed the lowest range while Douglas-fir gave the highest estimates.

Germination capacity (GC), the percentage of seeds that had germinated at the end of the test, varied among seed parents within species and ranged from 26–97%, 90–99%, 59–97%, and 27–68% for Douglas-fir, Sitka spruce, western redcedar, yellow-cedar, respectively

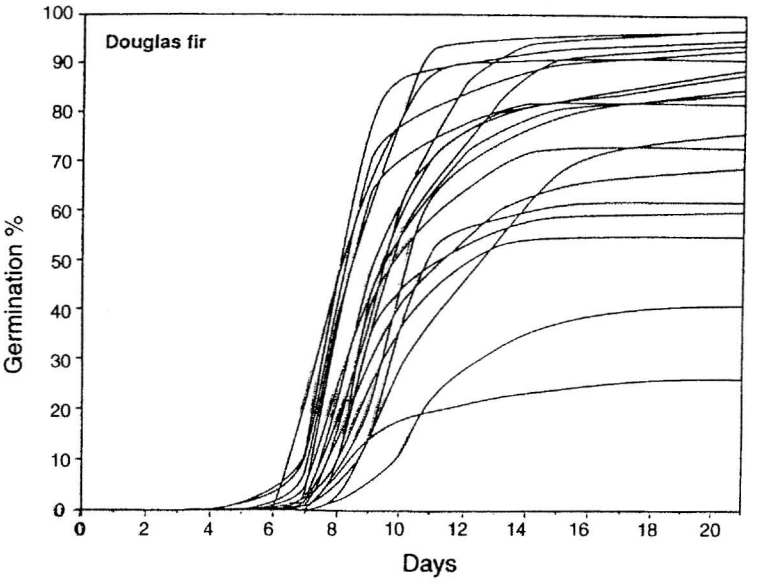

Figure 1. Germination curves for 19 Douglas-fir families. (Source: El-Kassaby *et al.* 1992)

Table 2. Estimates of variance components, significance level, and broad-sense heritabilities (h_b^2) for germination parameters of Douglas-fir, Sitka spruce, western redcedar, and yellow-cedar.

Species	Source of variation	Degrees of[1] freedom	Germination parameters[2] GC	PV	GV
Douglas-fir	Among trees	t-1	0.058**	3.602**	1.681**
(Df)	Residual	t(r-1)	0.005	0.348	0.134
	h_b^2		0.92	0.91	0.93
Sitka spruce	Among clones	c-1	0.003**	0.710**	19.537**
(Ss)	Residual	c(r-1)	0.004	0.204	6.744
	h_b^2		0.42	0.78	0.74
Western	Among clones	c-1	0.020**	0.521**	23.055**
redcedar	Residual	c(r-1)	0.005	0.151	5.733
(Cwr)	h_b^2		0.79	0.78	0.80
Yellow-cedar	Among trees	t-1	0.014**	0.327*	2.427
(Cy)	Residual	t(r-1)	0.025	0.829	3.603
	h_b^2		0.36	0.28	0.42

* = P <0.05, ** = P <0.01

[1] t = # of trees (Df, t = 19, Cy, t = 12),
c = # of clones (Ss, c = 18, Cwr, c = 22),
r = # of replications = 4.

[2] GC = germination capacity; the percentage of seeds that had germinated at the end of the test (transformation = arcsin).

PV = peak value (Czabator 1962); a mathematical expression of the break of a sigmoid curve representing a typical course of germination (no transformation).

GV = germination value (Czabator 1962); a mathematical expression combining speed and completeness of germination into a single value (transformation = $\sqrt{(X + 0.5)}$ for Df).

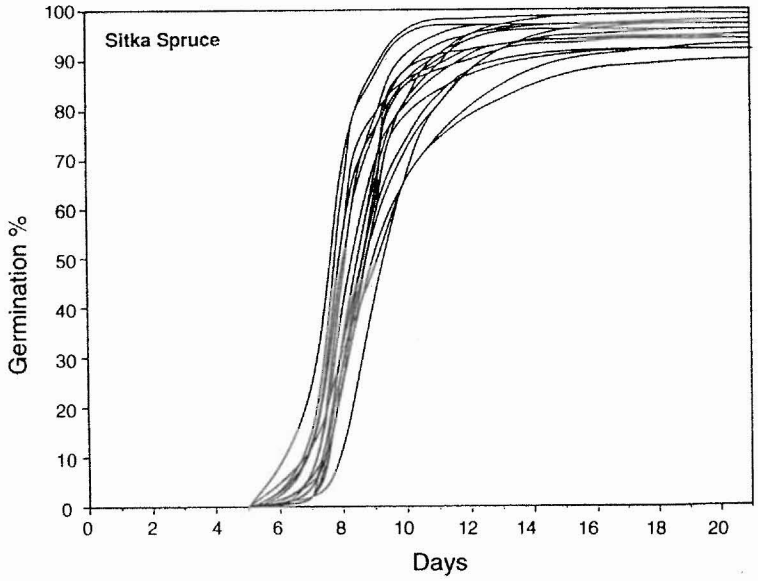

Figure 2. Germination curves for 18 Sitka spruce clones. (Source: Chaisurisri *et al.* 1992)

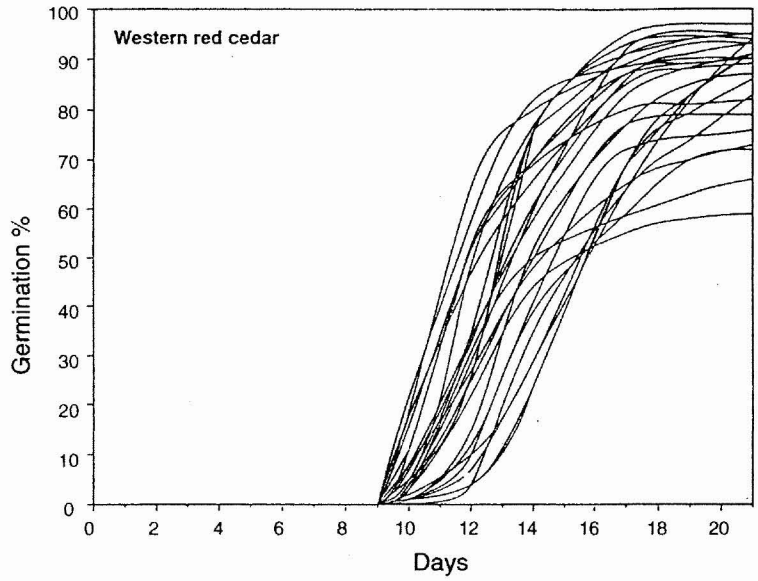

Figure 3. Germination curves for 22 western redcedar clones

(Figs. 1–4). Yellow-cedar showed the lowest germination capacity and germination rate.

Discussion

Numerous studies have shown a large maternal genetic effect on the germination of several plant species (see El-Kassaby *et al.* 1992, for review) including conifers (Bramlett *et al.* 1983; Hoff, 1987; Davidson 1990; El-Kassaby *et al.* 1992; Chaisurisri *et al.* 1992). Germination in conifers is the end product of several complex metabolic activities involving three genomes, viz., the seed coat (2n), megagametophyte (1n), and the embryo (2n) (Fig. 5). The maternal contribution of these three genomes is 80% compared to the 20% contributed by the male (Fig. 5). It is to be expected, therefore, that an appreciable amount of variation in seed germination controlled by the dominant, maternal genome will be observed. This variation has been considered to be an adaptation for survival under the temporally heterogeneous environment that is a common feature during germination periods (Jain 1982). The relationships among these genomes (i.e., between maternal-genotype via the seed coat and the other two genomes or between embryo-genotype and the maternal-genotype via the megagametophyte tissue) are sometimes of a conflicting nature. These conflicts have been shown to control the time of germination in Douglas-fir (i.e., the degree of dormancy) (De Matos Malavasi *et al.* 1985).

Seed dormancy varies among species so different seed pretreatments are required. Seed-dormancy differences among families within a species has been reported for western white pine (*Pinus monticola* Dougl.) (Hoff, 1987). In his study, Hoff (1987) also found that improvement in germination varied with duration of stratification. Heit (1961), on the other hand, concluded that stratification (and chemical pretreatment) is not required for germination of Sitka spruce seeds. Yet the present study demonstrates (i) that stratification is essential for Sitka spruce, as well as for Douglas-fir and yellow-cedar, and (ii) that a stratification period of 21 days is inadequate for Douglas-fir (see also Sorensen 1991, Edwards and El-Kassaby in preparation), but that it is adequate for Sitka spruce (Figs. 1-2, 4). In fact, the 1 month warm and 3 months cool stratification used on the yellow-cedar seeds was probably inadequate also, hence the poorer germination.

Sowing prescriptions for any particular seedlot in a container nursery are governed by two major

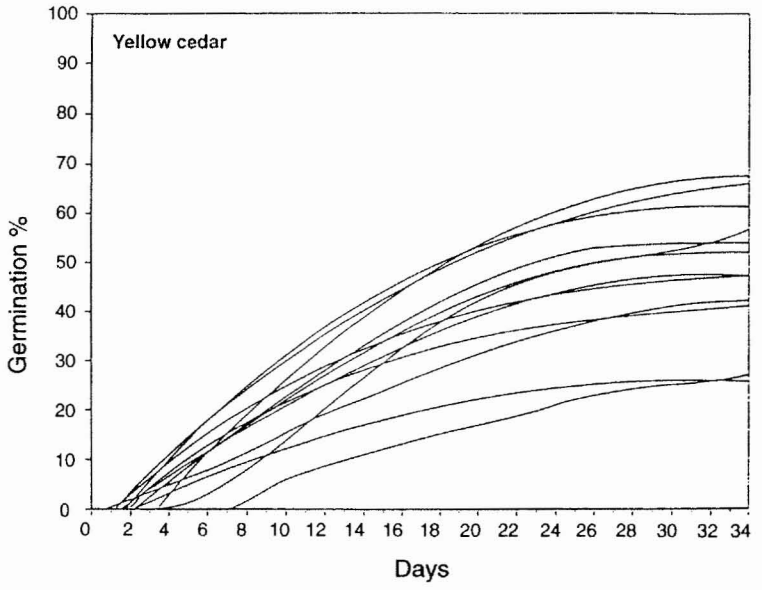

Figure 4. Germination curves for 12 yellow-cedar clones

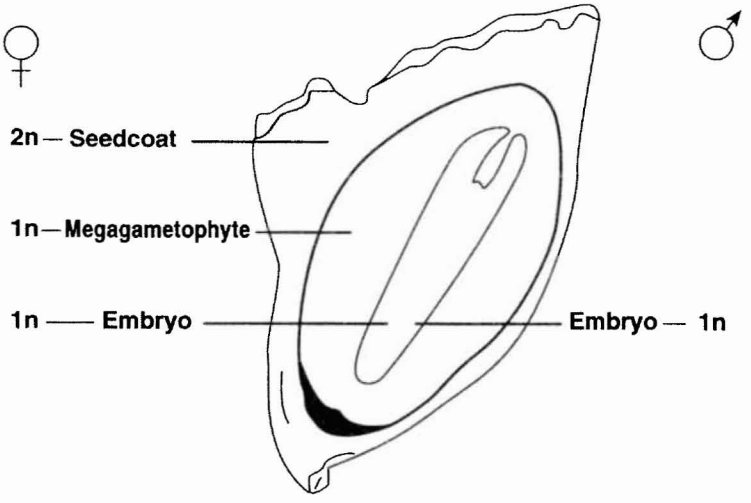

Figure 5. The genomes of a mature Douglas-fir seed.
(Source: El-Kassaby *et al.* 1992)

considerations, viz., a) the expected proportion of empty cavities and b) the expected proportion of cavities with multiple germinants. When seed viability is low, cavities may be double or triple sown to minimize empty cavities. Yet empty cavities continue to occur. Both empty and multiple-sown cavities represent economic and biological problems to the nursery industry. Empty cavities represent a loss in productivity, while cavities that were sown with more than one seed may have multiple germinants that require thinning by hand to leave a single germinant.

Thinning practices in container nurseries represent the first step in attaining seedling crop uniformity. In most cases, the germinants that remain after thinning are the largest ones because they are generally the earliest to germinate in the cavity. If seed parents (clones or families) exhibit variation in their germination behaviour, such thinning exerts an inadvertent selection pressure in favor of fast-germinating seed parents.

This selection pressure is also being affected by several other factors. These include: i) the reproductive output of the seed parent in the seed orchard (see El-Kassaby *et al.* 1989), ii) the relationship between reproductive energy and reproductive success (Reynolds and El-Kassaby 1990; Chaisurisri and El-Kassaby 1992), iii) variation in germination capacity (GC, Table 2), and iv) germination speed (represented in this study by PV, Table 2). In nature, all of these factors can be expected to work in concert. For example, if the reproductive output (i.e., the number of viable seeds) of a seed parent is high and these seeds germinate rapidly, then the contribution of this seed parent to the seedling crop can be expected to be high. This has not yet been established and empirical studies are needed for validation.

The high estimates of broad-sense heritabilities obtained in this study demonstrate the presence of genetic variation of germination parameters among the various seed parents for the four species studied and we have speculated on their effect in the nursery. The common practice of harvesting cone crops from seed orchards, extracting the seeds and sowing them on a bulk basis needs to be re-evaluated in light of these findings.

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Loblolly pine seed dormancy: the relationship between protein synthesis in the embryo and megagametophyte and the loss of seed dormancy

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Abstract

Embryos from the mature unstratified loblolly pine (*Pinus taeda* L.) seeds used in this study were nondormant, however, they failed to germinate *in situ*. The megagametophyte played a major role in the maintenance of embryo dormancy in the unstratified seeds. During the cold stratification period, a sharp increase in seed germination, from 30% to 66% percent, occurred between 14 and 21 days. Pulse-label studies of embryos and megagametophytes showed that the pattern of protein synthesis in the embryo remained constant during the 14 to 21-day period. In contrast, a shift in the pattern of protein synthesis by the megagametophyte was observed. The synthesis of a protein set with molecular mass of 27-29 kD decreased significantly following 14 days of cold stratification, whereas a set of proteins with molecular masses of 31-33 kD showed increased synthesis after this period.

Résumé

Des embryons obtenus à partir des graines mûres non stratifiées du pin à encens (*Pinus taeda* L.) qui ont servi dans la présente étude n'étaient pas en dormance, mais elles n'ont pas germé in situ. Le mégagamétophyte a eu un rôle déterminant dans le maintien de la dormance des embryons obtenus à partir des graines non stratifiées. Au cours de la période de stratification froide, il s'est produit une nette augmentation de la germination des graines, de 30 à 66 %, entre le 14e et le 21e jour. L'étude par marquage d'embryons et de mégagamétophytes a montré que le déroulement de la synthèse protéinique dans l'embryon est resté constant pendant la période de 14 à 21 jours. Par contre, une modification du déroulement de la synthèse protéinique chez le mégagamétophyte a été observée. La synthèse d'un ensemble de protéines ayant une masse moléculaire de 27 à 29 kda a été significativement réduite après 14 jours de stratification à froid, alors qu'un accroissement de la synthèse d'un ensemble de protéines à masses moléculaires de 31 à 33 kda a été observé après cette période.

Introduction

In nature, seed dormancy is an adaptive mechanism to ensure the survival of the species and that germination only occurs at the appropriate time. In the nursery situation, however, seed dormancy contributes greatly to production shortfalls and increased nursery costs. Current practices to overcome seed dormancy involve diverse seed pretreatments: light, plant hormones, temperature, and osmotic shock, for example. While various hypotheses have been proposed to account for the action of these agents (for a review see, Cohn 1987), there is little understanding of their mechanism of action at the cellular or molecular level. Consequently, current dormancy-breaking mechanisms are empirical and their application is limited. Previous studies have indicated that the regulation of protein synthesis may play a role in the transition from a dormant to a non-dormant state (Bewley and Black 1982). For example, specific enzymes may be required for the dormancy-breaking process, or the inhibition of the synthesis of specific germination-inhibiting proteins may have to occur. We have, therefore, begun to evaluate changes in gene expression that may be occurring in the conifer seed as dormancy is lost. In this paper we report that, while no evidence of a change in the pattern of protein synthesis was observed for the embryo of loblolly pine, the megagametophyte showed a shift in the pattern of protein synthesis that coincided with the loss of seed dormancy.

Materials and methods

Seed material and germination

Loblolly pine seed from a first generation select tree was a gift from Westvaco, Summerville SC, U.S.A. (Clone 11-9, open pollinated, and collected in the fall of 1984). Prior to stratification, seeds were sterilized

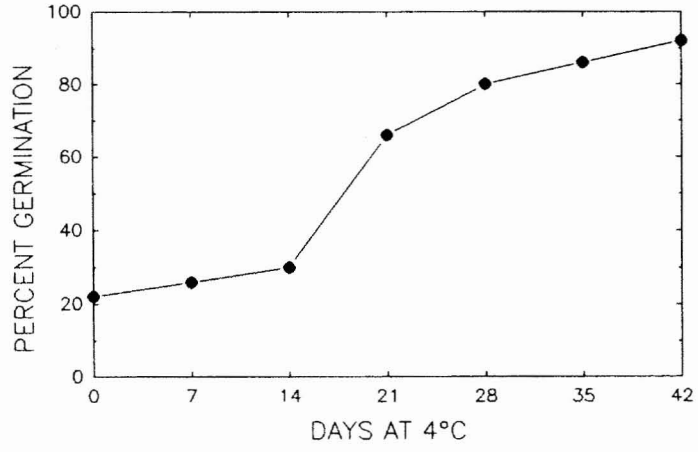

Figure 1. Changes in percent germination of seed taken at different times of cold stratification at 4°C. Percent germination at 30°C in the light (19 µmol m^{-2} s^{-1}) was evaluated after 7 days. Germination was said to be completed when the radicle had just emerged from the seed coat.

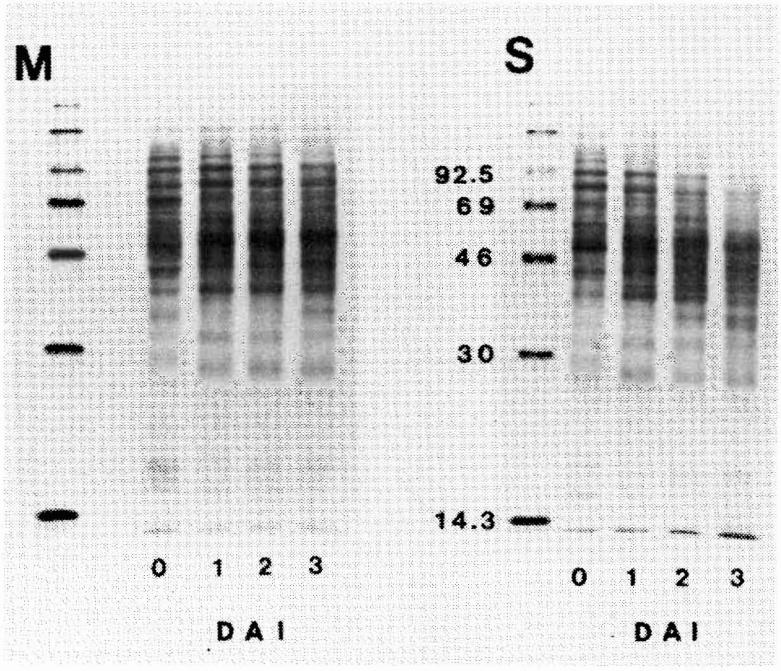

Figure 2. Fluorograph of SDS-PAGE profiles of changes in ^{35}S Methionine incorporation into embryo buffer-soluble proteins following days of imbibition (DAI) at 30°C in the light (19 µmol m^{-2} s^{-1}). M, embryos from mature unstratified seed, S, embryos from 42-day cold-stratified seed. In each lane 10 000 dpm was loaded in 10 µL. Numerical values adjacent to the fluorograph are molecular masses in kD of [^{14}C]methylated protein standards.

according to Molina and Palmier (1982), with the following modifications: seeds were placed under running tap water for 30 minutes, shaken in a dilute Tween 20 solution for 30 minutes, followed by 30 minutes under running tap water. Seeds were transferred between layers of moist Kimpak (Seedboro Equipment, Chicago, IL, U.S.A.) in an autoclaved germination tray (Spencer Lemaire Ind., Edmonton, Alberta, Canada), and imbibed at 4°C in the dark for up to 42 days to overcome dormancy. For the *in vitro* experiments, isolated embryos were excised from cold stratified and unstratified seeds and imbibed in Petri dishes containing 1% agar in an illuminated germinator (19 µmol m^{-2} s^{-1}) at 30°C for up to 3 days.

In vivo *protein synthesis*

Ten freshly harvested megagametophytes or embryos were labelled with 3.8 GBq ^{35}S Methionine (40 TBq/mmol; Amersham, Canada) for 3 hours at 4°C in the dark. Tissue was then rinsed with deionized water and buffer soluble proteins were extracted in 1 mL 50 mM sodium phosphate pH 7.5 containing 0.1 mM Leupeptin at 4°C. Radioactivity was determined by TCA precipitation (Gifford and Bewley 1984).

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described in Gifford and Bewley (1983) was carried out under reducing (+ 2-mercaptoethanol; ME) conditions on a BioRad mini gel system, using a 0.75-mm 12% slab gel. For two-dimensional electrophoresis, isoelectric focusing (IF) was carried out on a BioRad mini gel system. Samples, treated with 9.5 M urea, were loaded at the cathode in a tube gel containing 9.2 M urea, 4% acrylamide, 1.6% 5/8 ampholyte (Pharmalyte) and 0.4% 3/10 ampholyte (Pharmalyte). The cathodic (upper) buffer used was 20 mM NaOH and the (lower) anodic

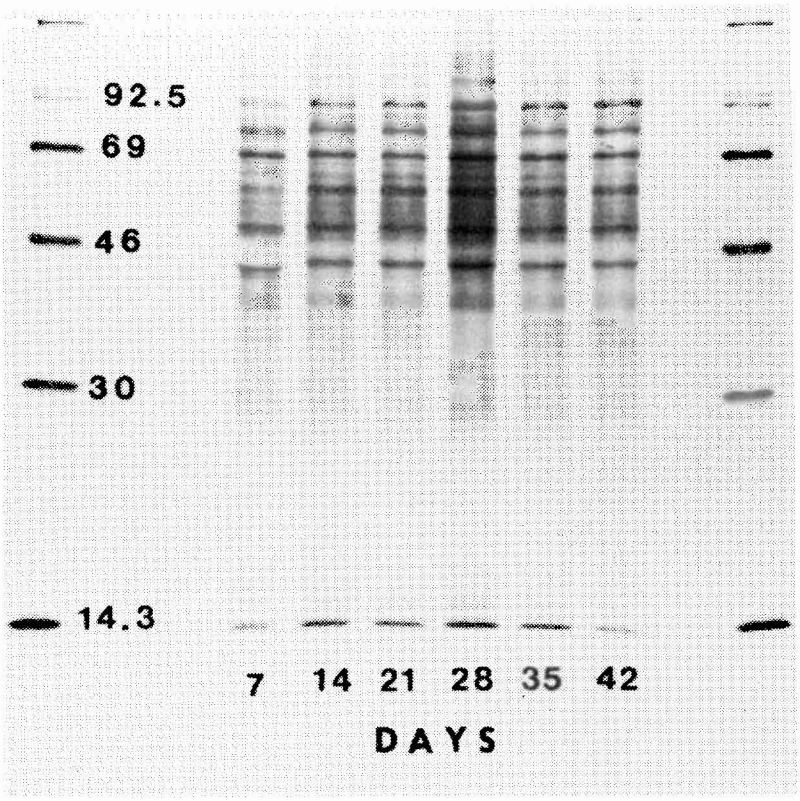

Figure 3. Fluorograph of SDS-PAGE profiles of changes in ^{35}S Methionine incorporation into buffer-soluble proteins of embryos taken at different days of cold stratification. In each lane 10 000 dpm was loaded in 10 µL. Numerical values adjacent to the fluorograph are molecular masses in kD of [^{14}C]methylated protein standards.

buffer was 10 mM H_3PO_4. Following IEF, SDS-PAGE, under reducing conditions, was as above. Gels were dried and subjected to fluorography (Gifford and Bewley 1984). Molecular weight of proteins was determined by the method of Weber and Osborne (1969). ^{14}C-methylated molecular weight markers were: myosin, 200 000; phosphorylase b, 92 500; bovine serum albumin, 69 000; ovalbumin, 46 000; carbonic anhydrase, 30 000; lysozyme, 14 300 (Amersham, Canada).

Results

The mature loblolly pine seeds used in this study showed 20% germination prior to cold stratification, and 90% germination following this procedure (Fig. 1). During stratification, the relative water content of whole seeds rose from 13.5 ± 0.4% to 30.4 ± 0.1%. Also during this period, no significant changes in the total lipid, protein or nucleic acid content of either megagametophyte or embryo were observed (Gifford, D.J., unpublished results).

Embryo germination, in vitro *studies*

Embryos, isolated from mature unstratified seeds, were nondormant. At 30°C, they germinated on agar and during a 3 day growth period mobilized their stored protein and lipid reserves. This growth and mobilization was similar to that observed following germination of embryos from 42-day cold-stratified seeds (Gifford, D.J., unpublished results). The pattern of protein synthesis during germination of embryos from mature unstratified seeds was determined and compared to that of embryos from 42-day cold-stratified seeds (Fig. 2). No significant differences were observed.

Protein synthesis in the embryo and megagametophyte during cold stratification

The patterns of protein synthesis in embryos taken at different times of cold stratification are shown in Figure 3. With the exception of low molecular mass proteins (<20 kD), one dimensional electrophoresis revealed no changes in protein synthesis, regardless of time taken. The synthesis of the low molecular mass proteins was noticeably decreased by 35 days of cold stratification (Fig. 3). These observations were even more apparent when fluorographs of two-dimensional electrophoretic profiles, obtained for embryos taken before and after stratification, were compared (Fig. 4). The low molecular mass proteins are likely late-embryogenesis-abundant proteins (LEA). LEA mRNAs are conserved during seed maturation, translated for a brief period following rehydration, and then rapidly degraded (Dure *et al.* 1989). The megagametophyte also showed a decrease in the synthesis of low molecular mass proteins (< 20 kD) following 35 days of cold stratification (Fig. 5). In contrast to the embryo, the megagametophyte showed additional shifts in the patterns of protein synthesis during the cold stratification period. The synthesis of a protein set with molecular mass of 27-29 kD decreased following 14 days of cold stratification, whereas a set of proteins with molecular masses of 31-33 kD showed increased synthesis after 14 days of cold stratification (Fig. 5). These differences were clearly seen when fluorographs of two-dimensional electrophoretic profiles, obtained for megagametophytes taken before

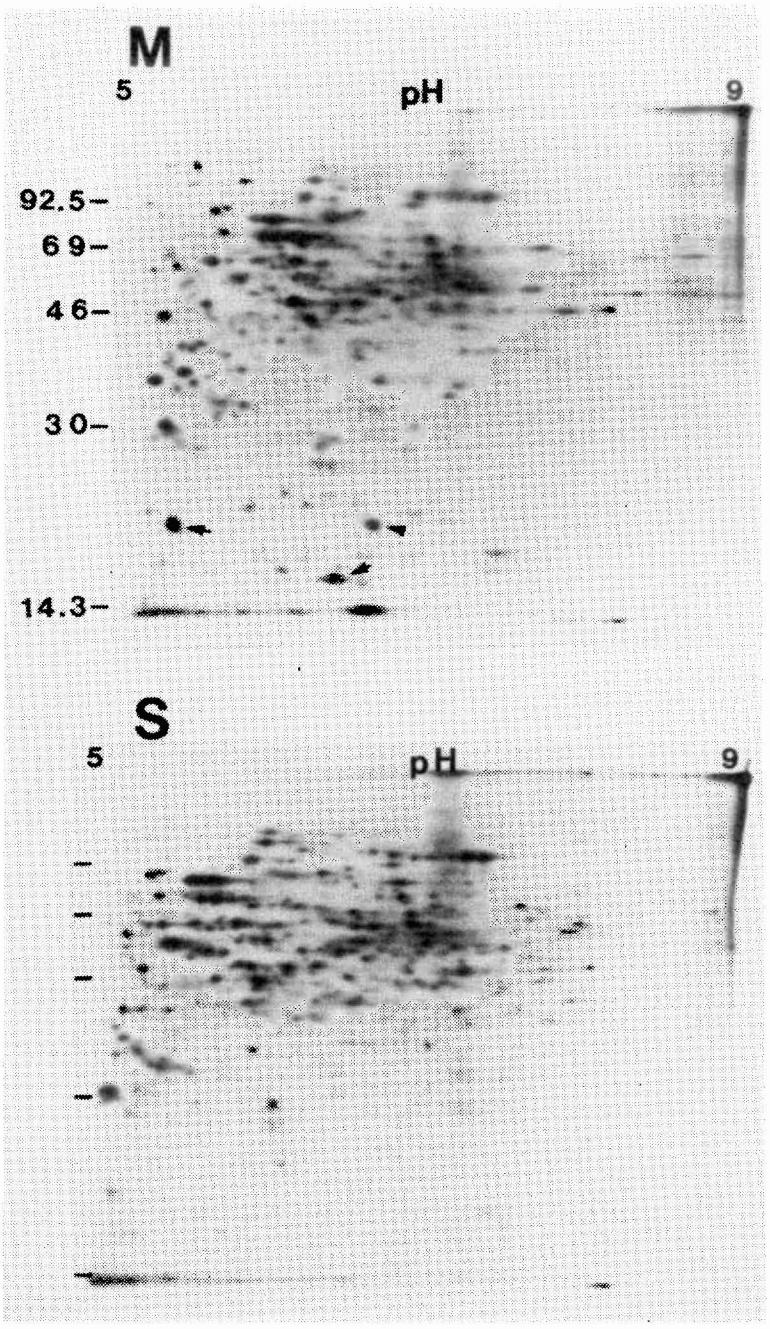

Figure 4. Fluorograph of ^{35}S Methionine labelled buffer-soluble embryo proteins taken at different days of cold stratification, subjected to two-dimensional electrophoresis. M, embryos from mature unstratified seed, S, embryos from 42-day cold-stratified seed. Range of pH for first dimension is indicated at top. Molecular masses in kD of methylated standards run in the second dimension are adjacent to the fluorographs. Arrows highlight low molecular mass proteins whose synthesis decreases.

and after stratification, were compared (Fig. 6).

Discussion

At 30°C, embryos from mature unstratified loblolly pine seeds used in this study failed to germinate *in situ*. However, when removed from these seeds, the embryos readily germinated *in vitro*. Thus, embryo dormancy in the mature unstratified seed is imposed by the surrounding tissues. Germination tests, following sequential removal of the surrounding tissues, indicate that the megagametophyte plays a major role in the maintenance of embryo dormancy in mature unstratified seeds. Only through its removal does complete embryo germination occur (Gifford, D.J., unpublished results). Similar observations have been made for *Pinus monticola* (Hoff 1987).

The capacity for protein synthesis of both embryo and megagametophyte is seen as an essential component of increased sugar pine seed germinability due to the loss of seed dormancy (Noland and Murphy 1986). Loblolly pine appears to be similar in this regard. Protein synthesis in both the embryo and megagametophyte increases 100-fold after 7 days of cold stratification, and this level is maintained as seed dormancy is lost (Gifford, D.J., unpublished results). In addition, it has been proposed that the synthesis of specific proteins may be needed to maintain embryo dormancy (Satoh and Esashi 1979). In pine, one can speculate that these proteins, synthesized by the megagametophyte, would move to the embryo where they would inhibit the germination gene set, the expression of which is an essential component of normal embryo germination and radicle growth (Gifford *et al.* 1991). In this model, the termination of synthesis of these germination-inhibition proteins would initiate the transition to nondormancy. Interestingly, while the pattern of proteins being synthesized by the embryo remains the same throughout the cold stratification period, the megagametophyte does show a significant shift in the pattern of protein synthesis that occurs between 14 and 21

days of cold stratification. At this time, the synthesis of a protein set with molecular mass of 27-29 kD decreases markedly. Significantly, this coincides with a increase in seed germinability from 30% to 66%.

Acknowledgements

The technical assistance of S.R. Axler is gratefully appreciated. This work was supported by a Natural Sciences and Engineering Research Council of Canada grant, number 0GP002240.

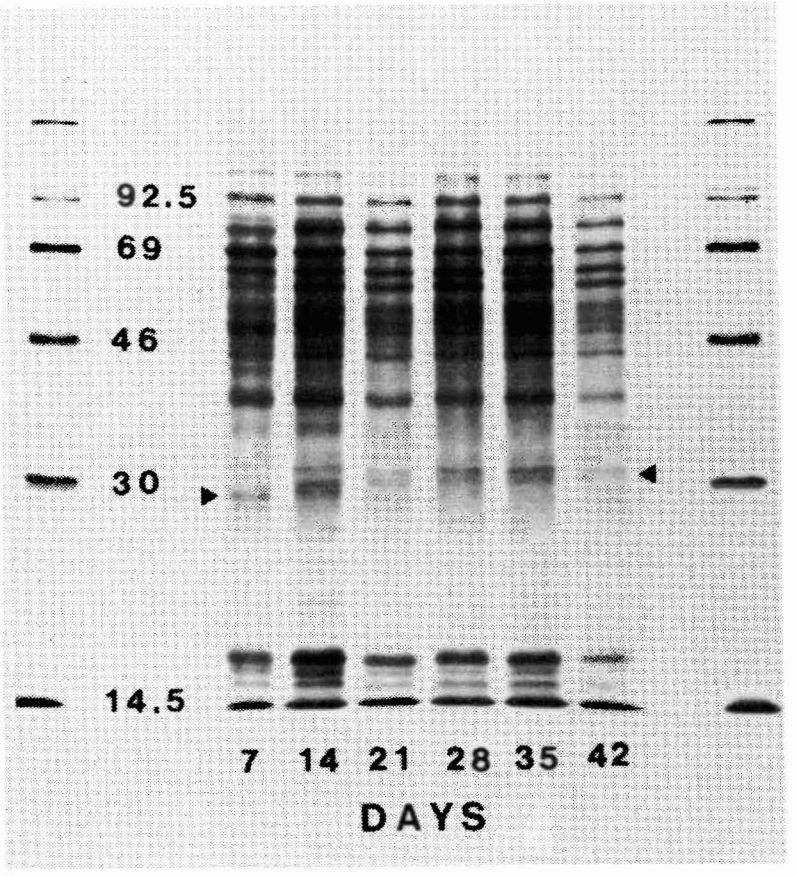

Figure 5. Fluorograph of SDS-PAGE profiles of changes in ^{35}S Methionine incorporation into buffer-soluble proteins of megagametophytes taken at different days of cold stratification. Arrows highlight proteins whose rate of synthesis is changing (see text for details). In each lane 10 000 dpm was loaded in 10 µL. Numerical values adjacent to the fluorograph are molecular masses in kD of [^{14}C]methylated protein standards.

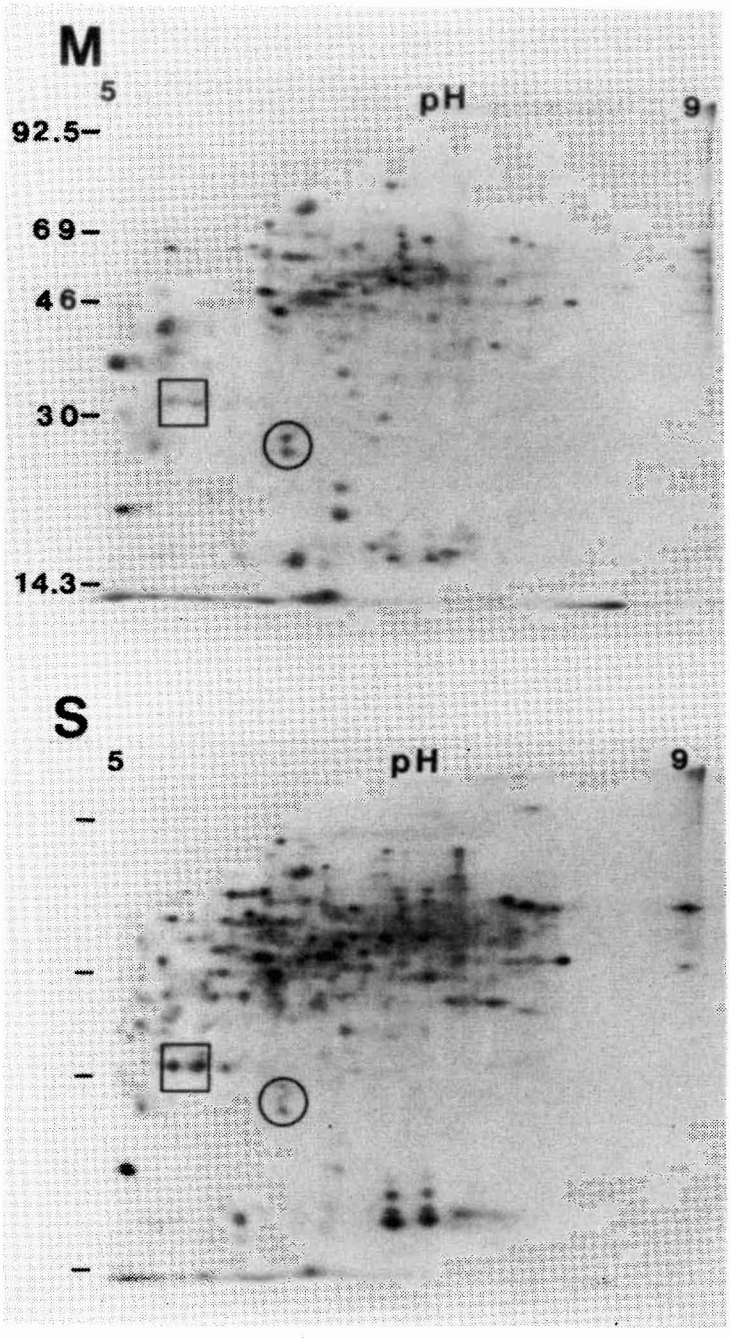

Figure 6. Fluorograph of ^{35}S Methionine labelled buffer-soluble megagametophyte proteins taken at different days of cold stratification, subjected to two-dimensional electrophoresis. M, embryos from mature unstratified seed, S, embryos from 42-day cold-stratified seed. ❑ Proteins whose apparent rate of synthesis is increasing, ○ proteins whose apparent rate of synthesis is decreasing. Range of pH for first dimension is indicated at top. Molecular masses in kD of methylated standards run in the second dimension are adjacent to the fluorographs.

hydrolysis of storage proteins is accompanied by an increase in activity of peptidases in cell free extracts of embryos and megagametophytes. Studies on angiosperm seeds have shown that changes in storage reserve hydrolase activities are due to *de novo* synthesis resulting from increased transcriptional activity of genes encoding these enzymes (Harada *et al.* 1988, Comai *et al.* 1989; Smith and Leaver 1986, Turley and Trelease 1989; Allen *et al.* 1988)). In conifer seeds to date, the molecular mechanisms regulating storage protein mobilization remains unknown. In order to understand the gene regulation during conifer seed germination we have recently isolated germination-specific cDNA clones from a cDNA library of 4-day germinated Douglas-fir mRNAs. The sequence analysis and further characterization of their expression during germination is in progress.

Conclusions

We have shown that major changes in gene activity in Douglas-fir seeds occur following incubation of stratified seeds. SDS-PAGE and Western blot analysis of storage proteins indicated that mobilization of storage proteins is rapid between 4-6 d of germination. Currently we are investigating the molecular mechanisms of gene regulation during course of germination, using homologous as well as heterologous gene probes.

Acknowledgements

This work was supported by a grant to Santosh Misra from the Natural Sciences and Engineering Research of Canada and the Forest Biology Fund (University of Victoria). The technical help of J.K. McLeod is acknowledged.

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Combination of dormancy-breaking and storage for tree seeds: new strategies for hardwood species

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Abstract

Globally, more than 60% of forestry seeds, especially hardwood species, possess a deep dormancy that impedes germination in the nursery. The long cold treatments (stratification) or combined cold and warm treatments that are required to eliminate this dormancy are usually applied after storage and before sowing. In this paper it is shown that such treatments can also be applied before storage. Results are reported for three hardwood species, *Fagus silvatica* L. (European beech), *Fraxinus excelsior* L. (European ash), and *Prunus avium* (wild cherry), which exhibit poor germination and low seedling yields in the nursery due to dormancy. Dormancy is usually eliminated by naked stratification (without medium) which involves hydrating the seeds to a precise moisture content (m.c.) (30% m.c. for beechnuts, 55%-60% m.c. for ash seeds, 27%-30% m.c. for cherry stones). Seeds are maintained in a refrigerator (2-5°C) at the precise m.c. for several weeks or months until dormancy is broken, but before germination occurs. After treatment, the seeds can either be sown, or dried to below 10% m.c. and stored for 1-6 years, depending on the species. This strategy enables a continuous supply of non-dormant dry seeds that are ready to germinate so that nurserymen can choose the sowing date according to favorable climatic conditions and other factors.

Résumé

Dans l'ensemble, plus de 60 % des semences d'arbres forestiers, notamment les essences feuillues, ont la faculté d'entrer dans un état de dormance profonde qui empêche la germination en pépinière. L'exposition prolongée à de basses températures (stratification) et les traitements combinant l'exposition au froid et à la chaleur qui sont nécessaires pour éliminer cette dormance sont généralement appliqués après la période d'entreposage et avant l'ensemencement. Le présent document démontre que de tels traitements peuvent également être appliqués avant l'entreposage des graines. L'auteur fait état de résultats qu'il a obtenus avec trois essences de feuillus, soit le hêtre européen (*Fagus silvatica* L.), le grand frêne (*Fraxinus excelsior* L.) et le merisier des oiseaux (*Prunus avium*), dont la germination et le rendement en plantules sont faibles en pépinière en raison de la dormance. La dormance est généralement levée par stratification à nu (sans milieu de culture) qui consiste à hydrater les graines jusqu'à une teneur précise en eau (30 % dans le cas des akènes de hêtre, 55 à 60 % dans le cas des graines de frêne et 27 à 30 % dans le cas des noyaux de merisier des oiseaux). Les graines sont conservées dans un réfrigérateur (2 à 5 °C) à une teneur en eau précise pendant plusieurs semaines ou mois jusqu'à ce que la dormance soit levée, mais avant que ne se produise la germination. Après le traitement, les graines peuvent être semées ou séchées jusqu'à une teneur en eau inférieure à 10 % et entreposées pendant un à six ans, selon l'essence. Cette stratégie permet de garantir un approvisionnement continu en graines sèches non dormantes et prêtes à germer de sorte que les pépiniéristes peuvent choisir la date d'ensemencement à la faveur des conditions climatiques et d'autres facteurs.

Introduction

The production of high quality forest tree seeds is hampered by a variety of factors. Although a growing proportion is being obtained from seed orchards, most forest tree seeds continue to be collected from natural stands. For the most part, forest tree seeds have an inherent high genetic variability which results in great heterogeneity in their behaviour, especially germination. Seed dormancy can vary from year to year, from seedlot to seedlot, and among seeds within a lot (Edwards 1980). The irregularity of good seed crops (sometimes 5-7 years between good mast in the case of beech or oak) makes long-term storage necessary to ensure that a consistent supply of high quality seeds available to nurseries. Also, the seeds of a majority of forest tree species worldwide, especially hardwood species, are dormant to some degree, and germination is impeded unless they are specially treated. Treatments to eliminate this dormancy can be long in duration.

Seed dormancy can have its origin either in the embryo or in the seedcoats, or in both and, according to Côme (1989), can be considered as a mechanism to regulate germination. Dormancy is a relative phenomenon which appears at low temperatures for seeds of warm regions, at higher temperatures for seeds of temperate regions, and sometimes under low conditions of oxygenation. Long cold treatments, or combined cold and warm treatments, are often necessary to break the embryo dormancy of hardwood species, e.g., 1-3 months for beechnuts, 5-6 months for wild cherries, or 6-8 months for ash. The elimination of dormancy leads to an increase in the range of conditions, especially temperature, under which germination can occur. Thus, some hardwood species such as *F. silvatica*, *F. excelsior*, *P. avium*, and maple (*Acer* spp.) can germinate under a wider range of temperatures after dormancy has been broken.

Dormancy-breaking treatments are usually applied after storage and before sowing, but the results reported in this paper demonstrate that such treatments can also be applied before storage. This new strategy which associates the breaking of dormancy with storage is convenient for nurserymen because it provides a continuous supply of dry, non-dormant seeds that are always ready to germinate.

Materials and methods

Dormancy-breaking treatments can be performed with a medium (classical stratification) or without a medium if seed moisture content is controlled, a procedure developed for hardwood species on the basis of Polish research (Suszka 1974). The procedure is similar to the stratification technique developed by Edwards (1986) for coniferous seeds, and which has become known as the "stratification-redry" method (Leadem 1986, 1989).

1. Stratification with a medium

This is the classical method whereby dormancy is eliminated by chilling seeds at 2-5°C in a moist medium (peat, sand or vermiculite). However, this treatment is not very effective in overcoming the heterogeneity within a seedlot, especially if seeds are able to germinate at low temperature. For example, stratification of beechnuts may be interrupted when less dormant seeds begin to germinate. The duration of treatment to this point, expressed in weeks, is represented as "X" and is generally taken to be the time when 10% of the seeds have germinated. The time period "X" is an indication of the degree of dormancy of the seedlot and it enables comparisons between different seedlots. However, since dormancy is not broken for most seeds after "X" weeks, subsequent germination is neither uniform or fast. Moreover, once the germination process has begun, there is no way to dehydrate the seeds without damaging them.

2. Pretreatment without medium

A new procedure has been developed for beechnuts by Suszka (1974) to overcome the drawbacks of classical stratification. The treatment involves hydrating the seeds to a predetermined moisture content, depending on the species, e.g., 30% m.c. for beechnuts, then maintaining the seeds at this m.c for X+2 weeks (2-5°C). At this m.c., this period is sufficient to break dormancy, but germination cannot occur. After treatment, the seeds can either be sown, or they can be dried to below 10% m.c. and stored for several years (Muller and Bonnet-Masimbert 1980, 1989; Muller *et al.* 1990).

This ability to dry and store seeds in a non-dormant state has opened new possibilities in the handling and preparation of deeply-dormant hardwood species, and it is especially advantageous for nursery growers who can sow seeds with little or no additional preparation.

Results

For *Fagus silvatica*, *Fraxinus excelsior*, and *Prunus avium*, seed dormancy is an important but poorly understood problem which has resulted in poor germination, a waste of seeds, low nursery yields, and probable loss of genotypes. The following describes the methodology developed in our laboratory that has greatly improved the seedling production potential of these important hardwoods.

1. Fagus silvatica *(European beech)*

Beechnuts are typical examples of seeds with a variable, but often deep, dormancy that may require up to 3 months of chilling to eliminate. Long-term storage is now achievable (Suszka 1974; Bonnet-Masimbert and Muller 1975) on a large scale (Muller and Bonnet-Masimbert 1980). However, nursery growers still have some seedling production difficulties, even with high quality seedlots, due to seed dormancy. Currently, no more than 1000 seedlings are obtained in the nursery from 1 kg of nuts, but with this new procedure the same quantity of seeds will produce 2500 seedlings or more.

There are three possible treatments for breaking the dormancy of beechnuts depending on whether the treatment is applied after, before, or during storage (Figure 1). Treatment 1 is the classical method in which dormancy is eliminated after storage by chilling at 2-5°C either with or without a medium. Treatment 2 is applied during storage, generally a few weeks or

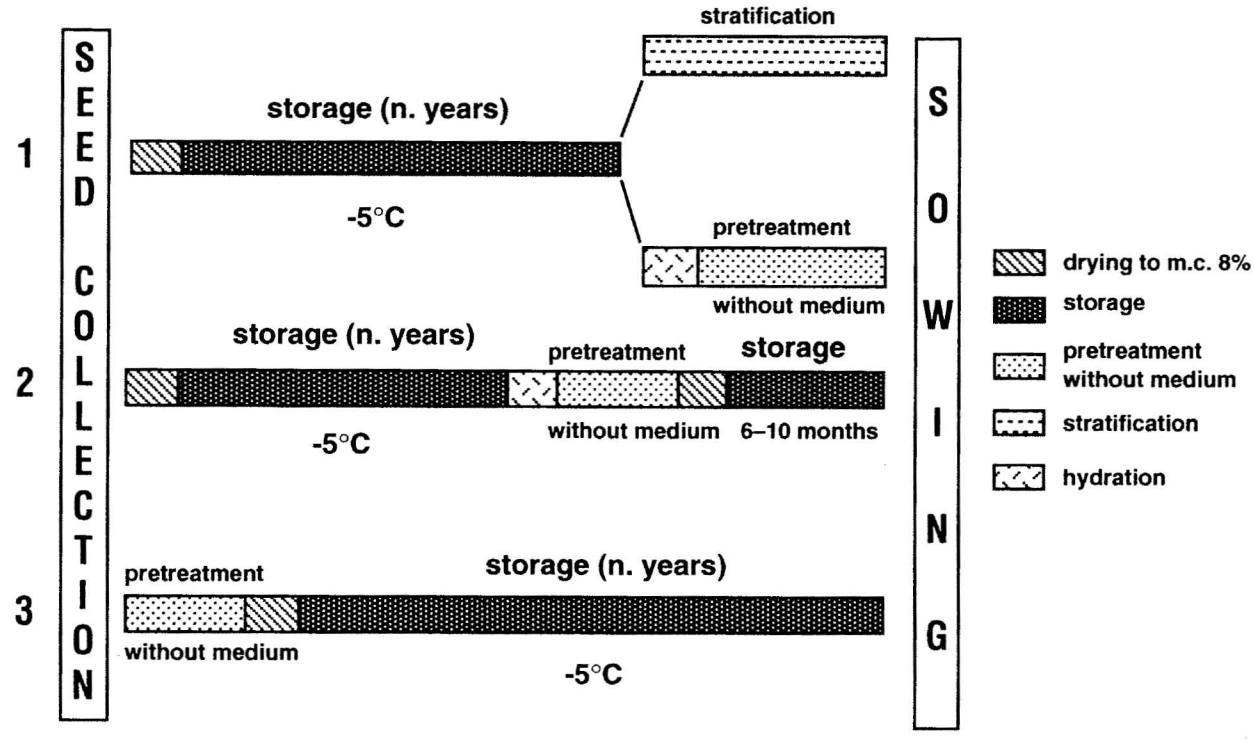

Figure 1. Three treatments for breaking dormancy in European beech, combining seed storage and stratification with and without a medium.

months before sowing. It differs from the classical method in that the seeds are dried and stored for periods up to 6-10 months (Suszka and Zieta 1976, 1977) after the chilling treatment. In treatment 3, dormancy is broken just after seed collection, then the seeds are dried and stored for several years (Muller and Bonnet-Masimbert 1989; Muller *et al.* 1990) (Figure 1).

Of the three methods, treatment 3 is the only one that ensures a continuous supply of dry, non-dormant seeds, able to germinate without any further treatment. This method allows a complete independence between dormancy-breaking treatment and date of sowing, such that late sowing-requests made by growers can be accommodated. This is is a significant improvement in seed handling since, for very dormant seedlots of beechnuts, up to 3 months of treatment would otherwise be necessary. The procedure has been shown to be effective in overcoming the problems of variable dormancy within a seedlot, as well as among different lots. This new methodology is being applied on a large scale by the French "Office National des Forêts" and by seed companies. Last year, Vilmorin

Table 1. Germination at 5°/15°C (16 h/8 h) of beechnuts (*Fagus silvatica*) stratified (in the laboratory) without medium, bèfore and after storage.

Lot	Duration of storage (months)	Stratification (without medium)	
		Before storage	After storage
A	72	61.3	71.0
B	42	72.5	72.0
C	30	86.7	70.0
D	6	88.0	78.0
E	6	86.7	89.0
F	6	92.0	90.0
Mean		81.2	78.3

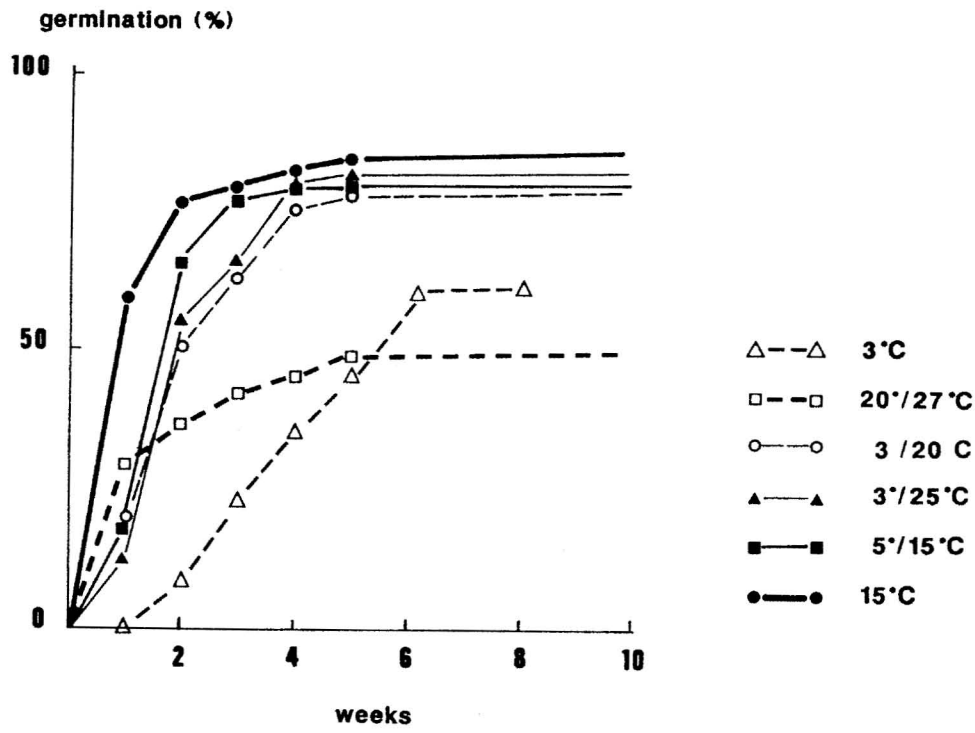

Figure 2. The effect of temperature on the germination of non-dormant European beechnuts.

(one of the larger French seed companies) applied the procedure to 10 tons of beechnuts and achieved an average germination (among the different seedlots) of about 60-70%. Some were sown in the nurseries last spring, i.e., 6 months after collection, with good results, and next spring, after 18 months of storage the remaining seeds will be sown. While breaking dormancy prior to storage is preferred, classical stratification (treatment 1, above) can still be applied particularly if the dormancy-breaking treatment is carried out without medium. Germination is comparable between the two methods (Table 1).

Stratifying seeds in a medium is not advisable because they often germinate before they can be sown. Recent tests have shown that seedlings grown from pregerminated seeds are often deformed at the base of the stem (root collar), which adversely affects subsequent seedling growth.

Temperature effects on germination

Nurserymen must consider temperature conditions when they choose their sowing dates. Dormant beechnuts germinate well only at 3°C, but non-dormant beechnuts can germinate in a wider range of temperatures. However, non-dormant beechnuts do not germinate properly either at constant warm or cold temperatures and better results are obtained at alternate temperatures such as 5°/15°C, 3°/20°C, 3°/25°C (16 h/8 h), or even at 15°C, than at 20°/27°C, or 3°C (Figure 2).

Storage of non-dormant beechnuts

More than 35 non-dormant seedlots have been stored for up to 8 years. A comparison with dormant seedlots showed that non-dormant lots maintained their germinative capacities as well as the dormant ones, and generally had higher germination rates (Table 2). Especially encouraging were the results for nursery bed emergence, which indicated that treated seeds had adapted well to the irregular environmental conditions in the nursery. Very good yields have been obtained in the nursery with these seeds that have been stored in the non-dormant state (around 3000 seedlings for 1 kg of seeds, compared to 1000 seedlings per kg obtained previously). However, after 4 years of storage, nursery results for some seedlots have been better for seeds that had been stored in the dormant state and then stratified by the classical method (without medium).

2. Fraxinus excelsior *(European ash)*

Ash seeds exhibit embryo as well as morphological dormancy due to the underdevelopment of the embryo at the time of the fruit collection. To break dormancy in these seeds, a warm incubation is followed by a cold treatment, altogether encompassing a 22-32 week

Table 2. Germination of dormant and non-dormant beechnuts (*Fagus silvatica*) after storage. GP - germination (%); GMT - mean time to germinate (days).

Lot	Duration of storage (months)	Seed condition	Before storage		After storage	
			GP (%)	GMT (days)	GP (%)	GMT (days)
A	66	Dormant	72.0	49.9	64.0	48.4
		Non-dormant	68.0	14.3	70.0	15.3
B	48	Dormant	69.0	65.2	72.7	63.5
		Non-dormant	77.3	13.6	77.3	15.5
C	30	Dormant	91.5	57.5	96.0	53.2
		Non-dormant	94.5	14.6	91.0	15.8
D	30	Dormant	76.0	62.1	69.0	58.5
		Non-dormant	80.0	12.1	73.5	16.2
E	12	Dormant	94.0	62.4	87.0	62.6
		Non-dormant	80.0	12.6	90.0	14.7
Mean		Dormant	80.5	59.4	77.7	57.2
		Non-dormant	80.0	13.4	80.4	15.5

Table 3. Germination of *Fraxinus excelsior* seeds before and after 4 years of storage at -5°C.

Lot	Warm + cold before storage	Warm before, cold after storage	Warm + cold after storage
A	87	82	89
B	71	73	67

warm phase (20°C for 6-16 wk)
cold phase (3°C for 16 wk)

period. The warm phase (20°C for 6-16 weeks) allows for the growth of the embryo which, at the time of collection, usually fills only half of the embryo cavity. Embryo growth may be fast or slow, depending on the seedlot. The cold phase (3°C for 16 wk) eliminates embryo dormancy. Treatment can be applied with or without a medium at 55-60% seed m.c.

Possible treatments after collection

As for beechnuts, dormancy of ash seeds can be removed before, after, or during storage (Muller *et al.* 1990). To remove dormancy prior to storage, our experimental data suggest it is possible to eliminate morphological dormancy by warm treatment (which enhances embryo development), or both morphological and physiological (embryo) dormancy by applying a warm plus cold treatment just after collection. The fact that dormancy may be removed in a stepwise fashion indicates some degree of independence between the two types of dormancy release. Whatever the method, good results have been obtained (Table 3). The flexibility of the treatments is a distinct advantage relative to the classical stratification in which sowing date is largely determined by the date that the chilling treatment commences, generally 6 to 8 months in advance.

Table 4. Seedling emergence (%) in the nursery from stored *Prunus avium* stones stratified with or without medium.

	Stratification after storage			
	3 warm phases + 3 cold phases		2 warm phases + 2 cold phases	
Lot	With medium	Without medium	With medium	Without medium
A	9.0	71.0	0.7	11.0
B	49.2	70.0	25.5	56.0

warm phase (20°C for 6-16 wk)
cold phase (3°C for 16 wk)

3. Prunus avium *(wild cherry)*

Wild cherry is also characteristically dormant which results in poor germination, particularly in the nursery. Because seed dormancy cannot be overcome by a classical cold treatment, seedling yields typically have not exceeded 15-20%, even in seedlots with 80% viable seeds.

We have found that it is necessary to interrupt the cold phase with one or several warm phases at 20° or 25°C. These warm phases, which induce a secondary dormancy, reduce the heterogeneity within the seedlot and result in fast and synchronized germination (Suszka 1976,1979). Experiments during the past several years have concentrated on determining the treatment most effective for the majority of seedlots. The best results have been obtained with a treatment that combined three warm and three cold phases. After collection, cherry stones should be dried to about 8% moisture content and stored at -5°C. This same treatment also works very well on seedlots stored for several years (Muller 1987).

Dormancy-breaking treatments can be applied either after or before storage (Muller *et al.* 1990) but until now, only the classical treatment (after storage) has been used in nurseries. Better results have been obtained when classical stratification has been applied without a medium (27-30% m.c. of the stones), especially for seedlots of low germinative capacity (Table 4). In the laboratory, non-dormant dried stones have been stored for 1 year without any loss of viability.

Conclusions

For the past several years, forest seed technology has progressed considerably as a result of contributions from a number of research programs that have introduced modifications and improvements to the classical techniques for removing seed dormancy. The objective of the research reported here was to try to utilize seeds more efficiently and this has been achieved, thanks to both improved storage and seed preparation techniques. Variations in dormancy between seedlots is typical of forest tree seeds, but this variation can be substantially reduced by applying new seed treatment methodology and stratifying seeds without a medium at a controlled moisture content. Moreover, for the three hardwood species reported in this paper, the integration of dormancy-release treatments with seed storage protocols ensures the availability of non-dormant seeds that are able to germinate well, even after prolonged storage (up to 5 years). This represents a substantial advance in an area in which there has been little recent progress. Nursery growers can now choose sowing dates according to favourable climatic conditions, rather than being restricted to the date that treatment was initiated. The recent increase in the number of options for breaking seed dormancy represents a significant improvement in the handling of forest tree seeds since it brings a flexibility to a system where the constraints are numerous. These new and different methodologies allow treatments to be planned according to the capabilities and at the discretion of the seed supplier.

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Temperature effects on gene expression of dormant sugar pine (*Pinus lambertiana* Dougl.) seeds

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Abstract

The mechanism by which stratification alleviates seed dormancy remains enigmatic. We have compared the effects of stratification at 5°C and incubation at 25°C (non-dormancy- breaking) on gene expression and certain metabolic activities in the embryos and megagametophytes of sugar pine (*Pinus lambertiana* Dougl.) seeds. The temperature treatments resulted in substantial differences both in the content of extracted poly(A)$^+$RNA and in its capacity to support *in vitro* translation. Embryos from seeds stratified for 90 d exhibited the highest content of poly(A)$^+$RNA which also supported the greatest *in vitro* translation activity. Comparison of the translation products by SDS-PAGE showed primarily quantitative differences. During stratification, embryos exhibited a sucrose to starch conversion that was absent in seeds at 25°C. Megagametophytes from stratified seeds showed an increase in isocitrate lyase activity that was absent in seeds at 25°C. Isocitrate lyase was also found by immunoblotting to be one of the *in vitro* translation products of poly(A)$^+$RNA from stratified megagametophytes. Thus, there appear to be tissue-specific metabolic pathways which are induced in response to stratification and are active prior to germination. While these metabolic activities are important features of germination, it is not yet clear whether they are related to the breaking of dormancy. Further application of immunochemical and molecular techniques should provide basic information on the spatial and temporal appearance of key enzymes and their specific mRNAs, which will further our understanding of seed dormancy and germination.

Résumé

Le mécanisme par lequel la stratification lève la dormance des graines reste mystérieux. Nous avons comparé les effets de la stratification à 5°C, à ceux de l'incubation à 25°C (sans levée de dormance) sur l'expression des gènes et certaines activités métaboliques observables dans les embryons et dans les mégagamétophytes des graines du pin de Lambert (*Pinus lambertiana* Dougl.). Les traitements thermiques ont conduit à des différences substantielles tant dans la teneur du poly(A)$^+$ARN extrait que dans sa capacité d'assurer la traduction *in vitro*. Les embryons des graines stratifiées pendant 90 jours avaient la plus forte teneur en poly(A)$^+$ARN qui, lui aussi, assurait le plus haut niveau de traduction *in vitro*. La comparaison des produits de traduction par la technique PAGE en présence de SDS a fait ressortir principalement des différences d'ordre quantitatif. Au cours de la stratification, il y a eu conversion du sucrose en amidon chez les embryons, ce qui n'a pas été observé chez les graines à 25°C. Les mégagamétophytes des graines stratifiées manifestaient une augmentation de l'activité de l'isocitrate lyase qui n'a pas été observée chez les graines à 25°C. Il a été établi par transfert électrophorétique suivi d'immunodétection que l'isocitrate lyase était l'un des produits de traduction in vitro du poly(A)$^+$ARN extrait des mégagamétophytes stratifiés. Il semble donc exister des voies métaboliques caractéristiques de certains tissus qui sont exploitées en réaction à la stratification et qui sont actives avant la germination. Bien que ces formes d'activité métabolique soient des caractéristiques importantes de la germination, il n'a pas encore été établi si elles sont en rapport avec la levée de la dormance. De nouvelles applications de techniques immunochimiques et moléculaires devraient nous apporter des renseignements fondamentaux sur l'apparition dans l'espace et dans le temps d'enzymes essentielles et de leurs ARNm spécifiques qui nous aideront à comprendre davantage la dormance et la germination des graines.

Introduction

Ultimately, a complete understanding of the mechanisms by which dormancy is imposed and germination is regulated will depend on unraveling the mysteries of the regulation of gene expression. While significant advances have been made in demonstrating the expression of specific genes during seed development, much less has been determined concerning gene expression which controls germination and dormancy (Bewley and Marcus 1990). The latter is particularly true for coniferous seeds. To date, efforts

Table 1. Reported effects of stratification on various metabolic activities of pine seeds

Increased levels of gibberellins and cytokinins	Paul *et al.* 1973 Taylor and Wareing 1979
Decreased level of ABA[1]	Murphy and Noland 1981
Increased PAL activity and phenolic acid content	Murphy and Noland 1980 Murphy and Noland 1981
Increased ICL activity and ATP levels	Noland and Murphy 1984
Increased protein synthesis and aminopeptidase activity	Noland and Murphy 1986
Increased ability to absorb and metabolize sucrose	Carpita *et al.* 1983
Increased SS activity and respiration	Murphy and Hammer 1988

[1]Abbreviations: ABA, abscisic acid; PAL, phenylalanine ammonia-lyase; ICL, isocitrate lyase; SS, sucrose synthase.

with pine seeds have been essentially limited to ascertaining what metabolic activities appear to be critical to germination and are responsive to stratification treatment (Table 1). An increase in growth-regulating gibberellins and cytokinins (Taylor and Wareing 1979) accompanied by a decrease in abscisic acid (Murphy and Noland 1981) results in a hormonal balance generally considered to be favorable for gene expression. The results of this expression can be seen in the increased activity of a number of metabolic processes in response to stratification (Table 1). A confounding factor in these studies, however, is that in some cases there is an initially higher metabolic activity in dormant seeds under non-dormancy-breaking conditions, e.g. respiration and protein synthesis (Murphy and Noland 1982; Noland and Murphy 1986), which does not culminate in germination. This suggests that there are genes involved in growth maintenance which are common to most vegetative cells and activated simply by hydration of the seeds, and growth-regulatory genes which are unique to a particular developmental process and induced specifically by particular environmental stimuli. Even growth-maintenance genes may exist as a family of genes which are differentially expressed either spatially or temporally during development. Techniques of molecular biology now allow us to directly determine specific gene expression and to differentiate which genes are only expressed in response to a change in development.

Our objectives in this study were to analyze the effects of either stratification at 5°C or incubation at 25°C (non-dormancy-breaking) on the poly(A)$^+$RNA (putative mRNA) populations of dormant sugar pine seeds. Additionally, we wanted to determine if we could relate these effects to changes in certain metabolic activities felt to be critical to the germination process.

Materials and methods

Seed Material

Seeds of sugar pine were obtained from Schumachers (Sandwich, MA, U.S.A.). These seeds were from a 1988 collection and were stored at 5°C until used. Seeds were imbibed at either 5°C or 25°C in aerated water, then surface-dried before treatment with 0.1% (w/w) Arasan 50-Red (DuPont, Wilmington, DE, U.S.A.). Treated seeds were then placed on moist germination blotter paper (Anchor Paper, St. Paul, MN, U.S.A.) overlaid on Kimpak (Burrows Co., Evanston, IL, U.S.A.) in Petawawa-style germination boxes (Wang and Ackerman 1983), and incubated at either 5°C or 25°C for up to 90 d. They were sampled following 14, 30, 60, and 90 d of incubation. At sampling times, embryos were excised from the surrounding megagametophytes and both were stored at -80°C until analysis.

Poly(A)$^+$RNA Extraction

Frozen tissue (50 embryos, 1.0-1.5 g) was pulverized in liquid nitrogen with a mortar and pestle to a fine powder. An RNA homogenization buffer (Baker *et al.* 1990) was added (10:1 v/w) containing an RNase protein degrader (0.02:1 v/v; Invitrogen FastTrack Kit) and the powdered tissue was thoroughly homogenized. The homogenate was centrifuged at 32 000 x g for 15 min at 4°C. The supernatant was filtered through Miracloth (Calbiochem, San Diego, CA, U.S.A.). Poly(A)$^+$RNA was purified from the supernatant by oligo (dT) cellulose absorption using a FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, U.S.A.) and quantified by its absorbance at 260 nm (Davis *et al.* 1986).

Integrity and size distribution of the purified poly(A)$^+$RNA was determined by electrophoresis (Mini-Sub system, Bio-Rad, Richmond, CA, U.S.A.) in a denaturing agarose (1.25%) gel (Davis et al. 1986). A 0.24-9.5 kb RNA ladder (GIBCO BRL, Gaithersburg, MD, U.S.A.) was run to determine size distribution.

In vitro *Translation*

Equal amounts (2.0 µg) of poly(A)$^+$RNA from each treatment were translated *in vitro* in a rabbit reticulocyte lysate system (GIBCO BRL) incorporating ^{35}S methionine (ICN, Irvine, CA, U.S.A.).

SDS-PAGE and fluorography.

Aliquots of 5.0 µL from the translation mixture were separated by SDS-PAGE (4% stacking, 12% running gel) in a Protean II (Bio-Rad). Following electrophoresis, the gel was fixed in 40% methanol:10% acetic acid for 2 h followed by a 30 min water rinse. The gel was then dried for 2 h at 80°C in a model 543 gel dryer (Bio-Rad). The dried gel was exposed to Hyperfilm β-max (Amersham Corp., Arlington Heights, IL, U.S.A.) for 72 h at -80°C. The film was developed by hand (Maniatis *et al.* 1982). Autoradiographs were scanned on an Ultrasan XL laser densitometer (LKB-Pharmacia, Piscataway, NJ, U.S.A.) and evaluated with Gelscan software (LKB-Pharmacia).

Western Blotting

Following SDS-PAGE as above, gels were transblotted onto Immobilon PVDF transfer membranes (Millipore Corp., Bedford, MA, U.S.A.) in a Mini Trans-Blot cell (Bio-Rad). The blotting buffer used was 25 mM Tris, 192 mM glycine, in 20% methanol. The Immobilon membranes were immunoblotted according to the procedure of Harlow and Lane (1988). Following blocking for 2 h in 3% BSA, the membranes were treated overnight with primary antibody [anti-ICL of pinyon (*Pinus edulis* Engelm.) which was raised in a rabbit], then reacted 1 h with alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit IgG, Sigma). The reaction was visualized with bromochloroindoyl phosphate/nitro blue tetrazolium substrate (Sigma).

Enzyme Analyses and Starch Determination

Sucrose synthase and isocitrate lyase (ICL) activity were assayed by coupled reactions (Sung *et al.* 1989; Giachetti *et al.* 1984). Protein content was determined by the dye-binding method (Bradford 1976). The starch assay was modified from that of MacRae (1971) and Haissig and Dickson (1979). Samples for starch determination were freeze-dried prior to extraction. Potentially interfering compounds were removed by repeated (3X) extraction with methanol:chloroform: water (15:6:3; Mangat *et al.* 1990). The residual pellet

Table 2. Amount of extractable poly(A)$^+$RNA from embryos of sugar pine seeds stratified at 5°C or incubated at 25°C. Data represent results of a single extraction (50 embryos) per treatment

		Days of Incubation			
	Dry Seed	14	30	60	90
Temp.		µg poly(A)$^+$RNA/embryo			
5°C	.670	0.283	0.240	0.259	0.315
25°C		0.227	0.270	0.160	—*

*Due to microbial contamination, insufficient seeds survived to take a sample.

was vacuum-dried at 50°C, resuspended in 10 mL water, then boiled for 10 min to gelatinize the starch. Aliquots of 0.5 mL were added to 4.5 mL water and 5.0 mL of 0.1 M Na acetate buffer (pH 4.5) containing 5 U/mL of amyloglucosidase (from *Aspergillus niger*; Sigma, St. Louis, MO, U.S.A.). Starch was digested for 28 h at 35°C, followed by glucose determination on a 0.5 mL aliquot using a glucose oxidase/peroxidase/o-dianisidine dihydrochloride system (Sigma). Absorbance at 540 nm was measured after a 1 h incubation at 35°C. A starch sample without added amyloglucosidase served as the control.

Results and discussion

Seed Lot Behavior

Once into this study, we discovered that this lot of seeds appeared to be of much reduced vigor relative to all earlier lots with which we have worked. This was evidenced in three different ways: 1) when seeds were stratified in plastic bags, our standard procedure, they consistently molded, despite the Arasan treatment; 2) when stratified in germination trays to avoid molding, subsequent germination upon transfer to 25°C was extremely slow and prolonged; 3) isocitrate lyase activity was much lower and exhibited a greatly reduced response to stratification compared to earlier seed lots. The seeds were viable as they eventually germinated to a high percentage, just at a much slower rate. Despite this inherent problem, some promising results were obtained.

Poly(A)$^+$RNA Levels and Translation Activity

The highest level of extractable poly(A)$^+$RNA occurred in the embryos of dry seeds (Table 2). Since this level declined by an average of 62% by day 14 at the two temperatures, this may represent considerable residual mRNA from the seed development period. At 25°C, poly(A)$^+$RNA content at day 60 had declined an additional 30% from the day 14 level. At 5°C, the poly(A)$^+$RNA content remained stable until day 90, by which time it had increased about 21%. Denaturing agarose electrophoresis verified the quantitative differences between treatments. It also showed that the size distribution was essentially the same in all treatments, being polydisperse with a range from below 1.4 kb to around 4.4. kb (Fig. 1).

Poly(A)$^+$RNA from the dry seeds, although present at high levels, was almost totally inactive in supporting *in vitro* translation (Fig. 2). This suggests that it may indeed be residual mRNA from seed development which has been modified in some way to prevent its translation upon seed imbibition. At 25°C, *in vitro* translation activity was higher at days 14 and 30 than at these times from 5°C embryos. This verifies previous findings on *in vivo* protein synthesis activity of sugar pine embryos under the same conditions (Noland and Murphy 1986). By day 60 at 25°C, *in vitro* translation activity had declined significantly, so both the level of poly(A)$^+$RNA and its translation capacity declined after this lengthy period at 25°C. A similar loss of translation capacity has been reported in response to accelerated aging treatments of seeds of several species (Gidrol *et al.* 1988; Gidrol *et al.* 1990). At 5°C, *in vitro* translation activity remained essentially the same until day 90, at which time it had increased substantially. So the increase in poly(A)$^+$RNA level by 90 d was associated with an increase in its translation capacity. This suggests that the breaking of dormancy by stratification results in the increased transcription of active poly(A)$^+$RNA. Extracted poly(A)$^+$RNA may contain co-extracted rRNA and tRNA (as evidenced by bands in Fig. 1), but these are assumed to be a minor and constant component.

The size distribution of the peptides following SDS-PAGE, ranging from around 14 kD to over 97 kD (Fig. 2), verified the integrity and size distribution of the poly(A)$^+$RNA.

Differences between the 25°C and 5°C treatment appeared to be primarily quantitative. However, densitometric comparison of the 14-day 25°C with the 90-day 5°C translation products revealed a few bands in each treatment which appeared to be unique, or at least in much higher concentration (Fig. 3). The increased resolution of a two-dimensional separation will be required to determine more definitely whether unique proteins were produced in response to the different temperatures. Breaking of dormancy in *Corylus avellana* L. seeds was associated with a change in the poly(A)$^+$RNA population and the appearance of peptides which were not produced by dormant seeds (Shannon *et al.* 1981).

Isocitrate Lyase Activity

Isocitrate lyase activity of the megagametophyte normally increases during stratification and declines at 25°C (Noland and Murphy 1984). In the present study, ICL activity declined initially at both temperatures (Fig. 4). At 5°C, ICL activity then increased about 60%, while at 25°C its activity continued to decline and remained at a significantly lower level. Immuno-blotting against an anti-ICL of pinyon revealed the presence of an approximately 66 kD peptide which showed essentially the same pattern (Fig. 5). This is the molecular weight found for the ICL subunit in *Pinus pinea* L. (Pinzauti *et al.* 1986) and *Pinus edulis* (Murphy and Hammer, unpublished results).

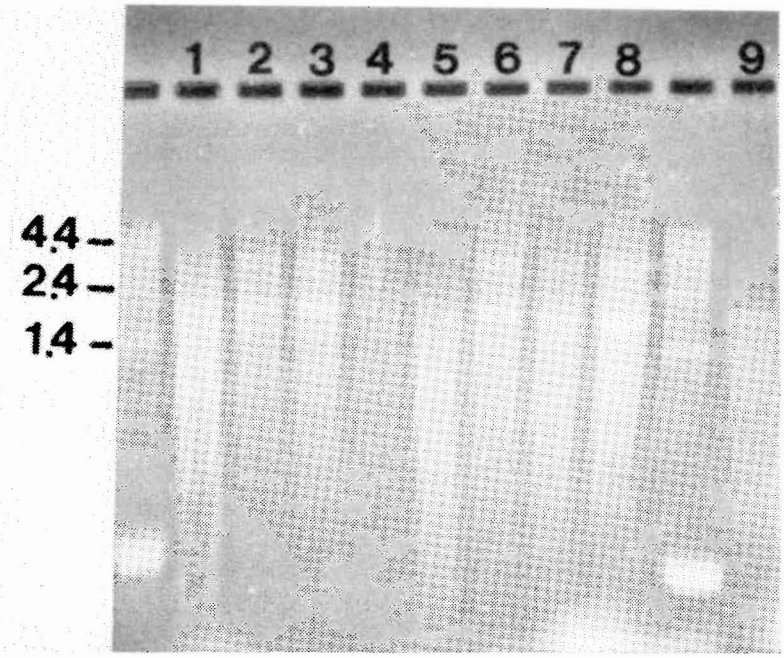

Figure 1. Sugar pine embryo poly(A)$^+$RNA separated on a reducing agarose gel. Lanes: 1) dry seeds; 2-4) 25°C, days 14, 30, and 60; 5-8) 5°C, days 14, 30, 60, and 90; 9) 90-d 5°C megagametophytes. Unmarked lanes are an RNA ladder whose sizes in kb are indicated on the left.

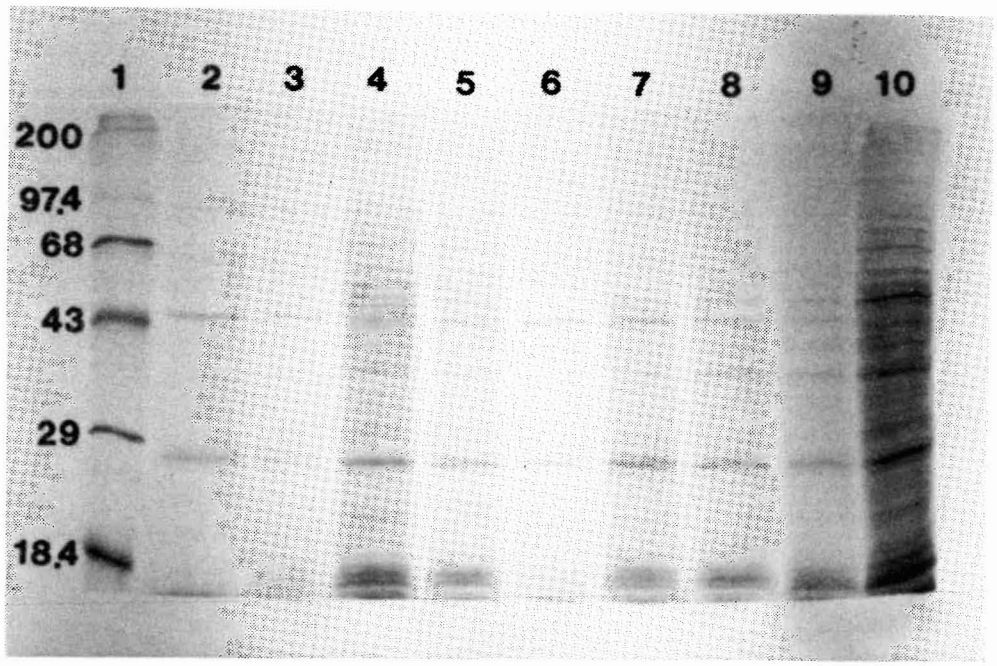

Figure 2. Autoradiograph of SDS-PAGE of *in vitro* translation products of poly(A)$^+$RNA from sugar pine embryos. Lanes: 1) MW markers, 2) blank (no RNA added); 3) dry seeds; 4-6) 25°C, days 14, 30, and 60; 7-10) 5°C, days 14, 30, 60, and 90.

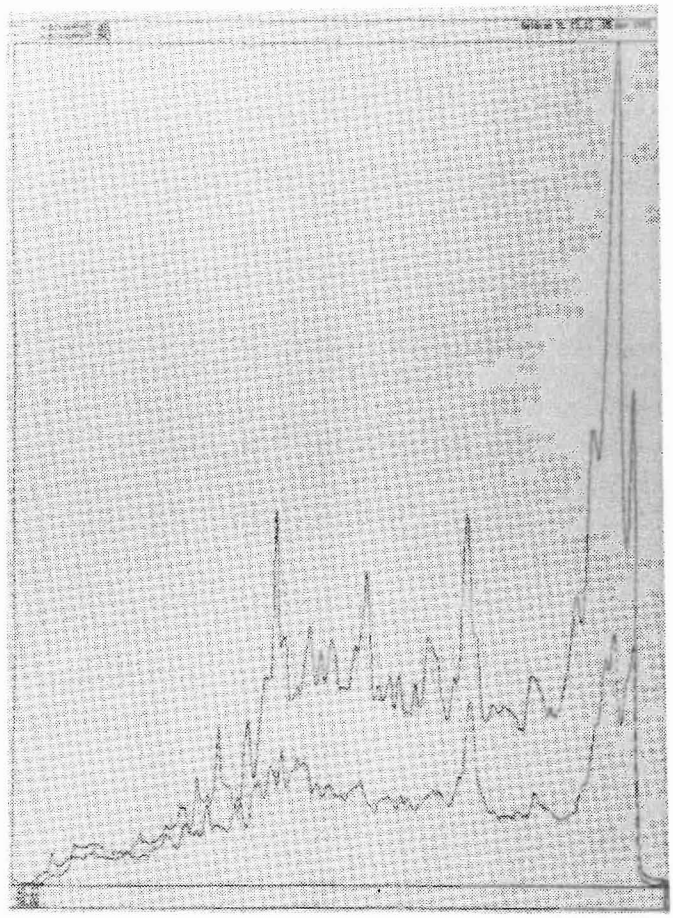

Figure 3. Densitometric scan of SDS-PAGE of *in vitro* translation products of poly(A)$^+$RNA from sugar pine embryos stratified at 5°C for 90 d (upper curve) or incubated at 25°C for 14 d (lower curve).

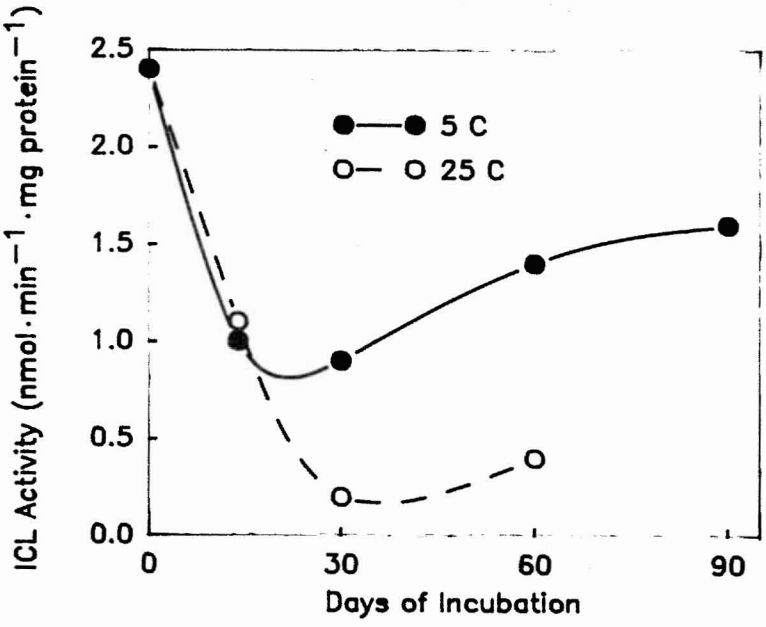

Figure 4. Isocitrate lyase activity in megagametophytes of sugar pine seeds stratified at 5°C or incubated at 25°C. Data points represent the results of a single extraction.

Immunoblotting of the *in vitro* translation products showed that an approximately 66 kD peptide was synthesized from the 90-d stratified megagametophyte poly(A)$^+$RNA (Fig. 6). This peptide was also evident on the autoradiograph of the *in vitro* translated material (Fig. 7).

Starch Content and Sucrose Synthase Activity

At 5°C, starch content increased 75% by day 14 and remained at this level through day 90 (Fig. 8). At 25°C, starch content showed no significant change over 60 d, remaining significantly lower than the level in embryos from the 5°C treatment. This indicates that there is significant carbohydrate metabolism occurring at 5°C and that sucrose-starch conversion does not occur under non-dormancy-breaking conditions. A similar increase in starch content occurs in pinyon (*Pinus edulis*) embryos in the first 4 d of imbibition, prior to radicle emergence (Murphy and Hammer, unpublished results). Thus, sucrose-starch conversion appears to be an important part of the earliest germination processes.

In pinyon, the observed sucrose-starch conversion is closely associated with the level of sucrose synthase activity, which cleaves sucrose to UDP-glucose, a precursor in the starch synthesis pathway (Murphy and Hammer, unpublished results). In dormant sugar pine embryos at 5°C, there is also a close correlation between sucrose synthase activity and the starch content (Fig. 9). This suggests that sucrose synthase activity and carbohydrate metabolism may be key elements which are induced in response to stratification. Work is currently underway to purify sucrose synthase for antibody production, in order to immunocytochemically determine the spatial and temporal pattern of its appearance, and eventually its specific mRNA by *in situ* hybridization.

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A comparison of 5°C and 15°C as dormancy breakage treatments for Sitka spruce seeds (*Picea sitchensis*)

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Abstract

In the U.K., the usual way to overcome conifer seed dormancy is to use a moist prechill at 5°C. In Sweden, seeds are invigorated at 15°C and a controlled moisture content (m.c.). A combination of the U.K. prechill plus the Swedish invigoration technique was used to study the effects of 5°C and 15°C as dormancy breakage treatments on Sitka spruce (*Picea sitchensis* Bong. Carr). Dry-stored (8% m.c., fresh weight basis) Sitka spruce seeds were raised to 30% m.c. Sixteen treatments incubating the moist seeds at 5°C for 0, 7, 14, or 21 days followed by incubation at 15°C for either 0, 5, 7, or 11 days were tested. Control seeds (8% m.c.), had a germination of 65% and mean germination time (MGT) of 17.1 days. The best treatment was a combination of the longest prechill and longest invigoration times studied (i.e. 21 days at 5°C followed by 11 days at 15°C), which achieved a germination capacity of 92% and MGT of 7.9 days. Moist seeds were also redried to storage moisture contents (5-7%) after treatment. Seeds were not harmed by redrying and the trends were similar, although the best treatment after redrying was 14 days at 5°C followed by 11 days at 15°C giving 89% germination and a MGT of 9.2 days. Incubation at 5 or 15°C were both effective at overcoming dormancy in Sitka spruce, but the similar end results may be achieved via different metabolic routes.

Résumé

Au Royaume-Uni, la façon habituelle de lever la dormance des graines des conifères est de pratiquer une stratification froide et humide à 5°C. En Suède, les graines sont revigorées à 15°C et en contrôlant la teneur en humidité. Une combinaison de la technique britannique de stratification froide et de la technique de revigoration suédoise a servi à étudier les effets d'un traitement à 5°C et à 15°C pour la levée de la dormance chez l'épinette de Sitka (*Picea sitchensis* [Bong.] Carr.). Des graines de l'épinette de Sitka entreposées à sec (8 % d'humidité, en poids frais) ont été portées à un taux d'humidité de 30 %. Seize traitements avec incubation des graines humides à 5°C pendant 0, 7, 14 ou 21 jours, suivis d'une incubation à 15°C pendant 0, 5, 7 ou 11 jours, ont été essayés. Les graines qui servaient de témoins (8 % d'humidité) ont eu un taux de germination de 65 % et une période moyenne de germination de 17,1 jours. Le meilleur traitement s'est révélé être une combinaison de la plus longue période de stratification froide et de la plus longue période de revigoration qui ont été essayées (c'est-à-dire le traitement de 21 jours à 5°C, suivi d'un deuxième traitement de 11 jours à 15°C); la faculté germinative atteinte a été de 92 % et la durée moyenne de germination de 7,9 jours. Des graines humides ont aussi été séchées à nouveau, jusqu'à la teneur en humidité de stockage (5 à 7 %), après le traitement. Elles n'ont pas été endommagées par ce nouveau séchage et les tendances sont restées les mêmes; mais le meilleur traitement qui a suivi le nouveau séchage a été celui de 14 jours à 5°C, suivi de 11 jours à 15°C; la faculté germinative obtenue a été de 89 % et la durée moyenne de germination a été de 9,2 jours. Que ce soit à 5°C ou à 15°C, la levée de la dormance est obtenue chez l'épinette de Sitka. Cependant, des résultats semblables peuvent être obtenus par différentes voies métaboliques.

Introduction

Commercially available temperate conifer seeds rarely germinate quickly or to a high final germination percentage without some form of pre(germination)-treatment. This phenomenon is widely termed 'dormancy'. Throughout most of the world the commonest nursery pre-treatment is to chill moist seeds at about 4°C prior to sowing (Allen and Bientjes 1954; Hosner *et al.* 1959; Gosling 1988; Edwards 1986).

The so-called "prechill" consists of three stages. First, the dry-stored seeds (6-8% m.c., fresh wt basis) are soaked in excess water for about 48 hours at 4°C; the seeds must be raised to a moisture content at which they can respond to the prechill (Gosling and Rigg 1990). Secondly, most of the excess water is drained

off to prevent waterlogging. Thirdly, the damp seeds are placed in a loosely tied polythene bag and incubated at 4°C for 3-8 weeks. Because the seeds are only drained of excess water a thin film of moisture remains adhered to each seed during the cold treatment, providing a reservoir of water which would enable non-dormant seeds to germinate. However, the low temperature prevents this and careful control of the seed moisture content is only necessary if prechill periods are scheduled to exceed 10-12 weeks.

A less common technique to improve the performance of tree seeds is "invigoration". This has been developed and is applied almost exclusively in Sweden (Bergsten 1987, 1988, 1989). In the invigoration method, higher temperatures (15-20°C) are used and seed moisture content and treatment duration become critical. Dry seeds are imbibed to predetermined moisture contents which must be carefully controlled throughout treatment to prevent premature germination. Incubation times are normally shorter for invigoration treatments than for prechill methods.

Accurate moisture content control is crucial to the success of invigoration, but this was considered unlikely to reduce the efficacy of prechilling. This paper reports a comparison between the germination of a dormant Sitka spruce seedlot raised to a controlled moisture content of 30%, incubated for up to 21 days at 5°C (prechilled) and up to 11 days at 15°C (invigorated). Combinations of 0, 7, 14, or 21 days at 5°C followed by 0, 5, 7, or 11 days at 15°C were also compared. The effect of redrying treated seeds to storage moisture contents was also studied.

Materials and methods

Seed material

Sitka spruce seeds, United Kingdom Forestry Commission identity number 80(30), collected in 1980 from a registered seed stand in North Wales and stored at a moisture content of 7.5% (fresh wt basis) were used. U.K. Official Seed Testing Station (OSTS) results showed untreated seed had 65% germinable, 21% fresh, 3% abnormal and 10% dead, while prechilled seed had 88% germinable, 0% fresh, 2% abnormal and 10% dead seed. This data indicated that the seeds were dormant.

Invigoration treatments

Seed samples (30 g) were placed in each of four invigoration tubes. "GORE-TEX" membranes (1.4 x 10^9 pores cm^{-2}) at each end of the tubes allowed movement of air and water vapour, but prevented water droplets crossing (Bergsten 1987). Seed moisture contents were adjusted to a target of 30% by adding water (9.6 g water per 30 g seeds). An average moisture content of 29% was achieved, with a range of 27-30% between treatments. The invigoration tubes were placed at 5°C and 100% relative humidity (RH) for either 0, 7, 14, or 21 days, then transferred to 15°C and 100% RH and samples taken after 0, 5, 7 and 11 days. The treatment referred to as 0 days at 5°C plus 0 days at 15°C was the untreated control seeds.

Drying treatment

As well as germinating seeds immediately after the treatments, samples were dried to storage moisture contents. Drying was done at 22±3°C and 10±5% RH until a moisture content of 6-8% was reached.

Moisture content (m.c.) determination

Moisture contents were determined by the low temperature oven method, 103°C for 16 hours (International Seed Testing Association 1985), using two replications of 100 seeds per treatment.

Germination tests

Four replications of 100 seeds per treatment were germinated on top of moist filter paper using the Copenhagen tank equipment in the Swedish laboratories. Lighting using Thorn T40 W/33 cool-white fluorescent tubes giving 20 µmol $m^{-2}s^{-1}$ was constant. Temperature was kept constant at 20°C. Germinants, counted when the radicles were at least 1 mm long, were recorded after 0, 3, 5, 7, 10, 14 and 21 days and converted to a percentage (%G). Mean germination time (MGT) was calculated from the germination counts. MGT is the time taken for an "average" seed to germinate and was calculated by accumulating the result of multiplying the number of seeds germinating per day (%G) by the day number and then dividing by the final germination percentage achieved. Thus,

$$MGT = \frac{(\%G \text{ on day } 0\times 0)+(\%G \text{ on day } 3\times 3).....+(\%G \text{ on final day} \times \text{final number of days})}{\%G \text{ on final day}}$$

Statistical analysis

Results for undried and redried seeds were analyzed separately using a Duncan's multiple range test; statistically significant differences were determined at P = 0.05 (SAS 1989). Before analysis of per cent germination, data for each replication were transformed using an angular function. The data for MGT were not transformed.

Table 1. Seeds were set to germinate immediately after treatment. Treatment 1 (5°C) was followed by the corresponding treatment 2 (15°C). O d 5°C + O d 15°C are the untreated control seeds. Untreated seeds m.c. = 8%, treated seeds 27-30%.

Germination percentage after 21 days at 20°C

Treatment 1 5°C	Treatment 2 15°C O d	5 d	7 d	11 d
O d	65e	78d	81c,d	83c,d
7 d	82c,d	88a,b	90a,b	91a,b
14 d	86b,c	90a,b	88a,b	92a
21 d	90a,b	90a,b	92a	92a

Data are actual means (analysis was performed on the angular transformed data). Means sharing the same letter are not significantly different (P = 0.05).

Mean germination time

Treatment 1 5°C	Treatment 2 15°C O d	5 d	7 d	11 d
O d	17.1a	14.7b	13.1c,d	12.3d,e
7 d	13.8c	11.0f	10.6f,g,h	9.1i
14 d	13.3c,d	10.1f,g	9.6h,i	9.7g,h,i
21 d	12.0e	10.3f,g,h	9.1i	7.9j

Data are actual means with analysis on untransformed data.

Results

The maximum germination percentage of untreated seeds (reached after 21 days) was 65% (Table 1). Most of the remaining 35% of the seeds were dormant. This is clearly shown because treated seeds reached a maximum of 92% germination, the remaining 8% of these seed were either dead or empty (Table 1). The untreated and treated results in this experiment confirm the OSTS results.

The incubation treatments also affected the mean germination time (MGT) by reducing the time taken to germinate. Untreated seeds had a MGT of 17 days, while seeds treated for 21 days at 5°C followed by 11 days at 15°C germinated quickest with the lowest MGT of 7.9 days (Table 1). The best treatment in terms of yielding high germination (92%) and low MGT (7.9 days), was 21 days at 5°C followed by 11 days at 15°C.

Similar effects on germination per cent and MGT were found when treated seeds were reduced to storage moisture contents (Table 2). In this experiment the untreated control seed reached 74% germination by the end of the test, which was still significantly lower than most, but not all, of the treated seed. For treated seeds, the highest germination was 90% with the remaining 10% being dead or empty. Untreated seeds were still the slowest to germinate having the highest MGT of 16.5 days. For the redried seeds, incubation for 14 days at 5°C followed by 11 days at 15°C prior to redrying was the best treatment, resulting in 89% germination and a MGT of only 9.2 days. Redried seed (5-7% m.c.) need to take up a lot more moisture than treated but undried seeds (27-30% m.c.), so an increase in MGT to 9.2 from 7.9 days for undried seed might have been expected. Sitka spruce seeds at a moisture content of about 7% usually need 2-3 days at 20°C to become fully imbibed (Jones and Gosling unpublished data).

Discussion and conclusion

About 25% of the seeds did not germinate until pretreated. Incubation at 30% moisture content for either 21 days at 5°C or 11 days at 15°C stimulated the

Table 2. Seeds were set to germinate after redrying to storage moisture contents of between 5 and 7%, untreated m.c. = 8%. Od 5°C + Od 15°C are the untreated control seeds.

Germination percentage after 21 days at 20°C

Treatment 1 5°C	Treatment 2 15°C O d	5 d	7 d	11 d
O d	74f	77e,f	77e,f	87a,b,c,d
7 d	83d,e	83d,e	89a,b,c	89a,b
14 d	83c,d,e	85b,c,d	90a	89a,b
21 d	83c,d,e	86a,b,c,d	87a,b,c,d	90a,b

Data are actual means (analysis was performed on the angular transformed data). Means sharing the same letter are not significantly different (P = 0.05).

Mean germination time

Treatment 1 5°C	Treatment 2 15°C O d	5 d	7 d	11 d
O d	16.5b	14.4c	13.4d,e	12.5e,f
7 d	14.2c,d	12.7e,f	11.8f	10.6g
14 d	14.9c	12.5e,f	10.6g	9.2h
21 d	21.4a	14.5c	12.4f	10.6g

Data are actual means with analysis on untransformed data.

germination of this dormant fraction. 21 days at 5°C followed by 11 days at 15°C was even more effective in breaking dormancy when considering the effects on both germination percentage and MGT.

It is somewhat surprising, given the temperature differences, that 7 days at 5°C and 7 days at 15°C were equally effective. Also 14 days at 5°C gave very similar results to 11 days at 15°C. Although undried seeds given 21 days at 5°C followed by 11 days at 15°C was the best treatment, it is possible that 32 days at 5°C or even 32 days at 15°C could be equally effective.

Redrying treated seeds to storage moisture contents after the 5 and 15°C treatments did not significantly reduce the germination per cent, or increase MGT beyond that explained by the time needed to take up moisture for germination. This confirms the report of the successful redrying of a different seed lot of Sitka spruce to storage moisture contents after soaking and prechilling (Jones and Gosling 1990).

The work reported here highlights potential similarities between dormancy and vigour, and dormancy breakage and invigoration. The invigoration treatment requires more elaborate equipment for adequate moisture content control, but the temperature of 15°C was just as effective in stimulating germination per cent and MGT as a prechill temperature of 5°C. Therefore the frequent assumption that low temperature is an absolute requirement to overcome dormancy in Sitka spruce is untrue. These observations raise the interesting question of whether dormancy breakage and invigoration progress via the same or different metabolic routes.

Acknowledgements

We would like to thank the U.K. Forestry Commission for funding the visit of SJ to Sweden. We would also like to thank Kerstin Abbing, Rune Johansson and Mia Sundberg for doing the germination tests, and Andrew Peace for statistical advice.

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Respiration of tree seeds

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Abstract

Respiration is essential to generate energy and carbon skeletons for all metabolic processes occurring in tree seeds. This paper discusses how respiration may be measured, and how respiration measurements may be used to rapidly and quantitatively monitor the physiological status of seeds. Examples are given for several different conifer species to demonstrate how respiration varies in tree seeds during imbibition, dormancy, and germination. The effects of moisture content and temperature are also shown. Additional applications of the method are suggested.

Résumé

La respiration est un mécanisme essentiel à la production de l'énergie et des hydrates de carbone qui sont utilisés dans tous les mécanismes métaboliques observés dans les graines d'essences forestières. Cet article examine comment la respiration peut être mesurée et de quelle façon cette mesure peut servir à la vérification suivie du développement physiologique des semences de manière rapide et chiffrée. Des exemples pris chez différentes espèces de conifères sont donnés et montrent comment la respiration varie chez les graines d'essences forestières durant l'imbibition, la dormance et la germination. Les effets de la teneur en humidité et de la température sont aussi décrits. D'autres applications de la méthode sont proposées.

Introduction

Germination commences as soon as dry seeds are imbibed and ends with the onset of radicle growth, but within the confines of these separate and distinct events the germination process can be separated into three identifiable stages. The first phase is imbibition during which water is taken passively, and then actively, into the seed. The second phase is variously called activation (Ching 1973), the lag period (Bewley and Black 1978), or germination *sensu stricto* (Côme and Thévenot 1982). Despite no apparent outward signs of growth or development, activation is probably the most significant phase for plant production. Activation is the stage when reserves are hydrolyzed and new enzymatic and structural components are synthesized for the developing seedling. The third and final phase commences with radicle emergence.

Germination capacity is widely employed as a measure of germination potential, but germination capacity and other terms derived from germination test data, such as germination value (Czabator 1962), are only empirical measures of seed performance. Such measures indicate how seeds will perform under prescribed conditions, but reveal little about the more functional, physiological properties of seeds, and thus provide limited understanding of why seeds may fail to perform as expected. A more promising approach to characterizing seed quality is to directly measure physiological attributes. The value of physiological measurements is that they quantitatively characterize metabolic function, and thus directly indicate how seeds respond physiologically to environmental and experimental variables.

Respiration is an essential biological process common to all living organisms; it plays a central role in metabolic function and is therefore an excellent process by which to monitor the physiological status of tree seeds. Essentially, respiration is an oxidation-reduction process in which substrates are oxidized to generate energy and carbon skeletons for other metabolic processes. Although respiration is a complex of many metabolic pathways, the overall reaction can be summarized as follows:

$$C_{12}H_{22}O_{11} + 12\ O_2 \rightarrow 12\ CO_2 + 11\ H_2O$$

The net result is that organic compounds are oxidized to carbon dioxide, and the absorbed oxygen is reduced to water. The energy which is released during

this process is either lost as heat, or trapped in chemical forms, such as ATP or NADH, as electrons are transferred along the electron transport chain (Lehninger 1970).

A major advantage to monitoring respiration in seeds is that changes in respiration rates (and imbibition rates, see Côme 1967) can be directly linked to each of the three stages of germination. Although little studied in gymnosperm seeds, respiratory patterns during germination have been well characterized in angiosperm tree seeds. In apple (*Pyrus malus* L.), for example, respiration increases exponentially immediately after imbibition, but soon levels off as germination enters the activation stage (Côme and Thévenot 1982). Respiration rates do not change appreciably during activation, but remain relatively constant until radicle emergence, at which time rates again increase exponentially.

Although several techniques may be employed, the Warburg apparatus is probably the most well-known device for monitoring respiration (Umbreit *et al.* 1964). The Warburg apparatus consists of several (usually 12) calibrated manometers maintained at constant temperatures with a large controlled temperature water bath. The sample to be measured is placed in a small reaction vessel connected to a manometer, and readings are taken as oxygen is displaced by Krebs' or Brodie's fluid in the manometer column. Apparent respiration is calculated from standard gas law pressure/volume relationships. The advantages of this method are that both oxygen consumption and carbon dioxide generation can be determined so that, if desired, respiratory quotients can also be calculated. The method suffers from several drawbacks: the apparatus requires a great deal of space; readings must be taken over a prolonged period; data must be corrected for changes in atmospheric pressure; manometers must be individually calibrated (a time-consuming process if they are broken or must be replaced); and relatively large amounts of material are required to obtain readings.

The Cartesian diver is a similar, but less often used, manometric device which is more sensitive than the Warburg apparatus, and is thus more appropriate for measuring respiration of small amounts of biological material. In common with the Warburg apparatus, however, is the need for calibration and adjustments for atmospheric pressure. The Gilson differential respirometer makes continuous accommodations for changes in barometric pressure, and avoids the need of calibrating reaction vessels, but otherwise shares many of the other drawbacks of the Warburg apparatus. Infrared gas analyzers, which measure the absorption of infrared radiation by carbon dioxide, have also been used to determine respiration, but they are generally not sensitive enough to detect the low respiration rates characteristic of many seeds.

The Clark O_2 electrode seems to overcome many of the limitations of earlier measurement methods. Initially used in animal studies (Burky 1978), the Clark O_2 electrode was subsequently used to a limited degree to measure respiration in tree seeds (Murphy and Noland 1982b; Murphy and Hammer 1988). The small volume of the cuvette and the sensitivity of this polarographic technique enables the experimenter to detect the low rates encountered when measuring seed respiration. Determinations can be made in as little as five minutes and are easily reproducible.

The purpose of this paper is to describe the procedures and practical advantages of the Clark O_2 electrode for measuring tree seed respiration. The paper also elaborates how respiratory monitoring can enable seed researchers and technologists to assess the manner in which various seed handling procedures may either inhibit or promote the basic metabolic processes occurring in tree seeds.

Materials and methods

A Yellow Springs Instruments Model 5300 Clark O_2 electrode was used to determine respiration rates. Seeds were placed in a cuvette containing 3 ml of 0.1 M potassium phosphate buffer (pH 7.0). The sample was allowed to equilibrate (with stirring) for 3 minutes. Care was taken to ensure that all air bubbles were removed as the O_2 probe was inserted into the cuvette and that there was thorough mixing of the cuvette contents to maintain constant O_2 levels at the surface of the electrode membrane. Readings were recorded on chart paper (calibrated in units 1 to 100) and continued for 5 minutes.

Respiration measurements were replicated three times on samples consisting of 10 seeds (about 0.5 g of material), but the number varied depending upon the size of the seeds. To maintain the material in good physiological condition until measurements were taken,

Table 1. Germination classes after incubation of seeds at 20°C or 30°C under 8 h photoperiod

Class	Description
1	No evidence of germination
2	coat split, radicle < 1 mm
3	1-4 mm radicle
4	5-10 mm radicle
5	> 10 mm radicle

imbibed seeds and germinants were always covered with moist towelling at room temperature (20°C) and stratified seeds were kept in an insulated container at 2°C. Given the nature of the Clark O_2 electrode technique, it was not possible to measure respiration of dry (6-10% moisture content) seeds. Measurements of "dry" seeds were actually determined after the seeds had been immersed in phosphate buffer for 5-8 minutes.

Seeds of many tree species, e.g., white spruce (*Picea glauca* (Moench) Voss), lodgepole pine (*Pinus contorta* Dougl.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), did not require special preparation, but with true firs (*Abies* spp.) it was necessary to remove the outer papery coat by rolling seeds gently between thumb and forefinger. This eliminated some of the loose debris which otherwise would have clogged or punctured the O_2 membrane.

Respiration of seeds which had been incubated at 20 or 30°C was measured after first separating samples into several germination classes (Table 1). Germinants in classes 4 to 5 were cut into 2 mm sections to eliminate large pieces of material which would otherwise tend to become caught in the cuvette and produce erratic measurements. Additional buffer solution was added when necessary, but this was avoided if possible because of corresponding reductions in sensitivity.

After oxygen consumption was measured, cuvette contents were emptied into a strainer, blotted with towelling, and transferred to a weigh boat. Dry weights were determined after drying in a convection oven for

Table 2. Volume of oxygen dissolved in water (microliters O_2 per milliliter at 1 atmosphere).

Temp (°C)	Equilibrated with 100% O_2	Equilibrated with air (21% O_2)
0	48.7	10.23
10	37.9	7.96
15	34.4	7.22
20	31.0	6.51
25	28.2	5.92
28	26.9	5.65
30	26.1	5.48
35	24.5	5.15
37	23.9	5.02
40	23.1	4.85

Recalculated from Hodgman *et al.* (1961).

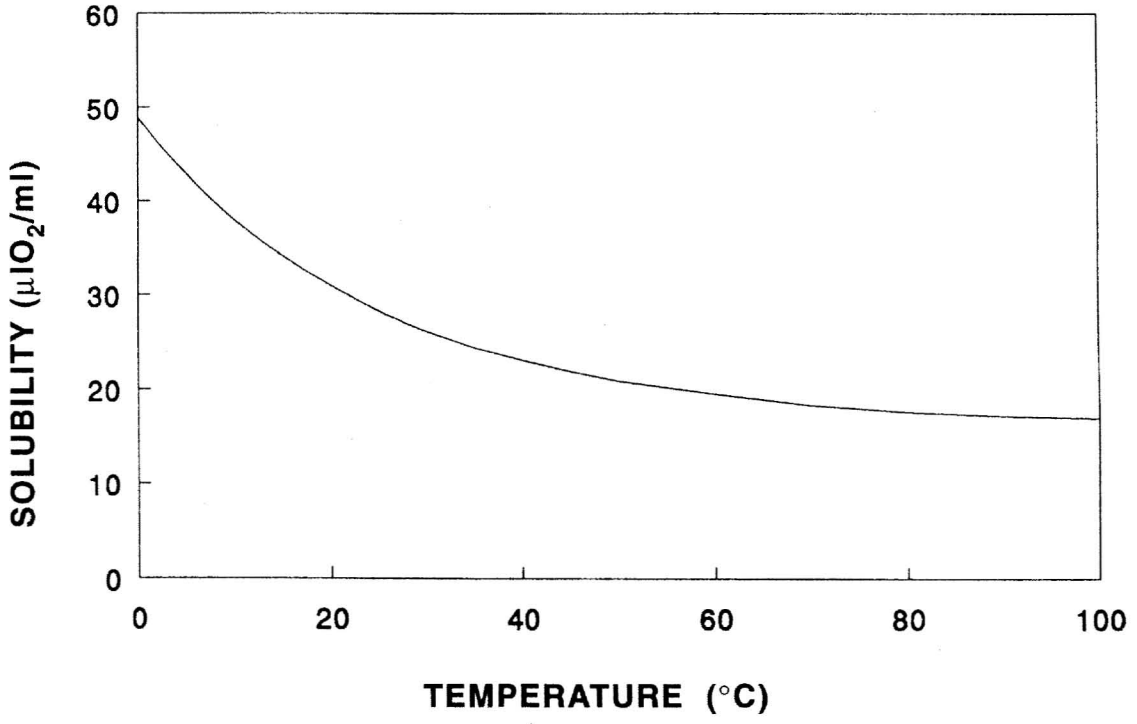

Figure 1. Solubility of oxygen in water in equilibrium with 100% oxygen and 1 atmosphere, as a function of temperature (Hodgman *et al.* 1961).

Table 3. Effects of imbibition and radicle emergence on respiration (μL O_2/min/g dry weight) of *Pseudotsuga menziesii* seeds.

Treatment	Resp. at 20°C
dry seeds	0.0
1 h soak	3.4
24 h soak	3.6
72 h incubation	4.2
6 d incubation	4.9
germinant < 1 mm	12.2
germinant 1-4 mm	15.0
germinant 5-10 mm	16.3
germinant > 10 mm	17.0

unstratified seeds incubated at 20°C

24 h at 100°C. Respiration rates were calculated according to the formula:

$$\mu L\ O_2/min/g\ dry\ wt = \frac{k \times mL\ solution \times ([O_2]_{initial} - [O_2]_{final})/100}{dry\ wt\ (g)/min}$$

k = 5.48 μL oxygen/ml solution at 30°C and 1 atmosphere, in equilibrium with 21% oxygen in air. For solubilities at other temperatures and partial pressures, see Table 2 and Figure 1.

For the experiment in which microbial respiration effects were examined, seeds were surface-sterilized by immersion in full-strength NaOCl (5.25% v/v) for 5 minutes, followed by three rinses with deionized water.

Results and discussion

Effects of incubation and radicle emergence

No measurable respiration was detected in dry seeds of *Pseudotsuga menziesii* seeds when measured at 20°C (Table 3), but within 1 h seeds achieved rates equivalent to those of seeds which had been imbibed for 24 h. Respiration of fully imbibed seeds remained relatively constant throughout incubation at 20°C, but rates immediately doubled as soon as radicles emerged through the seed coat. Respiration continued to rise as radicles elongated.

In *Pinus monticola* Dougl., low respiration rates were detectable after dry seeds were incubated for only 5-8 minutes in phosphate buffer (Table 4). Seeds were fully imbibed by 48 h, with a corresponding six-fold increase in respiration. Seeds were then transferred from 20°C to 2°C for stratification, and respiration was measured at weekly intervals. Rates remained constant during 8 weeks of stratification, with no increases until seeds had been incubated for some time under warmer temperatures.

Table 4. Effects of stratification on respiration (μL O_2/min/g dry weight) of *Pinus monticola* seeds.

Treatment	Resp. at 30°C
dry seeds	0.3
48 h soak	1.8
1 wk stratification	1.9
2 wk stratification	1.9
3 wk stratification	2.1
4 wk stratification	1.8
5 wk stratification	2.0
6 wk stratification	1.7
7 wk stratification	1.7
8 wk stratification	1.7
germinant 1-4 mm	3.3

Table 5. Effects of stratification, incubation, and emergence on respiration (μL O_2/min/g dry weight) of *Pinus monticola* seeds.

Treatment	Resp. at 30°C
8 wk strat	1.7
48 h @ 30°C/20°C	1.5
21 d @ 30°C/20°C	1.5
germinant < 1 mm	2.5
germinant 1-4 mm	3.3
germinant 5-10 mm	4.6
germinant > 10 mm	10.8
cotyledon not separate	14.7
cotyledons separate	16.0
coat shed	87.0

Increased respiration was not simply due to transferring seeds from 2°C to 30°/20°C (Table 5); even after warm incubation for 3 weeks, respiration was no greater than that observed during stratification. The plateau in respiration observed during the stratification period in these conifer seeds seemed to be analogous to the constant rates maintained during the activation stage of nondormant angiosperm seeds (Côme and Thévenot 1982).

No further gains in respiration were achieved until the radicles emerged through the seed coats. By the

Table 6. Effects of measurement temperature on respiration (μL O_2/min/g dry weight) of *Pseudotsuga menziesii* seeds.

	Incubated/measured @ 20°C		Incubated/measured @ 30°C	
Treatment	Unstrat	Strat 3 wk	Unstrat	Strat 3 wk
germinant < 1 mm	12.2	10.5	11.5	12.0
germinant 1-4 mm	15.0	18.4	14.4	11.6
germinant 5-10 mm	16.3	15.0	16.6	16.0
germinant > 10 mm	17.0	20.6	20.3	19.7

Table 7. Effects of stratification and measurement temperature on respiration (μL O_2/min/g dry weight) of *Abies lasiocarpa* seeds

Treatment	0°C	10°C	20°C	30°C
Seedlot 3414				
dry seeds	-	0.39c	0.74c	0.88b
48 h soak	1.10	1.51b	2.10a	3.07a
8-wk strat	0.56	2.11a	2.20a	2.85a
strat-redry	-	0.68c	1.38b	2.49a
Seedlot 3417				
dry seeds	-	0.22b	0.38b	1.14b
48 h soak	-	0.99a	1.95a	2.50a
8-wk strat	1.50	1.16a	2.13a	2.48a
strat-redry	-	1.12a	2.38a	2.80a
Seedlot 28838				
dry seeds	-	0.76c	1.28b	1.47b
48 h soak	-	2.03ab	2.29a	3.54a
8-wk strat	-	2.60a	2.32a	4.68a
strat-redry	-	1.24bc	1.83ab	3.22a

Means with the same letter in the seedlot and column are not statistically different at P = 0.05 when evaluated by Duncan's (1965) multiple range test.

time radicles had grown to 1-4 mm in length, rates had doubled. When cotyledons were apparent, rates were 10 times those of ungerminated seeds. By the time seed coats had been shed, respiration had increased nearly sixty-fold relative to rates observed in seeds during stratification.

Effects of measurement temperature

Respiration is known to be strongly temperature dependent (Salisbury and Ross 1969), so it was of interest to determine how the temperature conditions under which the seeds were measured would affect respiration patterns. Stratified and unstratified *Pseudotsuga menziesii* seeds were measured at 20°C and 30°C, temperatures under which seeds are commonly incubated. Respiration was measured at the same temperature under which seeds had been incubated (Table 6).

Unstratified germinants achieved rates of 12.2 μL O_2 $min^{-1}g^{-1}$ dry weight at 20°C, and 11.5 μL O_2 $min^{-1}g^{-1}$ dry weight when measured at 30°C (Table 6). Due to the greater solubility of oxygen in water at lower temperatures (Table 2), differences in respiration due to temperature were not as great as might have been

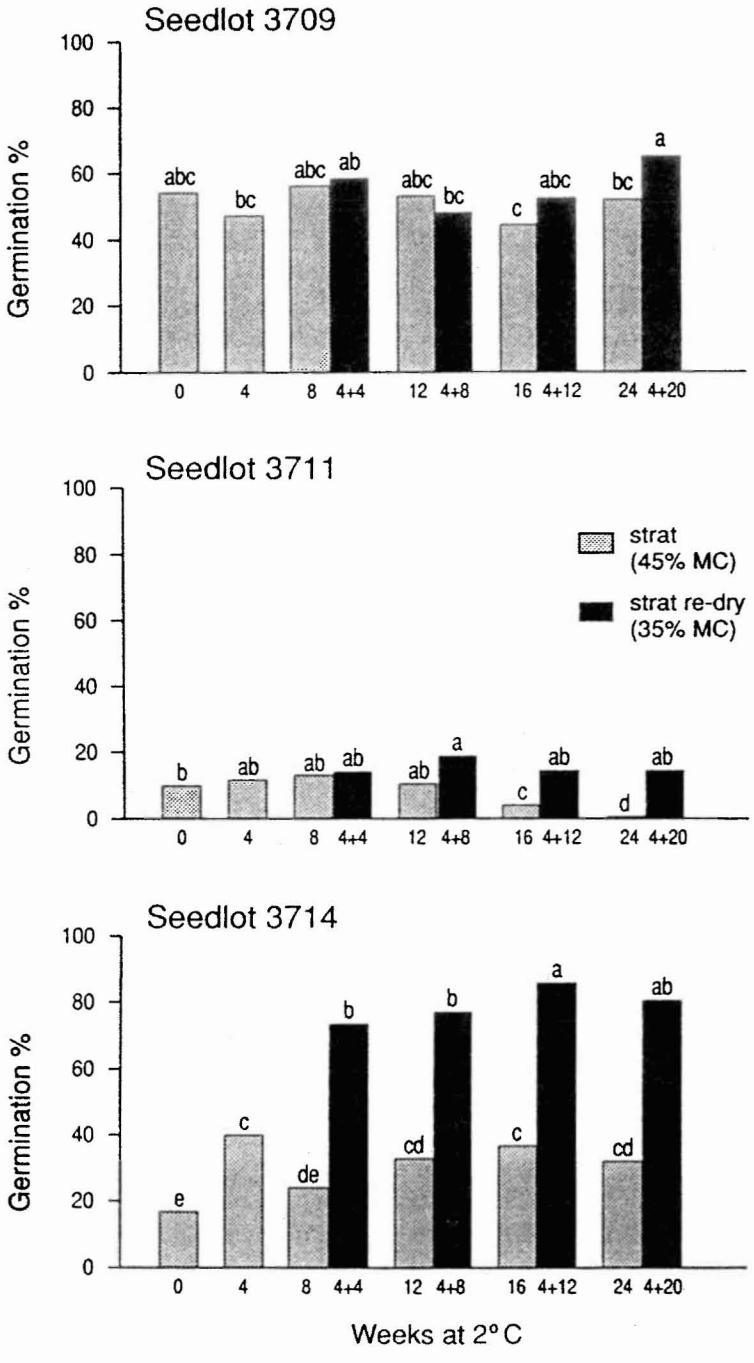

Figure 2. Effects of stratification treatment on the germination of three seed sources of *Abies lasiocarpa* after incubation at 30°C/20°C for 28 days (from Leadem 1989). Data are the means of six replications of 50 filled seeds. For each seedlot, treatments with the same letter are not significantly different at P = 0.05 as determined by Duncan's (1965) multiple range test.

expected. Thus, at equivalent stages, respiration rates at 20°C and 30°C were relatively similar. It is not clear why higher temperatures did not result in higher respiration rates, but the same trends were observed throughout all stages of incubation and radicle elongation.

Effects of stratification

Comparisons between stratified and unstratified seeds revealed no apparent effects of stratification on respiration, regardless of the temperature at which rates were measured (Table 6). The most significant determinants of the magnitude of respiration were the developmental stage during which the seeds were measured. It should be noted, however, that although respiration of germinants in the same size class did not differ, seeds which had been previously stratified germinated more rapidly, and consequently progressed through each developmental stage more quickly than unstratified seeds.

The effects of stratification and temperature on seed respiration were also examined in *Abies lasiocarpa* (Hook.) Nutt. (Table 7). Stratification-redry is a treatment known to be especially effective for *Abies* seeds (Edwards 1981, 1985; Leadem 1986). Seeds of this genera require prolonged chilling to break dormancy, but tend to germinate at stratification temperatures (2-5°C) if moisture levels are too high (> 40% moisture content). With stratification-redry treatment, seed moisture is maintained at about 30% moisture content during prolonged stratification so that seeds have sufficient moisture for metabolic processes associated with dormancy release, but not enough moisture to promote radicle emergence. In this way, seeds receive the lengthy chilling they require, but limited moisture contained in seed tissues effectively prevents germination during treatment.

Within a treatment, respiration increased in all three lots as

temperature was increased from 0° to 30°C, but rates increased on average only about 1.5 times for each 10°C increase in temperature. In two of three lots, the lowest respiration at any single temperature was attained by seeds which received stratification-redry treatment.

Effects of sterilization and seed coat removal

Bacteria and other microbes are present in tree seeds, as well as in all natural systems. A critical question which must be addressed in relation to respiratory measurements is the relative contribution of microbial respiration to total apparent respiration. In an experiment that compared the effects of surface sterilization on total apparent respiration, major differences were found when comparisons were made between intact seeds and seeds without coats (Table 8). In all cases, respiration was significantly higher in seeds with coats removed.

As to the effects of sterilization, no significant differences were noted when equivalent treatments were compared; respiration of sterilized and unsterilized intact whole seeds was essentially the same. Similarly, respiration rates of sterilized-decoated seeds were about the same as those of unsterilized-decoated seeds.

The fact that no differences were noted between sterilized and unsterilized seeds indicates that the observed respiration was primarily attributable to seed tissues, rather than that of microbes naturally present on the seeds. The existence of microbial respiration, however, cannot be discounted in respiratory measurements, especially in seeds known to be infected with pathogens. It is advisable to compare respiration of unsterilized seeds to that of sterilized controls to determine whether compensations must be made for microbial respiration. Alternately, an antibiotic such as streptomycin sulphate might be added to buffer solutions

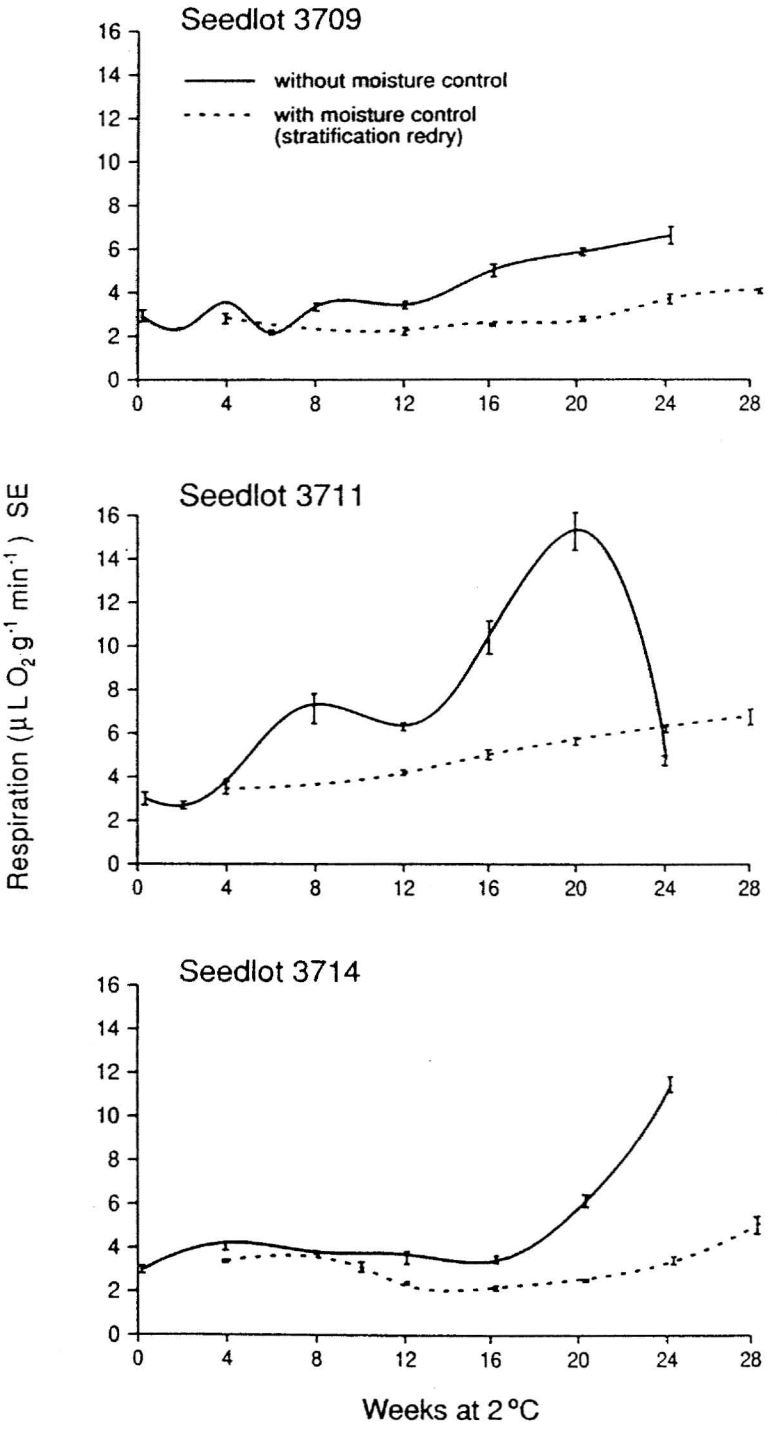

Figure 3. Respiration of *Abies lasiocarpa* seeds during stratification, with moisture control (stratification-redry) and without (from Leadem 1989). Data are means of three replications of 10 seeds, and standard error bars have been given for each data point. All measurements were made at 30°C.

Table 8. Effects of sterilization and coat removal on respiration (μL O_2/min/g dry weight) at 30°C of *Abies lasiocarpa* seeds.

	Sterilized		Not sterilized	
Treatment	+ Coat	- Coat	+ Coat	- Coat
Seedlot 3414				
dry seeds	0.86b	3.64b	0.88c	2.68c
48 h soak	2.74a	5.80a	3.40a	7.75a
8-wk strat	2.47a	7.46a	2.85ab	6.46b
strat-redry	2.63a	7.20a	2.49b	5.88b
Seedlot 3417				
dry seeds	1.06c	4.75b	1.14c	1.72b
48 h soak	3.56a	7.60a	3.45a	8.10a
8-wk strat	2.23b	7.08a	2.48b	6.22a
strat-redry	1.91bc	7.76a	2.80ab	6.84a

Means with the same letter in the seedlot and column are not statistically different at P = 0.05 when evaluated by Duncan's (1965) multiple range test.

Table 9. Effects of seed structures on respiration (μL O_2/min/g dry weight) of *Pinus monticola* seeds.

Treatment (mg)	Resp. at 30°C	Dry
dry seeds	0.3	—
48 h soak, with coat	1.8	110.5
48 h soak, w/o coat	3.1	—
48 h soak, gametophyte	2.0	89.6
48 h soak, embryo	14.3	10.7

(Murphy and Noland 1982b) if no phytotoxicity to seeds is demonstrated.

Partitioning of respiration between embryo and megagametophyte

As observed previously, respiration increased substantially once seeds were imbibed (Table 9). Removal of the seed coat resulted in an additional increase in respiration. Since seed coats are relatively impermeable to gases, increased oxygen availability usually results in higher respiration when radicles emerge or seed coats are removed (Côme and Tissaoui 1973; Bewley and Black 1978).

Rates observed after separating the megagametophyte and embryo tissue showed that respiration of the megagametophytic tissue accounted for the major portion of total seed respiration. This is reasonable since the megagametophyte tissue comprises about 90% by weight of total seed tissue (Table 9). Although embryos respire at high rates, they contribute relatively little to total respiration because the embryos represent only a small proportion of the total seed mass.

Application of respiratory measurements

Judging from the preceding evidence, respiration in conifer seeds appears to vary in a predictable manner during different stages of germination, in much the same manner observed in seeds of angiosperms (Bewley and Black 1978). This is an important point, because knowledge of basic respiratory patterns could prove to be very beneficial to furthering our understanding of tree seed physiology. Once familiar with the respiration rates typical of a given development stage, deviations from usual respiratory activity might be used to ascertain the effects of experimental treatments on basic metabolism. Respiratory monitoring might also be used to differentiate between good and poor vigour seeds. Since seeds generate energy for growth and development by respiring stored reserves (Bewley and Black 1982; Ross 1984), it is reasonable to assume that the rate at which seeds utilize reserves indicates, to some degree, their physiological vigour. The following examples illustrate how monitoring respiration may clarify some relationships between essential physiological processes and seed performance.

The primary purpose of the experiment was to test the effects of several stratification treatments on germination (Fig. 2) of *Abies lasiocarpa*, but respiration (Fig. 3) was also measured at various times during the prolonged stratification period. In seedlot 3714, a good-quality but dormant lot, respiration remained low in seeds in which moisture was controlled throughout the chilling period (stratification-redry treatment). Without moisture

control (>45% moisture content), respiration was initially low, but after about 16 weeks, rates increased exponentially.

In the low-germinating seedlot 3711, respiration was relatively low for the first 4 weeks, but seeds stratified at >45% moisture content respired at increasingly higher rates when treatment continued beyond 4 weeks. In comparison to the seeds at high moisture content, the respiration rates of seeds which had received stratification-redry treatment maintained much lower rates throughout the chilling period.

Examination of germination data (Fig. 2) shows that treatments in which respiration remained low (i.e., stratification-redry) during stratification were associated with high germination. Dormant species such as the true firs require long stratification periods to achieve optimal performance (Leadem 1986), yet the data (Fig. 3) indicate that if seed moisture is kept at high levels, respiration tends to rise with increasing time of chilling at 2°C. It appears that high rates of respiration during stratification may be detrimental to subsequent seed performance, possibly because reserves respired during stratification will not be available for the developing seedling during germination. If, however, stored products are slowly respired during stratification, more energy supplies will be available for the critical emergence and establishment period. Some support for this view was recently given in a study of sugar pine (*Pinus lambertiana* L.) seeds in which high seed respiration rates were shown to be related to poor germination at higher temperatures (Murphy and Noland 1982 a,b).

There is also some evidence that different levels of respiratory activity may signal changes in dormancy status. If respiration rates in Figure 3 are examined in reference to the germination data in Figure 2, it can be seen that the rise in respiration at approximately 16 weeks coincides with the length of stratification necessary to overcome the dormancy of the seedlot. Considering the increase in respiration usually associated with the onset of the final stage of germination, it is possible that this observed rise in respiration may be related to the breaking of seed dormancy, and may presage imminent radicle emergence.

The ability to identify when the breaking of dormancy occurs could prove to be a valuable physiological assessment technique. Usually, stratification requirements are determined by subjecting seeds to a series of chilling times, and then incubating the seeds under favourable conditions for germination. Such testing generally takes at least 3-4 weeks. Employing seed respiration as an alternative means of assessing performance would greatly simplify the search for optimal stratification treatments. Respiration can be easily monitored throughout the treatment period, and with the Clark O_2 electrode, determinations can be obtained in as little as 5-10 minutes.

Another potential application is the systematic monitoring of tree seeds retained in long-term storage. Observation of higher than usual rates of respiration rates may signal that seeds are beginning to deteriorate, and possibly indicate that seeds should be discarded or sown before further declines in vigour occur.

A final word of caution may be in order here. The interpretation of respiration data in the context of the physiological condition of the seeds is essential, for high rates of respiration do not necessarily imply high energy capture. If respiration is impaired for some reason, energy capture will occur at less than maximum capacity, even though oxygen consumption is high. Under some conditions, respiration may be less efficient because electrons are transported via the alternate (cyanide-insensitive) pathway in which energy is not captured in ATP or NADH (Yentur and Leopold 1976; Murphy and Noland 1982b).

Summary

Seed respiration measurements with the Clark O_2 electrode made on seeds of *Abies lasiocarpa*, *Pinus monticola*, and *Pseudotsuga menziesii* revealed that, in all three species, respiration followed the general three-stage process noted for angiosperm seeds (Côme and Thévenot 1982) i.e., a rapid increase in respiration soon after imbibition, followed by a period in which rates remain relatively constant, and another increase at the time of radicle emergence.

The Clark O_2 electrode has been demonstrated to be suitable for measuring respiration of tree seeds under a variety of conditions, and good possibilities exist for diagnostic applications. Measurements are obtained within 5-10 minutes, and the technique can be learned with minimum training. The greatest advantage, of course, is that the technique employs a physiological basis for assessment with which to utilize and expand our understanding of seed metabolism in practical applications.

Acknowledgements

The capable assistance of Sue Baker and Joanne Clark is gratefully acknowledged. The research reported in this paper was partially funded under the Canada-British Columbia Federal Provincial Resource Development Agreement.

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Delayed germination and seedling emergence of *Pinus albicaulis* in a high elevation clearcut in Montana, U.S.A.

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Abstract

In a field study of whitebark pine (*Pinus albicaulis* Engelm.), delayed germination was examined by comparing seedling emergence after three growing seasons under three light levels (0% - open, 25% and 50% shade cover), two seedbed conditions (mineral and litter), and two sowing depths (surface sown and buried 2-4 cm beneath surface). Results show that delayed germination of whitebark pine occurs for up to 3 yr following sowing and is influenced by microsite factors. Emergence from buried seeds was 11% the first year, 45% the second yr, and 11% the third yr. Emergence from surface-sown seeds was 1% the first yr and 6% the second yr, with no germination occurring in the third season after sowing. Cumulative emergence after 3 years was higher on 25% and 50% shaded plots than on non-shaded plots for surface-sown and buried seeds. Mineral seedbeds showed significantly higher cumulative emergence from surface-sown seeds than did litter seedbeds. Litter seedbeds had slightly higher cumulative emergence from buried seeds than did mineral seedbeds.

Résumé

Dans une étude sur le terrain qui portait sur le pin albicaule (*Pinus albicaulis* Engelm.), la germination retardée a été examinée en comparant l'émergence des semis après 3 saisons de croissance sous 3 niveaux d'éclairement (couvert offrant 0 (ouvert), 25 et 50 % d'ombre), dans 2 types de lits de germination (minéral et litière), dans 2 conditions d'ensemencement des graines (en surface et enfoncées de 2 à 4 cm). Les résultats montrent que la germination retardée du pin albicaule se produit jusqu'à 3 ans après l'ensemencement et qu'elle est influencée par des facteurs observés sur les microsites. L'émergence des graines enfouies a été de 11 % la première année, de 45 % la deuxième et de 11 % la troisième. L'émergence des graines déposées en surface a été de 1 % la première année, de 6 % la deuxième et d'aucune germination la troisième année et après l'ensemencement. L'émergence cumulative au bout de 3 ans a été supérieure dans les parcelles ombragées à 25 % et à 50 % à ce qu'elle était dans les parcelles non ombragées, dans les 2 conditions d'ensemencement. Avec les graines semées à la surface, les lits de germination minéraux ont donné une émergence cumulative significativement supérieure à celle des lits constitués de litière. Chez ces derniers, on a observé une émergence cumulative légèrement supérieure à celle des lits minéraux dans le cas des graines enfouies.

Introduction

Prior to the early 1980's little was known about the basic biology of whitebark pine (McCaughey and Schmidt 1990). Interest in this high elevation pine increased dramatically after whitebark seeds were found to be an important food source for grizzly bear (*Ursus arctos horribilis*) (Craighead *et al.* 1982; Kendall 1983; Knight *et al.* 1987). Information is limited on germination and establishment rates for whitebark pine under natural conditions (Eggers 1986). Most whitebark pine studies have only reported first-year emergence results, with little attention to delayed emergence and actual establishment (McCaughey and Weaver 1990).

Delayed emergence of broadleaf tree species is a common phenomenon (Marquis 1975), but appears to occur in considerably fewer conifer species. Several North American pines such as jack pine (*Pinus banksiana* Lamb.), eastern white pine (*Pinus strobus* L.), and western white pine (*Pinus monticola* Dougl. ex D. Don). have been shown to possess varying degrees of delayed emergence (Haig *et al.* 1941; Thomas and Wein 1985). *Pinus cembra* L., a European stone pine similar to whitebark pine, can delay emergence for up to 3 years (Krugman and Jenkinson 1974).

Managers are becoming more interested in reforesting whitebark pine through artificial (planting and seeding) and natural regeneration methods. Pitel (1981) and Pitel and Wang (1990) have reported on some of the mechanical methods for breaking dormancy of whitebark seeds. This information is being used by nurseries for producing planting stock. But information on delayed emergence is needed to accurately assess the emergence capabilities of whitebark pine if seed planting is to be used as a reforestation method.

This paper reports the results of a study of delayed germination and emergence of whitebark pine after 3 years under field conditions. The objectives were:

i) to determine how long after sowing emergence occurs;
ii) to compare cumulative emergence between surface-sown seeds and seeds buried 2-4 cm under the surface level; and
iii) to compare cumulative emergence between shading (0%, 25%, and 50% shading) and seedbed (mineral and litter) treatments.

Study area and methods

The study area was in the Gallatin National Forest in south central Montana, 3 km north of Yellowstone National Park and near the southwestern corner of the Absaroka Beartooth Wilderness. The study site was on the northeast aspect of a high ridge at an elevation of 2652 m MSL, approximately 9 km east of Gardiner, Montana, U.S.A..

Study plots were established within a 6 ha clearcut on Forest Service land on the west side of a 20 ha clearcut on adjoining private property. The study area is bordered by mature timber of similar composition on its south, west, and north sides. Both clearcuts were harvested during the winter of 1985-86. The species and proportions of volume harvested were: live lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) 75%, dead lodgepole 13%, Engelmann spruce (*Picea engelmannii* Parry [Engelm.]) 4%, subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) 4%, and whitebark pine 4%.

The experimental study area is classified as an *Abies lasiocarpa-Pinus albicaulis/Vaccinium scoparium* habitat type (Pfister *et al.* 1977). Its soils are classified as Typic Cryorthent, sandy skeletal, and well drained, occurring on slopes of 0% to 25%. Soil pH values range from 4.7 to 5.5.

Table 1. Whitebark pine study design factors and factor levels for each sowing depth (surface-sown and seeds buried 2-4 cm beneath the surface.

Factor	Levels
1. Shade level	3
a. 0% shade	
b. 25% shade	
c. 50% shade	
2. Seedbed condition	2
a. Mineral	
b. Litter	
3. Replication	3

Study Design

A factorial design (Table 1) was used to evaluate the effects of microsite factors (light levels, seedbed conditions, and replications) on delayed emergence of surface-sown and buried (2-4 cm beneath the surface) seeds of whitebark pine. Three block replications were subjectively chosen within the 6 ha clearcut as representative, similar, and suitable for plot establishment. The replications had minimal amounts of logging slash, large areas of undisturbed litter, and were representative of the study area. Plots were randomly located in each replication to sample each shade level, seedbed condition, and sowing depth combination. Mineral seedbed plots were located on scarified skid trails or hand-scalped areas when scarified areas could not be found.

Plot Layout

Within each replication 12 plots were randomly established to represent all combinations of three shade levels (0%, 25%, and 50%), two seedbed conditions (mineral and litter), and two sowing depths (surface sown and buried 2-4 cm beneath the surface). Plots were rectangular (0.5 x 1 m) and oriented north-south.

Each plot was subdivided into 40 subplots measuring 9.9 x 10.9 cm. Within each subplot two seeds were planted, one surface sown and one buried. The surface-sown seeds were placed on the ground surface in the north half of each subplot. The buried seeds were placed 2-4 cm below the surface in the south half of each subplot and was covered by the appropriate seedbed material (mineral soil or forest litter).

A total of 2880 whitebark pine seeds were sown in 1987. Seeds had been collected in 1985 from trees 0.4 km away from the study area and at the same elevation as the study area. All seeds were x-rayed, and only filled seeds were sown.

Shade levels were imposed with slatted roofs. Four 1.9 m-tall steel posts were installed at the corners of an imaginary 1.2 x 2.4 m rectangle overtopping but slightly to the south of each plot to be shaded. A 1.2 x 2.4 m-long wooden frame was constructed with 5.1 x 10.2 m lumber and attached to the steel posts 1.2 m above the ground. A 1.2 x 2.4 m section of wood snow fence was suspended on the wood frame. The 50% and 25% shade levels were simulated by leaving all the wood slats in the snow fence or eliminating every other slat, respectively.

Screen wire (0.6 cm hardware cloth) was used to enclose each plot to exclude seed predators. The bottom edges of the screens were buried 10-15 cm deep with a 5 cm lip bent outward to discourage rodents from tunneling under. Screening techniques were suggested by Curt Halverson, Fish and Wildlife Service, U.S. Department of the Interior, Fort Collins, Colorado, U.S.A.

Measurements

Counts of whitebark pine seedlings were made periodically on all plots during the summers of 1988, 1989, and 1990, and were recorded weekly until August, and twice monthly from August through September. Seedlings were marked with colored plastic toothpicks to indicate the week they emerged. Total seedling counts for each year were summarized by treatment combination.

Data Analysis

The proportion of whitebark seedlings on each subplot was used as the dependent variable for analysis of emergence differences between shade levels, seedbed conditions, sowing depth, and factor interactions. Proportion of emergence was defined as the number of emergents divided by the number of seeds sown. Plots with no seedlings were transformed by the formula [1/(4n) n=40] to prevent distortion of the analysis by zero proportion values (Mosteller and Youtz 1961). The transformation arc sine of the square root of the germination percentage was used to stabilize variation due to proportions (Snedecor and Cochran 1980).

Analysis of variance (ANOVA) procedures of the SAS statistical analysis software (SAS Institute 1987) were used to analyze whitebark pine emergence data. Analyses were conducted independently for buried and surface-sown seeds. ANOVA was used to test for significance of main factors (shade levels, sowing depth, and replicates) and their interactions on seedling emergence. The Ryan-Einot-Gabriel-Welsch multiple F test was used to statistically test differences in emergence among factor levels. Statistical significance was assumed at the alpha = 0.05 level.

Table 2. Percentage seedling emergence of whitebark pine for surface-sown, buried, and total (surface-sown + buried) seeds in the first, second, and third year after sowing. Percentage emergence for each year is based on the number of seeds that had not previously emerged. The three year total represents a cumulative, not an additive, value.

	Emergence			
	First year	Second year	Third year	Three year total
	percent			
Surface sown	1	6	0	7
Buried	11	45	11	56
Total	6	25	6	31

Table 3. ANOVA table for effect of shade cover, seedbed condition, and replication on emergence of surface-sown whitebark pine seeds.

Factor	df	SS	MS	F	Pr > F
Shade cover	2	0.0745	0.0372	2.71	0.0826
Seedbed condition	1	0.3841	0.3841	27.99	0.0001
Replicate	2	0.0337	0.0169	1.23	0.3072
Error	30	0.4117	0.0137		
Total	35	0.9040			

Table 4. Percentage emergence of surface-sown whitebark pine seeds as affected by shade cover and seedbed condition for each of the first 3 years following sowing and the 3-year total. Percentage emergence for each year is based on the number of seeds that had not previously emerged. The three year total represents a cumulative, not an additive, value.

		Emergence			
Factor	Factor level	First year	Second year	Third year	Three year total
		percent			
Shade cover (%)	0	<1	3	0	3a[1]
	25	2	6	0	8a
	50	1	9	0	9a
Seedbed condition	Mineral	2	10	0	12a
	Litter	0	2	0	2b

[1]Totals followed by the same initial are not significantly different ($\alpha = 0.05$).

Table 5. ANOVA table for effect of shade cover, seedbed condition, and replication on emergence of buried whitebark pine seeds.

Factor	df	SS	MS	F	Pr > F
Shade cover	2	0.1802	0.0901	2.49	0.0996
Seedbed condition	1	0.0944	0.0944	2.61	0.1166
Replicate	2	0.2750	0.1375	3.80	0.0337
Error	30	1.0844	0.0361		
Total	35	1.6340			

Table 6. Percentage emergence of buried whitebark pine seeds as affected by shade cover and seedbed condition for each of the first 3 years following sowing and the 3-year total. Percent emergence for each year is based on the number of seeds that had not previously emerged. The three year total represents a cumulative, not an additive, value.

		Emergence			
Factor	Factor level	First year	Second year	Third year	Three year Total
		percent			
Shade cover (%)	0	10	39	5	47a[1]
	25	13	51	14	61a
	50	12	46	15	60a
Seedbed condition	Mineral	13	40	7	51a
	Litter	9	50	15	60a
Replication	1	14	35	8	48a
	2	16	52	21	65b
	3	4	49	2	53ab

[1]Totals followed by the same initial are not significantly different ($\alpha = 0.05$).

Results

Over 30% of all whitebark pine seeds sown had emerged by the end of the third growing season, but emergence varied greatly among study factors (Table 2). Emergence was over 55% for buried seeds and less than 7% for surface-sown seeds. Emergence occurred over a 3-yr period for buried seeds but ceased after the second growing season for surface-sown seeds.

There were no significant differences between replications or factor interactions for surface-sown seeds. Seedbed condition was the only significant factor affecting emergence of surface-sown seeds of whitebark pine (Table 3). Emergence on mineral seedbeds (12%) was significantly higher than on litter seedbeds (2%)(Table 4). Although not statistically significant, emergence tended to be higher with increased shade (Table 4).

ANOVA showed that microsite factors did not significantly affect emergence of buried whitebark pine seeds (Table 5); there were no significant differences in emergence within the shade and seedbed treatments. However, emergence was numerically higher on shaded treatments and on litter treatments (Table 6). The only significant differences in emergence for buried seeds occurred between replications. There were no significant interactions for buried seeds.

Discussion and conclusions

Emergence of buried whitebark seeds can be expected to occur for up to three growing seasons after sowing. Field studies have shown that rodents consume all surface sown-seeds, and because counts of first-year emergence were low it was assumed that a high proportion of buried seeds were consumed when accessible to natural predation (McCaughey and Weaver 1990; McCaughey 1990). The proportion of buried seeds eaten by rodents may be less than first believed, and burying (spot seeding) whitebark seeds may be a viable reforestation option.

Emergence of buried whitebark seeds was not significantly higher on shaded plots. However, there appears to be a trend that emergence may be enhanced by increasing shade cover. Managers can increase shade levels through silvicultural systems such as partial, shelterwood, and seed tree cutting or by planting seeds on the north side of shade sources.

Reducing surface scarification will increase emergence of human- and nutcracker-planted whitebark seeds. Increasing the amount of undisturbed ground in harvested areas may heighten the probability of the Clark's nutcrackers (*Nucifraga columbiana*) caching within litter substrate. The nutcracker extracts seeds from whitebark cones, stores them in a sublingual pouch, and then carries them up to 23 km from the seed source, although typical caching sites are close to the harvest site (Hutchins and Lanner 1982; Tomback 1978; Vander Wall and Balda 1977). Nutcrackers cache groups of 1 to 25 seeds, 2-4 cm deep, on a variety of sites. No specific type of cache site is favored over others, and cache sites occur on both well-drained and moist substrate, including soil, forest litter, gravel rubble, cracks and fissures on exposed rock, and pumice (Hutchins and Lanner 1982; Tomback 1978, 1982).

It is not known why cumulative emergence from buried seeds differed significantly between replications after 3 years. It was speculated that the low emergence in replication 1 was due to below-average precipitation (82% of normal) in 1988 (first year) and a higher percentage sand content of the soils there than in the other replicates (McCaughey and Weaver 1990). Precipitation was average in 1989 (second year), which probably reduced the drought influence of the high sand proportion. In 1990 (third year) precipitation was 90% of normal which, along with a high sand content, may have again contributed to the low emergence in replication 1. Percentage sand content of each replication was not known until after first-year emergence was evaluated. Soil samples were collected and analyzed to try to explain differences in emergence between replications.

This study indicates that delays in whitebark pine germination may occur for up to 3 yr after seeds are buried. Successional models (Keane *et al.* 1989) used to simulate tree establishment of whitebark pine can be modified to account for delayed germination. Regeneration surveys should be delayed if seeding of whitebark pine is used as a reforestation tool. Further monitoring of the protected seeds in this study should yield more insight into the delayed germination phenomenon of whitebark pine.

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SDS-PAGE and Western blot analysis of storage protein mobilization following germination of Douglas-fir seeds

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Abstract

The storage protein composition of isolated protein bodies of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seeds was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot analysis. Crystalloids, the major storage proteins, constitute up to 86% of the storage reserve in the seeds. These proteins, in their non-reduced form, have molecular mass of 55-63 kD and in their reduced form migrate as two distinct groups of proteins in the molecular mass range of 32-35 and 20-23 kD. The soluble protein gel profiles show that a group of proteins in the 45-47 kD molecular mass region are the major proteins. Each group of proteins is mobilized upon seed germination. Mobilization of matrix proteins is rapid between 4-6 days whereas the crystalloids show a more gradual decline over the course of seed germination.

Résumé

La composition des protéines de réserve dans les masses protéiniques isolées dans les graines du douglas taxifolié (*Pseudotsuga menziesii* [Mirb.]) a été étudiée par électrophorèse de polyacrylamide en présence de sulfate de sodium dodécylique et par la méthode de transfert Western. Les cristalloïdes, principales protéines de réserve, constituent jusqu'à 86 % de la réserve des graines. Dans leur forme non réduite, ces protéines ont une masse moléculaire de 55 à 63 kda, et dans leur forme réduite elles migrent en deux groupes distincts de protéines dont les plages de masses moléculaires sont de 32 à 35 et de 20 à 23 kda. Les profils sur gel des protéines solubles montrent qu'un groupe de protéines dont la masse moléculaire les situe dans la région de 45 à 47 kda constituent les principales protéines. Tous les groupes de protéines sont mis à contribution au moment de la germination de la graine. L'utilisation des protéines de la matrice se fait rapidement, entre 4 et 6 jours, alors que la concentration des cristalloïdes diminue d'une manière plus progressive à mesure que se fait la germination de la graine.

Introduction

Storage organs of seeds synthesize large amounts of storage reserves during development and sequester them in the storage organelles (Bewley and Black, 1985). Following germination these reserves are rapidly mobilized providing a source of carbon and nitrogen for subsequent growth and development of the seedling. In angiosperms, the synthesis and degradation of storage reserves has been studied extensively and during the past several years a great deal of information has accumulated on the developmental regulation of gene activity during embryogenesis (Goldberg *et al.* 1989; Crouch and Sussex 1981; Dure and Galeu 1981; Higgins 1984) and seedling development (Harada *et al.* 1988; Comai *et al.* 1989; Smith and Leaver 1986; Turley and Trelease 1987; Allen *et al.* 1988). In contrast, the biochemical and molecular aspects of seed development and germination of gymnosperms remain largely unknown. We have recently initiated a research program aimed at elucidating the molecular mechanisms of gene regulation in haploid megagametophytic tissue as well as diploid zygotic and somatic embryos of white spruce (*Picea glauca* (Moench) Voss) (Misra and Green 1990, 1991) and Douglas-fir (Green *et al.* 1991). In this report we present characterization and mobilization of storage proteins during germination of Douglas-fir seeds. We also show that mobilization of storage proteins occurred following incubation of the stratified seeds. Matrix proteins were rapidly mobilized between 4-6 d followed by a gradual decline in crystalloid proteins during the course of germination.

Materials and methods

Plant Material

The Douglas-fir seeds used in this study were obtained from the Ministry of Forests (coastal Douglas-fir seed lot #3356). For germination experiments, seeds were stratified at 4°C for 2 wk within a sealed plastic bag. Stratified seeds were germinated on Whatman 3 mm filter paper with day/night temperatures of 30°C/20°C and 8 h light period. Germinated seeds were harvested after 2, 4, 6 and 8 d of incubation.

Protein Extractions and Sodium dodecyl sulphate (SDS)-PAGE

Protein extractions were carried out under non-reducing conditions at 4°C. All extraction buffers contained the protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and 10 μM leupeptin (Sigma). Fractionation of proteins into soluble matrix and insoluble crystalloids was carried out according to Misra and Green (1990). Briefly, soluble matrix proteins were extracted using 0.05 M sodium phosphate buffer (pH 7.5). Insoluble crystalloid proteins were solubilized by boiling the pellet for 5 min in 65 mM Tris-buffer (pH 6.8), containing 2% SDS and 10% glycerol. Extractions were performed using a tissue weight to buffer volume ratio of 100 mg:1 mL.

SDS-PAGE of proteins was performed according to the method of Laemmli (1970) with minor modifications. Prior to electrophoresis, extracts of both soluble (matrix) and insoluble (crystalloid) proteins were boiled for 5 min in sample buffer (65 mM Tris-buffer (pH. 6.8), 2% (w/v) SDS, 10% (w/v) glycerol) and when reducing conditions were required, 5% (v/v) βMSH. Electrophoresis was carried out using mini gels (Bio-Rad mini Protean II gel apparatus.) Approximately 2.5 μg total protein was loaded per lane.

The Bradford protein assay (Bradford 1976) was used for all protein determinations using Coomassie Brilliant Blue G250 (Serva) (Misra and Green 1990). After SDS-PAGE the gels were stained for total protein with Coomassie Brilliant Blue R250 (Serva) according to the method of Weber and Osborn (1969). Molecular weight protein standards (Bio-Rad) used were phosphorylase B, 97.4 kD; bovine serum albumin, 66.2 kD; ovalbumin, 43.7 kD; carbonic anhydrase, 31 kD; trypsin inhibitor, 21.5 kD; and lysozyme, 14.4 kD.

Preparation of Antigen/Antiserum

The electrophoretically purified SDS-Dissociated non-reduced crystalloid protein complexes with the apparent molecular weights of 43 and 57 kD were collectively used as antigen.

SDS-solubilized crude protein extracts of Douglas-fir crystalloids were electrophoretically separated in 1.0 mm 12% SDS-polyacrylamide gels under non-reducing conditions. The gels were equilibrated for 20 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 0.02T (w/v) SDS, 20% (v/v methanol) (Hurkmann and Tanaka 1987). Proteins were transferred to nitrocellulose membranes (pore size 0.45 mm; Schleicher and Schuel) using a mini-Trans-Blot Cell (Bio-Rad) for 1.1 hours at 100 volts.

With prestained molecular weight standards (Bio-Rad) as markers, the entire region where the 43 and 57 kD complexes were expected to be transferred was cut out of the membrane. Transfer efficiency was evaluated by staining the blotted gel with Coomassie Brilliant Blue R250 and the remaining (non-excised) membrane with Amido Black (0.05% [w/v] Amido Black, 10% [v/v] acetic acid, 10% [v/v] isopropanol; destain: 10% [v/v] acetic acid, 10% [v/v] isopropanol).

The excised nitrocellulose membrane was rinsed with dH_20 for 5 min to remove residual SDS and stored at -20°C. The membrane was pulverized to a fine powder using a mortar and pestle on dry ice. The ground membrane was resuspended in 1.5 ml PBS (10 mM sodium phosphate buffer, 0.85% [w/v] NaCl, pH 7.4), emulsified in 1.8 ml Freund's complete (for priming, i.e. first injection) or incomplete (for boosting) adjuvant and injected subcutaneously and intramuscularly into a New Zealand white male rabbit. Approximately 100 μg of blotted antigen was used for each priming and boosting session.

After priming, two boostings were performed using antigen prepared as above with injections spaced 30 d apart. Antiserum titers were checked 2-3 wk after each boosting using blood serum from ear bleeds and an enzyme-linked immunoabsorbent assay (ELISA). The ELISA was performed using the standard method (Engvall and Perlmann 1971) except that the antigen was diluted in PBS/0.1% [v/v] SDS and dried onto the wells. Immunodetection was done using the horse-radish peroxidase/ABTS conjugated enzyme/substrate system. Blood serum was collected by cardiac puncture 2 wk after the last test bleed (or 15 wk after priming). Blood was obtained by exposing the whole blood to air at RT for 1 h or until clotted, left overnight at 4°C, centrifuged at 1,000 rpm (800 x g), 15 min, and then the serum carefully drawn off. Serum was preserved with thimerosal (0.01% [w/v] (Sigma) and stored in small volumes at –70°C.

Western Blot (immunoblot) Analysis of Storage Protein Profiles

Polypeptides separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes at 100 v for 1.1 h using a mini-Trans-Blot Cell (Bio-Rad). The membranes were blocked with 1% [w/v] BSA, 0.5% [v/v] Tween 20 in PBS, pH 7.4, for 1 h at RT or overnight at 4°C with gentle shaking. Membranes were incubated with antiserum against the Douglas-fir dissociated crystalloid protein complex for 2 h at RT. Antiserum was diluted 1:5,000 in first antibody buffer (10% [v/v] bovine calf serum, 10% [v/v] glycerol, 1 M D-glucose, 0.5% [v/v] Tween 20 in PBS, pH 7.4). After washing 3 x 15 min with PBS/0.5% [v/v] Tween 20 the membranes were incubated for 1 h at RT with gentle shaking with goat

anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (AP) (Tago Inc., Burlingame, California) using a 1:4,000 dilution in blocking buffer. After washing (as above) the membranes were stained in the following manner: membranes were washed in AP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) then stained with nitro-blue tetrazolium (0.33 mg/mL; sigma) and 5-bromo-4-chloro-3-indolyl-phosphate (0.165 mg/mL; Boehringer Mannheim) in AP buffer without shaking. Colour development was halted by washing the membranes in stop solution (20 mM Tris pH 7.5, 5 mM EDTA) followed by running tap water.

Results and discussion

Ultrastructural observations of sections of the mature seeds of Douglas-fir has shown the presence of numerous lipid and protein bodies (Green *et al.* 1991). To analyze the protein composition, isolated protein bodies were extracted for the buffer-soluble (matrix) and the SDS-soluble (crystalloid) proteins and their electrophoretic pattern determined by SDS-PAGE analysis. The Coomassie Blue stain profiles obtained by separating these proteins in their non-reduced (–ME) and reduced (+ME) forms are shown in Figure 1 A-B. In the absence of ME, the SDS-soluble proteins comprise a major group of proteins with molecular mass in the range of 55-63 kD (Fig. 1A, lane C). In the presence of ME, this complex gave rise to two major groups of polypeptides; one ranging in molecular mass from 32-35 kD and the other from 20-23 kD (Fig 1B, lane C). The reduced gel also shows a prominent band in the low molecular range (< 14 kD) however, our analysis show that this is not a product of the 55-63 kD non-reduced protein complex. In the soluble protein fraction (Fig. 1A-B, lane M), the most abundant group of proteins have molecular mass of 45-47 kD (Fig. 1A, lane M). The soluble proteins do not appear to be affected by the mercaptoethanol treatment.

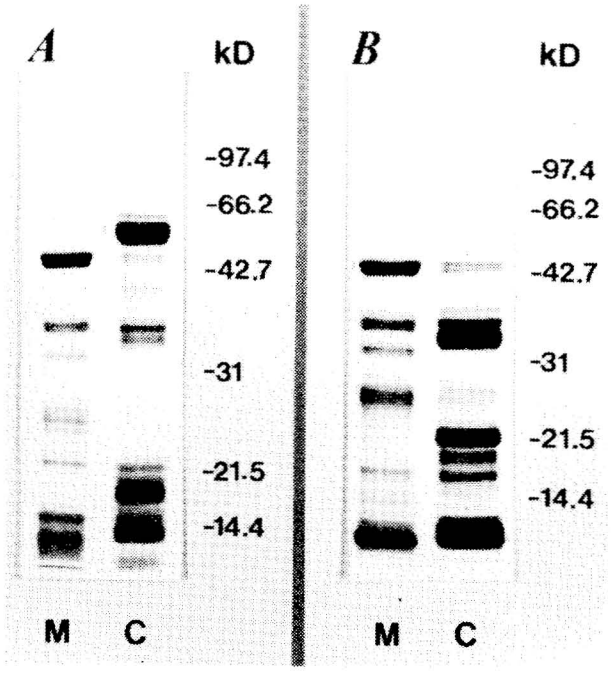

Figure 1. Coomassie blue stained SDS-PAGE profiles of proteins from isolated protein bodies of Pseudotsuga menziesii. (A), non-reducing (-ME); (B) reducing conditions (+ME). (C), insoluble crystalloid proteins, (M) soluble matrix proteins. In each case approximately 2.5 µg of insoluble or soluble protein (in 10 µl) was loaded onto 12% gels. Molecular masses in kilodaltons (kD) are shown as numerical values adjacent to protein profiles.

To determine the storage function of soluble and insoluble reserves, protein profiles were examined during germination and growth. Figures 2A and 2B show that by day 8 of germination, most of the soluble proteins had disappeared. For example, proteins of 42-45, 35-37, 32 and 22-23.5 kD disappeared after 6 d of germination. Several new polypeptides were visible in the soluble fraction, the most pronounced being a group of polypeptides in the 20-25 kD range. In the insoluble fraction, the crystalloid proteins have significantly decreased by 8 DAS (days after germination of stratified seed). The results were confirmed by Western blot analysis of the crystalloid proteins (Figure 3 A-B).

For immunodetection, proteins were first separated by SDS-PAGE and then transferred to nitrocellulose membrane by electrophoresis. Proteins were detected using an antibody specific for the Douglas-fir crystalloid protein complex. In the matrix fraction an immunoreactive band at ~ 42 kD is seen which appears to break down to form a band of ~18 kD between 2– 4 d after germination of stratified seeds. Within the crystalloid fraction the immunoreactive bands decrease steadily after 4 d. These results are consistent with storage function of these proteins, as the mobilization of these proteins coincided with radicle emergence and elongation. No changes in protein profiles were apparent between mature dry seeds and those of seeds undergoing stratification for 2 wk. In pine (Gifford *et al.* 1989; Pitel and Cheliak 1988) and in white spruce (Gifford and Tolley 1989) the rapid

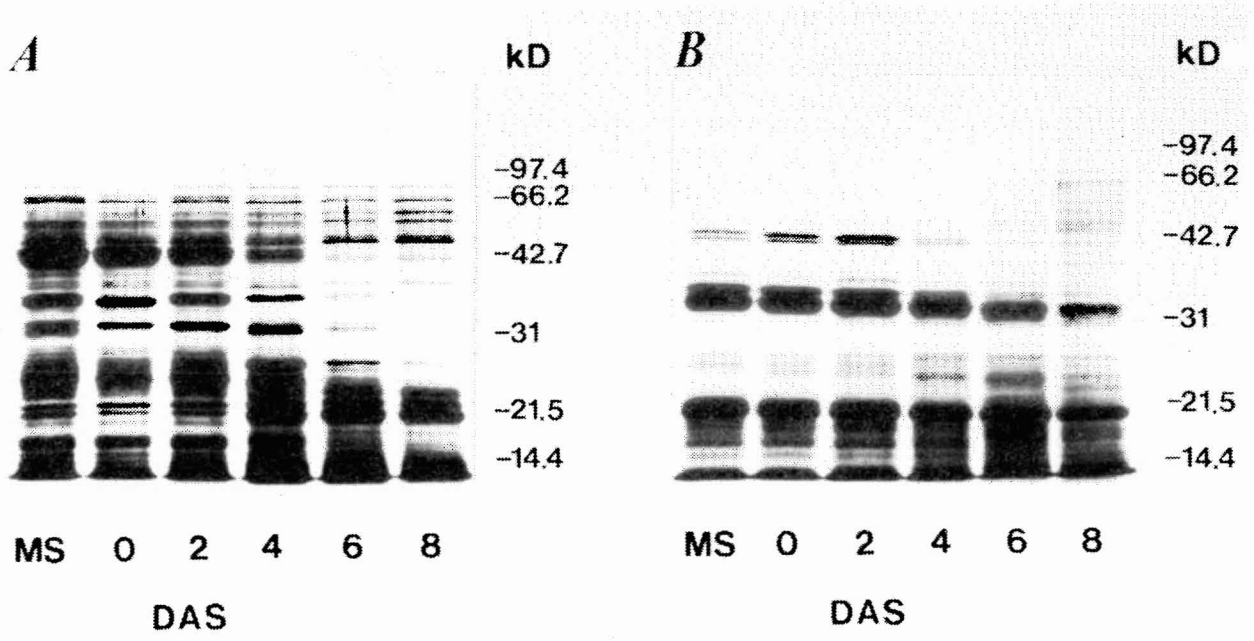

Figure 2. Coomassie blue stained gels of soluble (A) and insoluble (B) proteins extracted from germinated seeds. MS (mature dry seeds), DAS (days after germination of stratified seeds). In each case 2.5 µg of protein was applied in 10 µl. β mercaptoethanol was added to all samples. Molecular masses in kD are shown.

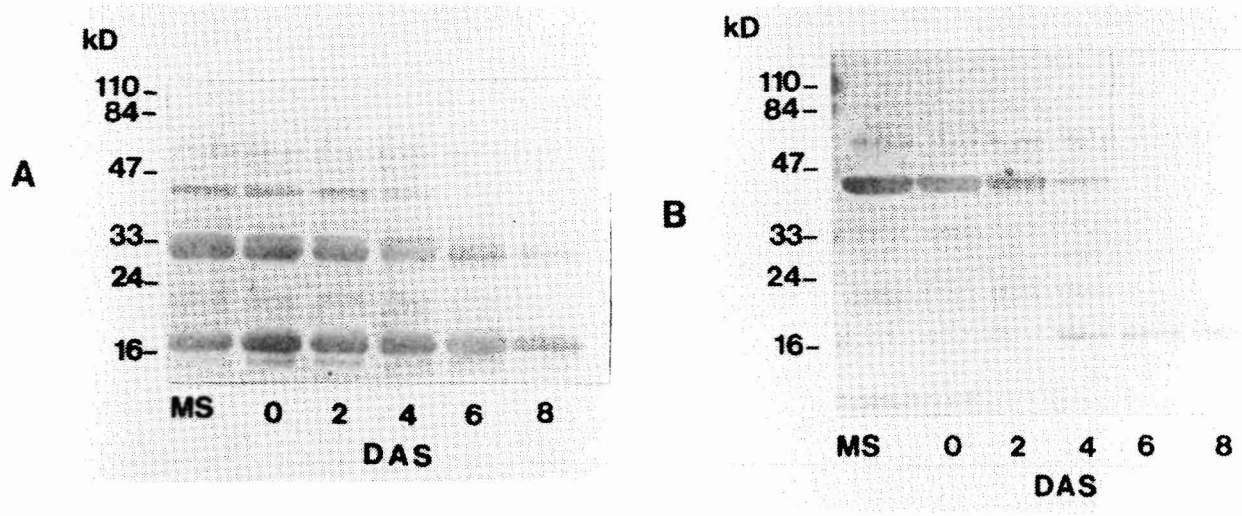

Figure 3. Immunoblot analysis of soluble (A) and insoluble (B) proteins extracted from the seeds during course of germination and separated by SDS-PAGE. The membrane was immunoblotted with antibodies against Douglas-fir non-reduced protein complex as described in Methods.

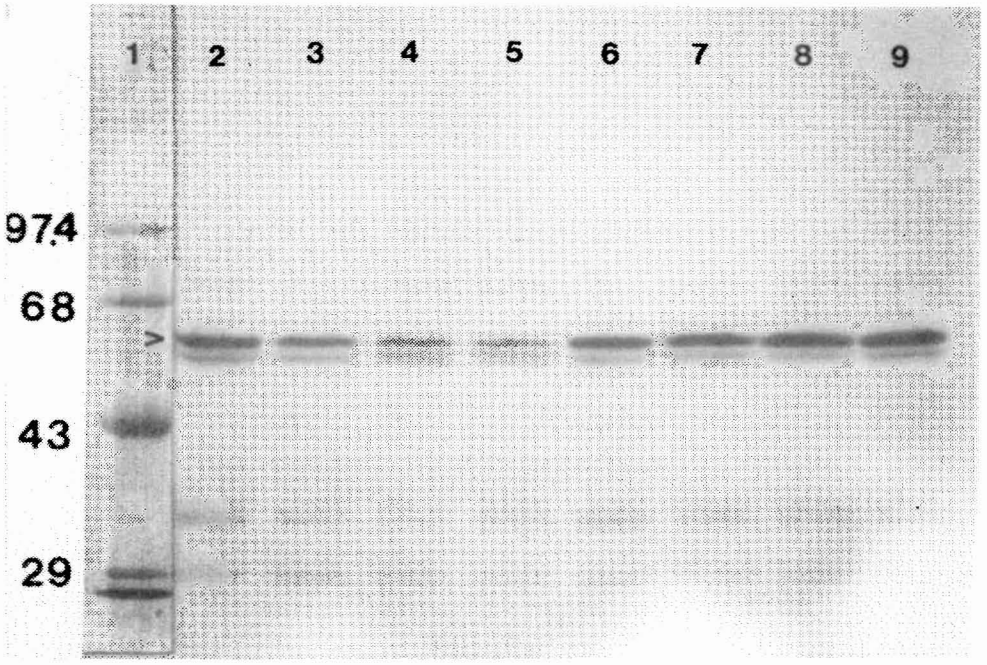

Figure 5. Immunoblot of megagametophyte extract from sugar pine seeds. Lanes: 1) MW markers; 2) dry seeds; 3-5) 25°C, days 14, 30, and 60; 6-9) 5°C, days 14, 30, 60, and 90. Arrow indicates iso-citrate lyase subunit (66 kD).

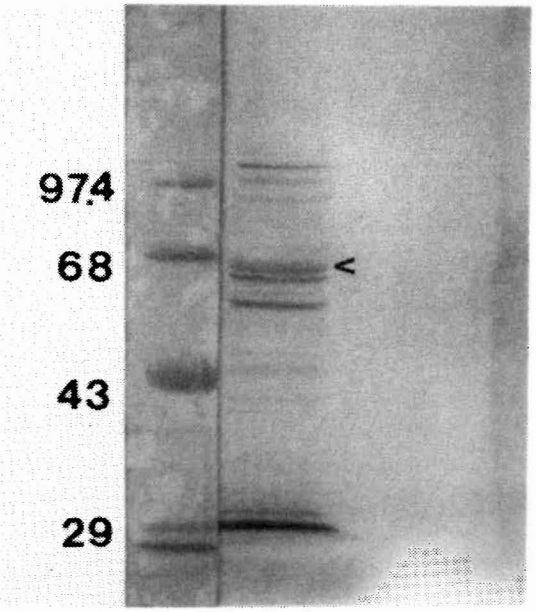

Figure 6. Immunoblot of *in vitro* translation products of poly(A)$^+$RNA from megagametophytes of sugar pine seeds which were stratified for 90 d at 5°C. Arrow indicates isocitrate lyase subunit (66 kD).

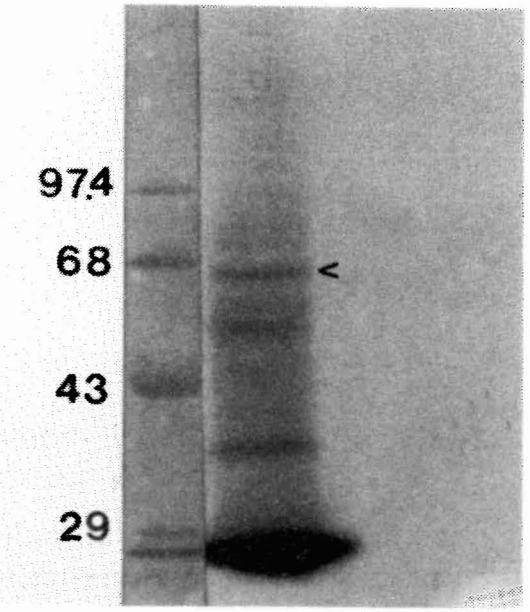

Figure 7. Autoradiograph of in vitro translation products of poly(A)$^+$RNA from megagametophytes of sugar pine seeds which were stratified for 90 d at 5°C. Arrow indicates 66 kD peptide.

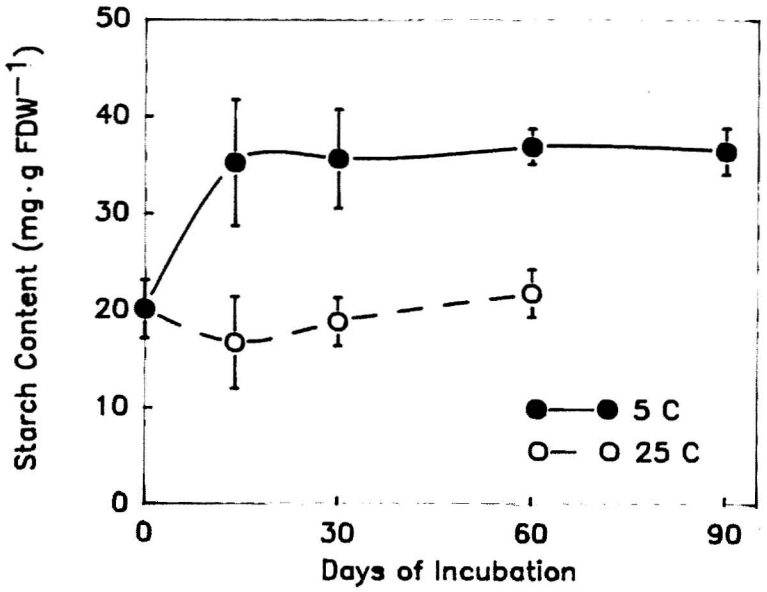

Figure 8. Starch content of embryos from sugar pine seeds stratified at 5°C or incubated at 25°C (non-dormancy-breaking). Data points represent three replications of 10 embryos each. Error bars are ± 1 s.d.

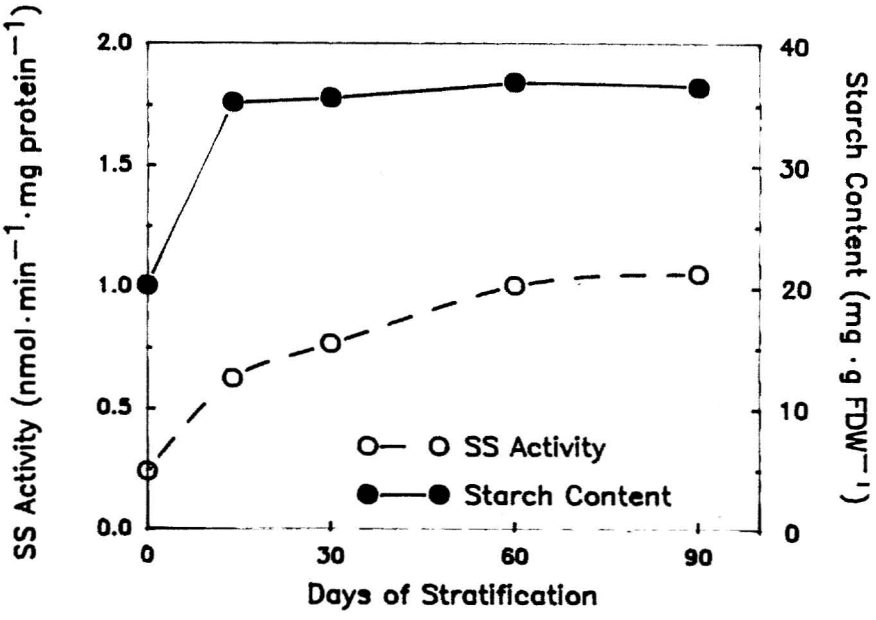

Figure 9. Starch content and sucrose synthase activity of embryos from sugar pine seeds stratified at 5°C. Data points for sucrose synthase are one replication (25 embryos) representative of the typically observed pattern. Starch data points as for Figure 8.

Conclusions

In response to stratification or a non-dormancy-breaking incubation, dormant sugar pine embryos differed in their content of poly(A)$^+$RNA and its capacity to support *in vitro* translation. This indicates that there has been an induction of gene expression in response to stratification resulting in increased mRNA content. Construction of a subtraction library based on the mRNA populations resulting from the two temperature treatments should screen for differentially expressed genes and enrich the rare messages. Library construction should be possible, as Pitel and Cheliak (1988) have successfully constructed a cDNA library from poly(A)$^+$RNA extracted from *Pinus banksiana* Lamb. seeds at various stages of germination.

Metabolic systems which appear to be induced by stratification include carbohydrate metabolism in the embryos, particularly sucrose synthase activity and starch synthesis, and lipid metabolism in the megagametophytes. The preliminary results reported here indicate that a combination of immunological and molecular approaches should provide additional information on the spatial and temporal patterns of gene expression and hopefully elucidate more clearly the complex regulatory factors involved in controlling pine seed dormancy and germination.

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Determination of seed moisture content in *Pinus contorta* L. by low resolution pulsed NMR

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Abstract

Measurement of seed moisture is often slow and destructive of the sample. Nuclear magnetic resonance (NMR) spectroscopy offers an alternative which is both rapid and non-destructive. We evaluated the performance of a low-resolution, benchtop pulsed NMR spectrometer (the Bruker MINISPEC) to determine moisture in seeds of lodgepole pine (*Pinus contorta* L.). Seeds were brought to a range of moistures by equilibrating in chambers of known relative humidities. The exact moisture contents were determined by oven-drying subsamples, and other subsamples were used to prepare a calibration curve of NMR signal per gram of sample. The NMR method proved to be rapid (less than one minute per sample), and typically used a 0.5 g sample that could be reclaimed for other uses, such as germination testing. There was close agreement between results obtained by the NMR and oven-drying methods, and accuracy and precision were completely acceptable for routine application.

Résumé

Souvent la mesure de l'humidité des graines est lente et conduit à la destruction de l'échantillon. La spectroscopie par résonnance magnétique nucléaire (RMN) constitue une solution de rechange qui est à la fois rapide et non destructive. Nous avons évalué l'efficacité d'un spectromètre RMN de table à pulsation, de faible résolution (le MINISPEC de Bruker) afin de déterminer la teneur en humidité des graines du pin tordu (*Pinus contorta* L.). On avait conféré différents niveaux d'humidité aux graines en les plaçant dans des chambres d'équilibrage dont l'humidité relative était connue. La teneur précise en humidité a été déterminée par séchage au four de sous-échantillons, et d'autres sous-échantillons ont servi à dresser la courbe d'étalonnage du signal RMN par gramme d'échantillon. La méthode par RMN s'est révélée être rapide (moins d'une minute par échantillon) et, le plus souvent, n'utilisait qu'un échantillon de 0,5 g qui pouvait servir à d'autres usages, par exemple, des essais de germination. Les résultats obtenus par RMN et par séchage au four étaient en étroite corrélation et l'exactitude ainsi que la précision étaient parfaitement acceptables en vue d'un usage courant.

Introduction

Seed moisture plays a critical role in all aspects of seed science, technology, and trade. Roberts (1973) introduced the terms orthodox and recalcitrant to distinguish between different types of seeds as regards their ability to withstand desiccation and dry storage conditions. When mature, orthodox seeds can withstand desiccation to a low moisture content (approximately 3-4% fresh wt. basis) without losing viability. Contrary to this, recalcitrant seeds loose their viability if dried below some relatively high (approximately 20% fresh wt. basis) moisture content.

Seeds of boreal coniferous tree species are orthodox and can be dried to low moisture content and subsequently stored at low temperature without losing viability for a number of years. Long-term storage of the seeds of Scots pine (*Pinus sylvestris* L.), for example, is a necessity in harsh climatic conditions in northern Finland where mature seed crops rarely occur.

Since seed longevity in storage largely depends on moisture content as well as the temperature, accurate seed moisture monitoring is needed. Current ISTA rules (International Seed Testing Association 1985) prescribe the low constant temperature oven method to be used for moisture determinations of all tree species. A number of other methods exist, including different distillation techniques, electronic meters, etc. (Bonner 1979; Grabe 1989).

Two spectroscopic techniques, nuclear magnetic resonance (NMR) and infrared reflectance (IR) offer the potential of non-destructive moisture determination. The NMR technique has been widely used for moisture determination in cereal and corn seeds (Kuzmina *et al.* 1980; Miller *et al.* 1980; Morley *et al.* 1984; Ratkovic 1987; Chambers *et al.* 1989; see also Preston and Rusk 1990). These methods may be a promising tool for measurement of seed moisture in gene banks and seed storage when limited amounts of seeds are available

and germination tests are needed for the same seeds. They are also rapid, making it possible to monitor physiological activity in seeds as a function of moisture content.

The NMR phenomenon occurs because some nuclei have an inherent magnetic moment or spin. In a magnetic field, they split into populations with different energies, the energy gap between adjacent levels being proportional to the applied field. For spin-1/2 nuclei such as ^1H there are two energy levels. At equilibrium, there is a slight excess population (determined by the Boltzmann equation) in the lower spin state, in which the magnetic moment is aligned with the external magnetic field. Perturbation of the spin system by a radiofrequency (RF) pulse whose energy corresponds to that of the energy gap (the Larmor frequency) induces an NMR signal.

This signal, which decays with time, is called a "free induction decay" or FID. The initial amplitude of this signal is proportional to the number of protons in the sample. For samples with protons in distinct chemical or physical environments, the signal due to each type of proton decays exponentially with a characteristic "spin-spin relaxation time" constant, T_2. The T_2 values for protons in solids are characteristically much shorter than those in liquids. The FID of a sample like a seed typically shows a very rapid initial decay corresponding to protons in the solid component, and this is followed by a slower decay due to the liquid component (Fig. 1). This can be composed of more than one component, including water in "bound" and "free" forms, and mobile lipids with the longest T_2 values. While more sophisticated NMR procedures have been developed, the simplest way of determining seed moisture is to measure the height of the NMR signal at a point in time after the signal from the solid component has died away.

In this study, we report a preliminary evaluation of the low-resolution pulse NMR technique for determining moisture content of lodgepole pine (*Pinus contorta* L.) seeds.

Materials and methods

Lodgepole pine seeds used in the study were from the Seed Laboratory of Pacific Forestry Centre, Victoria. Storage moisture content of seeds was 6.2% (fresh wt. basis). The first part of this study was to determine seed moisture isotherms, which was achieved by placing seed batches of approximately 1 g into sealed chambers of known atmospheric relative humidity at 12, 24, 42, 60, 80 and 98%. Humidity was regulated by using saturated salt solutions (Winston and Bates 1960; Day 1985) and measured at intervals using a Vaisala HMP-14A humidity probe. Chambers were kept at 11°C and the weight of the seed batches was monitored until it was constant. This condition was met within three days after placing the samples into chambers, except at 98% where seeds actually did not equilibrate with the prevailing atmospheric humidity; instead, their moisture content gradually increased during the whole monitoring period. The moisture content of five samples from each relative humidity treatment was

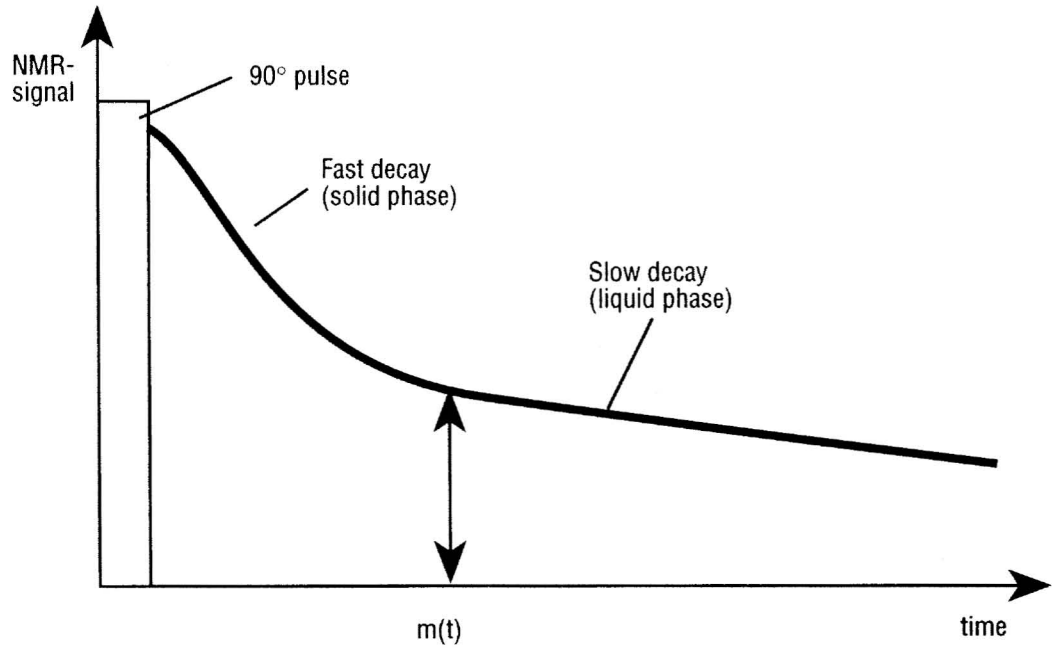


Figure 1. Schematic illustration of NMR-signal decay in solid and liquid phases.

determined by the low-temperature oven method after 17±1 hours at 103°C. Due to the limited number of chambers, moisture isotherms were determined in three replicated experiments using five seed batches per humidity treatment. Seeds of high moisture content (up to 30% fresh wt. basis) were obtained by soaking them in distilled water at 11°C for 24-36 h. After treatment seeds were surface dried and placed in sealed jars for an additional 6 h.

Sample moisture contents were measured using the Bruker MINISPEC PC120/100/13RTa NMR analyzer operating at 20 MHz for protons, and the EDM 320 (Experimental Definition Module). EDM 320 is a program designed to measure total proton quantity in samples, but can also be used for moisture determinations. A 90° pulse is applied to the sample, and the amplitude of the NMR signal measured at a fixed point (duration 1) after the pulse. To improve the signal-to-noise ratio, signal-averaging of 16-24 scans was used. The relaxation delay between pulses was 1.2 s, as measured T_1 values (spin-lattice relaxation times) were less than 0.15 s. As measurement is triggered by inserting the sample tube into the probe, an additional delay of 1-2 s was used between inserting the tube and the start of the measurement to allow the tube to be properly seated in the probe.

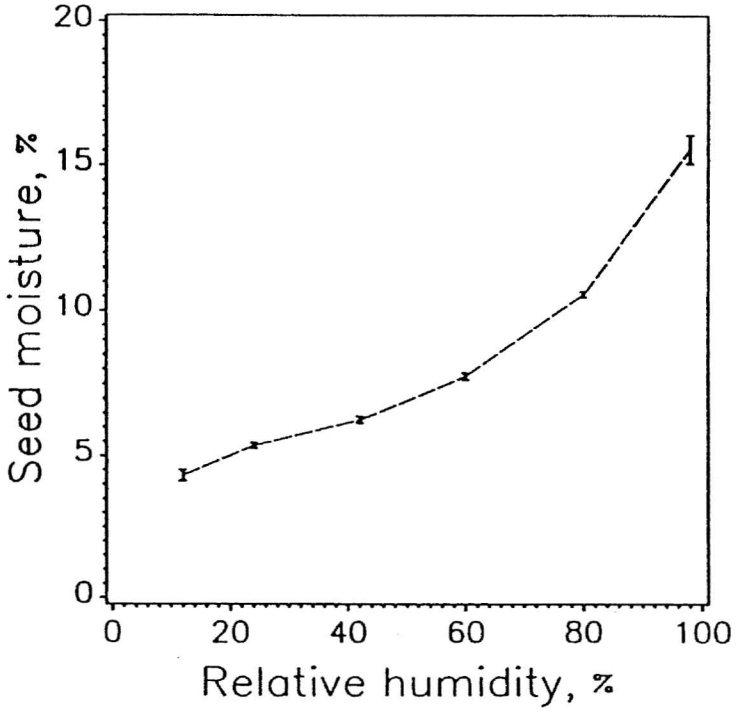


Figure 2. Moisture content of *Pinus contorta* seeds (% fresh wt basis) in equilibrium with different relative humidities. The vertical bars indicate standard error of the mean.

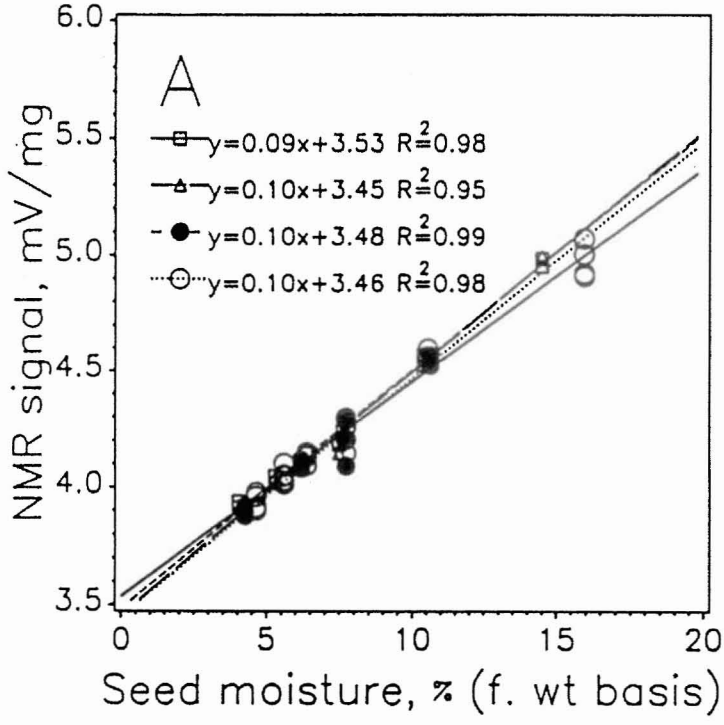
Calibration curves for the NMR signal *vs.* oven moisture content were prepared using three to six independent seed samples for each moisture level. Samples of approximately 0.5 g were weighed in sealed jars and put into 13 mm OD glass NMR tubes with caps. With the EDM in "calibrate" mode, the sample weight and oven-dry moisture value for each sample are entered, and the sample inserted. From three or more calibration samples, the program develops a linear regression of signal amplitude (voltage) *vs.* % moisture per gram of sample. Several calibration curves can be stored in memory for different sample types. In "measurement" mode, the sample weight and ID number are entered, the sample is inserted, the signal measured automatically, and the percent moisture printed out in less than one minute.

The value of duration 1 was not optimized for this application. The default value is 20 µs, which is appropriate for solids. A more suitable value for measuring the liquid component is 50-70 µs, by which time the solid component has decayed to an insignificant level. Duration 1 is reset to the default value each time calibrate mode is entered. During this preliminary study, it proved impractical to reset this parameter each time.

Two seed batches of approximately 10 g were equilibrated to different moisture contents in sealed chambers with relative humidity of 12, 60 and 80% as described above. Target seed moisture contents were 4.5, 7.5 and 10.5%, respectively; an equilibrium was met within a week after closure of seeds into the chambers. The moisture content of 30 samples, weighing approximately 0.5 g, was then measured independently by both low temperature oven method and NMR method.

Results and discussion

The moisture isotherm for lodgepole pine seeds (Fig. 2.) was similar to that reported earlier for other coniferous species (Bartels 1956). Due to high fat and lipid content, lodgepole pine seeds have lower equilibrium moisture content than carbohydrate- and protein-storing seeds. In high relative humidity at 98%, variance of equilibrium moisture content values increased considerably compared to the values obtained at lower relative humidities. Larger variance may arise from loss of water by evaporation during the sampling process or from the fact that seeds at high relative humidity actually did not



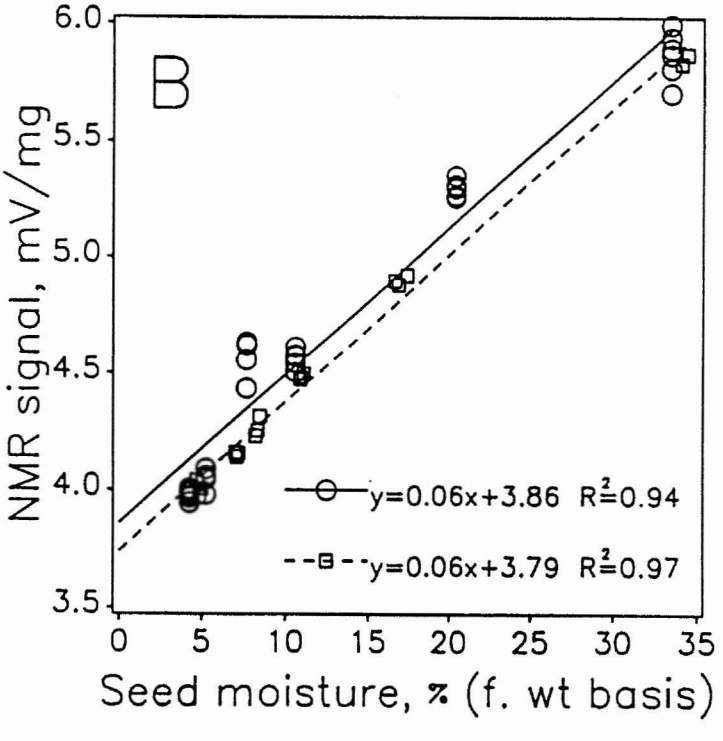


Figure 3. Linear correlation between seed sample moisture content (by low-temperature oven method) and the NMR-signal value. Results of four independent calibrations between 4-15% moisture content (A) and two calibrations between 4-33% moisture content (B).

equilibrate to a constant moisture; instead, their moisture content gradually increased during the monitoring period.

The calibration curves for the NMR signal were determined over a range of 4-33% moisture content (fresh wt. basis) as shown in Figure 3. A linear equation (by least squares method) fit the data with R^2-values ranging from 0.93 to 0.98. In some cases it was questionable whether a linear calibration line was an appropriate one. Generally, the fit was better at the lower moisture range from 4-15%. Coefficients of determination were somewhat lower than those reported for barley and wheat seeds, for example (Miller *et al.* 1980; Morley *et al.* 1984). Indeed, calibration may be more difficult for conifer seeds than for those seeds with more homogenous structure. In the former, the seed coat, female gametophyte and embryo hydrate to different extents during the hydration process, possibly increasing variance in a bulk seed sample. Improvements on calibration could be expected with using other reference methods such as Karl-Fischer titration (cf. Grabe 1989) and optimization of the NMR parameters used, especially duration 1.

Comparison of independent seed samples of three different target moisture levels showed close agreement between moisture values determined by the oven method and NMR method (Fig. 4). Mean as well as the median moisture values were within 0.2% units range with both methods. However, the coefficient of variation was larger among the samples measured by the NMR method.

Conclusions

The NMR method, being both rapid and non-destructive, is suitable as a routine method for the determination of moisture in seeds, both in production and gene resource conservation programs. A further study should be undertaken to establish standard protocol for a variety of seeds and moisture ranges. For the Minispec method to be adopted as a standard procedure, a slightly modified EDM 320 program should be developed in which the default value of duration 1 is longer, or in which it is not automatically reset each time the operator enters "calibrate". In future work, we hope to examine the proportions of free and bound water in relation to physiological activity in seeds.

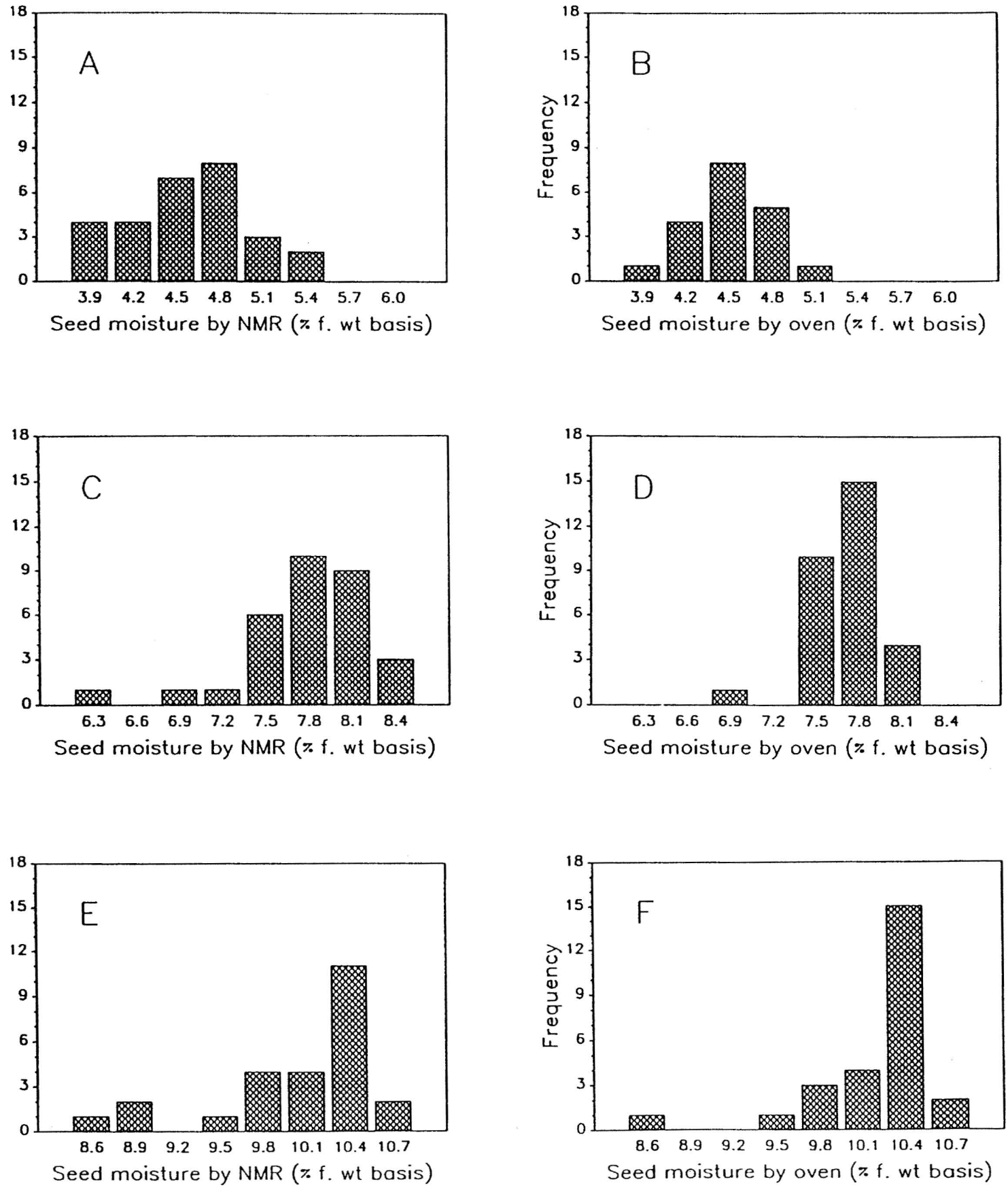


Figure 4. Frequency distributions of moisture contents in independent seed samples (n = 30) by both low-temperature oven and NMR methods. Target moisture levels in samples were 4.5% (A,B), 7.5% (C,D) and 10.5% (E,F) (fresh wt basis).

Acknowledgements

Doug Taylor, Toshi Panesar and Ann van Niekerk, all at Pacific Forestry Centre, helped in making the saturated salt solutions. M.N. acknowledges the grants from the Academy of Finland and the Foundation for Research of Natural Resources in Finland. We also thank G. Barlow of Bruker Spectrospin Canada for training and much helpful advice.

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A computerized, solid-state, controlled temperature gradient system for determining optimal seed germination temperatures

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Abstract

Temperature is a critical factor influencing conifer seed viability and germination success. The objective of this study was to design and construct a controlled thermal gradient system to determine the optimal temperature conditions for seed germination, and other biological processes influenced by temperature. The computer-controlled thermal gradient system consists of one to eight modules each containing 16 cells arranged in a 4 x 4 matrix. Each cell measures 12.5 cm square with a 10 mm aluminum plate at its base, and is thermally insulated from the surrounding cells. Both heating and cooling of each cell are provided by thermoelectric heat-pump units sandwiched between the aluminum plate and a large heat-sink common to all 16 cells. The temperature of each cell is monitored with a single diode temperature sensor. The computer determines whether heating or cooling is required for each cell to achieve the user-specified target temperatures, and schedules the power needed to do so, using a proportional integral derivative (PID) control algorithm. Using this approach, temperature control consistently to within ±0.1°C has been attained. The researcher may use the computer to examine current and historical cell temperatures and to change the target temperatures as required. The software also allows optional randomized arrangements of cell temperatures, so that experimental bias can be virtually eliminated. Results of preliminary tests on white spruce (*Picea glauca* [Moench] Voss) seeds are presented.

Résumé

La température constitue un facteur critique de la viabilité et du succès de la germination des graines de conifères. Cette étude avait pour objectif la conception et la fabrication d'un système à gradient thermique régularisé servant à déterminer les conditions optimales de température pour la germination des graines et pour d'autres processus biologiques sensibles à la température.Le système à gradient thermique régularisé par ordinateur est constitué de 1 à 8 modules, eux-mêmes composés de 16 cellules qui sont disposées en un bloc de 4 cellules de côté. Chacune mesure 12,5 cm^2 et comporte une plaque d'aluminium de 10 mm à sa base; chacune est isolée thermiquement des cellules contiguës. Le chauffage et le refroidissement de chacune sont assurés par des échangeurs de chaleur thermo-électriques coincés entre la plaque d'aluminium et un gros dissipateur de chaleur commun aux 16 cellules. La température de chaque cellule est contrôlée au moyen d'une seule sonde thermique à diode. L'ordinateur détermine s'il faut chauffer ou refroidir la cellule de manière à ce qu'elle garde la température établie par l'utilisateur, et il programme la quantité d'énergie nécessaire au moyen d'un algorithme du type proportionnel, intégral, dérivé (PID). Grâce à cette méthode, il est possible de garder la température à l'intérieur d'une plage de ±0,1°C. Le chercheur peut utiliser l'ordinateur pour connaître la température des cellules ou obtenir des relevés des températures antérieures ainsi que pour ajuster la température à ses besoins. Le logiciel permet aussi d'obtenir des combinaisons aléatoires de températures, ce qui supprime pratiquement les biais expérimentaux. Les résultats d'essais préliminaires sur des graines d'épinettes blanches (*Picea glauca* [Moench] Voss) sont présentés.

Introduction

Forest geneticists and seed physiologists recognize that temperature is a critical factor influencing conifer seed viability and germination success. In B.C. alone, it has been estimated that there are almost 4000 seedlots currently in use, for which optimal germination temperatures generally are not known to better than ±5°C (Rooke 1991, personal communication). Better knowledge of temperature optima should result in

higher germination success, and hence reduced seedling production costs for the nursery. By "optimum temperature" we refer to the temperature treatment at which the maximum and/or fastest seed germination occurs. The optimum temperature treatment may involve changes in temperature with time, and typically a night and day fluctuation is necessary (Heit 1958). Other treatments, such as stratification, also influence the optima for seed germination (Leadem 1986). When all possible factors are taken into account, a large number of germination tests are needed before proper understanding of the optimal germination treatment for a single seedlot can be achieved.

Standard seed germination tests typically require 21-28 days to complete (AOSA 1981, ISTA 1985), and determination of the optimum germination temperature usually requires repeating these tests at several controlled temperatures. In the past, seed researchers have had to choose between two approaches to do this. One was to use a series of controlled temperature units, such as controlled environment chambers or incubators, operating at different temperatures. The alternative was to use a thermogradient plate (e.g. Larsen 1971, Morgan 1980, McLaughlin *et al.* 1985). The former approach has the disadvantage that several units are required if acceptable results are to be obtained within a reasonable time, though samples from many seedlots can be tested at once. If budgets or work space are limited, fewer units may be used, with consecutive tests being run at different temperatures, but there is the risk that the test material will age between the beginning and end of the germination tests. Further problems include ensuring the test material is subjected to similar treatment from one controlled environment to the next, since lighting, ventilation and humidity control may differ, while human factors may also have an impact. Also, the temperature control inside a large enclosure such as a controlled environment chamber cannot be perfect. Small variations in temperature with time may be acceptable, but if consistent temperature gradients exist within the controlled environment chamber, the effect of these must be considered.

Of the few designs for thermal gradient plates that have been published, most are based on a single large thermally conductive plate, heated at one edge and cooled at the other (see McLaughlin *et al.* 1985 for a review). Cooling is usually achieved by circulating a refrigerated coolant through the plate, or pipes attached to its base, while heating is provided either using heated liquid, or more commonly, by electric heating elements. Temperature sensors positioned at various points on the plate are used to provide temperature control using simple feed-back loops. The seed researcher can run germination tests on small quantities of seed at several temperatures simultaneously, thereby eliminating some of the problems of using controlled temperature units described above. However, other problems are often introduced. Firstly, since the gradient is continuous, it is inevitably true that no two points on the plate (in the gradient direction) are ever at identical temperatures. Thus the researcher must either be content with very small samples of test material, or must accept that seeds within each sample are not actually being subjected to identical temperature treatments. Secondly, because the only sources of heating and cooling are at the edges of the plate, temperature control at any point in between is necessarily imprecise.

A more recent design was significantly different. It described a seed germinator with 100 individually temperature controlled cells (McLaughlin *et al.* 1985). Arranged in a 10 x 10 matrix, each cell was surrounded by styrofoam insulation and could accommodate a 10 cm Petri dish. Instead of a continuous temperature gradient, each cell was maintained at a single uniform temperature. The germination response could then be plotted as a curve or surface over a range of clearly-defined discrete temperatures. Both heating and cooling of each cell were provided by a thermoelectric device sandwiched between the lower surface of an aluminum pedestal at the base of the cell and a larger heat-sink, common to all 100 cells. Thermoelectric heat-pumps have the advantage that reversing the current direction switches them between heating and cooling modes. However, a significant drawback is that in cooling mode, excess heat is generated, requiring external cooling of the heat-sink.

The 100-cell germinator used an electromechanical timer to control a 24-hour cycle, during which as many as six separate control temperatures could be generated. The temperature control system was inflexible because it allowed a maximum of only six predetermined temperatures per cell, and required the construction of a new circuit board if a new cell temperature regime was required. McLaughlin *et al.* (1985) suggested that the electromechanical temperature control system could be replaced by a computer, which could monitor the cell temperatures continuously, and control relays to switch the heat pumps between heating and cooling, as required. However, the authors did not consider that the computer could also be used to schedule the demand on the power supply. In their system, it was assumed that all the heat pumps would sometimes need to be powered simultaneously, so a very large power supply was needed. The complete system was therefore large and heavy, and hence very inconvenient to move.

The project reported here arose because of an urgent need to identify temperature optima for seedlots in

British Columbia. The objective was to design and construct a thermal gradient system to generate a range of discrete biologically active temperatures simultaneously. It would be similar in concept to the McLaughlin *et al.* (1985) seed germinator, but with two major improvements. Firstly, the system was to be computer-controlled to enable any cell to be maintained to within ±0.1°C for any temperature in the range 0° to 50°C. Secondly, the system would be "modularized", with each module containing only 16 cells arranged in a 4 x 4 matrix. Combined with smaller power requirements, this would make the system more flexible and portable. For studies requiring larger numbers of cells, two or more modules would be connected to the same controlling computer.

Methods

Module Construction

Each module of the thermal gradient system consists of a square enclosure (650 x 650 x 150 mm) covered by a closely fitting clear double-glazed lid, hinged at the rear. Inside are 16 square (126 x 126 mm) cells arranged in a 4 x 4 matrix. The construction of a single cell is shown in Figure 1. Within each cell, the temperature is regulated independently by sets of five thermoelectric (Peltier) heat-pump units (model CP1.0-31-05L, Materials Electronics Products Corp., Trenton, NJ), connected electrically in series and thermally in parallel (see Anon. 1985, for discussion of Peltier heat-pump principles). The specification for these units was determined from consideration of the energy exchanges expected for worst-case heating and cooling conditions, discussed in more detail below. The five heat-pump units are sandwiched between, and in intimate thermal contact with, the lower surface of a 10 mm thick anodized aluminum "test-plate" at the base of each cell, and the upper surface of a single much larger aluminum "base-plate", common to all 16 cells. The heat-pump units are arranged in an 'X' pattern for more uniform energy distribution, with plastic foam insulation filling the gaps. The thermoelectric heat-pumps are very efficient (under optimum conditions, the ratio of heat pumped to electrical energy consumed exceeds 50%), and the base-plate facilitates heat transfer from cells being cooled, to those being heated, but some excess heat is inevitably generated and must be removed. In each module, a pair of extruded aluminum cooling plates (V&V Refrigeration Ltd., Richmond, B.C.) are bolted to the lower surface of the base-plate, so that liquid coolant (normally water) may be passed through the system to maintain the temperature of the base-plate, typically at about 15°C. Aluminum pipes welded to the ends of the cooling plates protrude from the sides of the enclosure, allowing the module (or modules) to be connected to a cold water source. Normally, a flow rate of 3-4 L min^{-1} (per module) at 15°C is sufficient to dispose of all excess heat.

The walls of each cell are 50 mm high, consisting of white plastic insulating foam approximately 25 mm

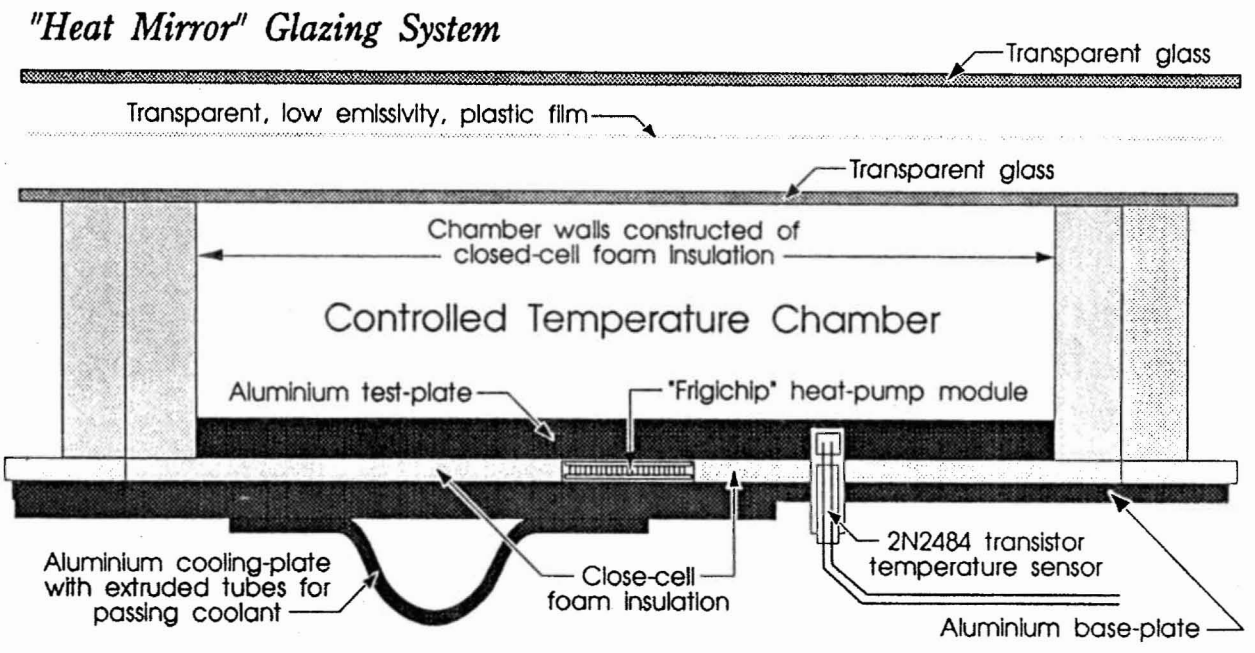

Figure 1. Cross-section through a single cell of a thermal gradient system module.

thick. When closed, the lid rests on the top edges of the cell walls, thereby insulating each cell from its neighbours, and the enclosure's surroundings. The lid features a proprietary glazing system (Heat Mirror™, Southwall Technologies Inc., Palo Alto, CA) consisting of two sheets of glass 22 mm apart, and a specially coated plastic film midway between. The plastic film filters strongly in the near infrared wave-band, thereby significantly reducing radiative heating of the cell or its contents (e.g. when used below incandescent lamps). The plastic film also reduces convective exchange between the two glass layers. The lower glass sheet is tempered for greater strength and resistance to heat stresses resulting from temperature differences between neighbouring cells.

A high current 12 VDC power supply is connected by pairs of #4 gauge welding cables to two pairs of terminals protruding from the rear of the module. Each terminal pair is mounted on a printed circuit board and allows up to 20 Ampere to be supplied to half the module (i.e. there are two such boards per module). Each circuit board carries 8 solid-state switches formed by sets of four power MOSFETs (Metal Oxide Semiconductor Field Effect Transistors), which allow power to be rapidly switched to the thermoelectric heat pumps in eight cells. Each cell typically draws about 2.2 A at 12 VDC, which the MOSFETs can dissipate without heat-sinking. The computer prevents more than eight cells from ever being powered simultaneously, so a 250 W power supply is typically needed for each module. Each of the 8 switches is controlled by two logic lines, one to determine whether the cell is powered, and the other the current direction. The 16 logic lines (two per switch) are toggled by two 8 bit ports of an 8255A programmable peripheral interface (PPI) controller, receiving its instructions and power from the computer via an "A-Bus" (Alpha Products, Darien, CT) ribbon cable connected to the board. Under computer control, each solid-state switch thus allows direct current to a single cell to be switched off or on, and in either direction. A panel of 16 bi-directional light emitting diodes (LEDs) connected across the MOSFET switches and mounted on the front of the module shows the status of the cells. When current passes in one direction, the heat-pumps cool the cell, while the corresponding LED glows green. Reversing the current causes the heat-pumps to heat the cell, and the LED glows red. When no power is supplied, the LED is off and the cell loses or gains heat as it returns toward ambient temperature (usually that of the base-plate).

Power is supplied under computer-control in pulses of approximately 0.05 s duration. The number of "on pulses" delivered in a given period therefore determines the amount of energy being supplied to the cell. For cells requiring much heating or cooling, power may be supplied more or less continuously, while those requiring only slight temperature adjustment need relatively few pulses per minute.

The temperature of each cell is measured every 10 s using a calibrated 2N2484 transistor (McNaughton 1989, personal communication) embedded near the upper surface of the test-plate. The voltage across the 2N2484 varies linearly with its temperature and, with careful calibration, it serves as a very accurate thermometer. All sensors were individually pre-calibrated in a stirred water bath over the range 0° to 60°C against a measurement-grade fine wire copper/constantan thermocouple connected to a data logger (model 21X Micrologger, Campbell Scientific Instruments, Logan, UT) operating in double-ended measurement mode. The r^2 was found to be typically better than +0.9999 with 50-100 readings over the calibration range.

The temperature sensors are connected via a 16-channel multiplexing circuit (one channel per cell) to a high quality instrumentation grade amplifier, mounted on a third circuit board located at the front of the module. A separate 500 mW power supply provides power to this analog sensing board because it is optically isolated from the computer's power supply, to eliminate digital noise that might contaminate measurements of the analog signals from the temperature sensors. For the same reason, the analog circuitry is also physically separated and electrically shielded from the digital switching circuitry at the rear of the module. The sensitivity of the sensors is about 2.2 mV $°C^{-1}$, but after amplification, the sensitivity is increased to about 200 mV $°C^{-1}$, over the range -25° to +75°C. The output voltage from the amplifier is multiplexed into an analog-to-digital (A/D) converter (ADC) circuit based on the ADC574A chip (Burr-Brown, Tucson, AZ). With the 12 bit resolution of the ADC574A, the measurement resolution is about ±0.025°C. Under computer control, the temperature of each cell is sampled, and the transistor output voltage multiplexed and amplified before being converted to a digital value by the ADC. Multiplexing and A/D conversion are controlled by the third of the 8255A interface controller's ports on one of the two digital boards at the rear of the module, while digital output from the ADC is returned to the computer via the third port of the 8255A on the other digital board. Each cell temperature measurement is completed in about 0.001 s. As many as eight modules may be connected to the computer, providing up to 128 individually controlled temperature regimes.

Energy Balance Analysis

To estimate the size of the thermoelectric heat-pump units required, a simple energy balance model was constructed. The model attempted to simulate the energy balance for a single cell in two extreme cases (Table 1). For both cases, heat exchanges between the cell and the base-plate (through the insulation separating the test-plate and the base-plate), the neighbouring cells (through the walls), and the surrounding air (through the double-glazed lid) were all estimated and summed. The net long-wave radiative exchange was also estimated from consideration of the radiative properties of the materials and suitable values for surface temperatures. In addition, for the "cooling worst-case", the short-wave radiative input from tungsten lamps was included.

Figure 2 shows that the amount of heat energy required to be pumped varies, with maximum demands under worst-case conditions of about 10 W when cooling to -5°C, and 11 W when heating to +60°C. The five thermoelectric heat-pump units used to heat and cool each cell, connected in series, are able to pump a total of about 27.5 W under optimum conditions (i.e. when the test-plate temperature is equal to the base-plate temperature). The heat-pumps decrease in efficiency as the temperature difference increases, but for the worst-case conditions identified above, the heat-pumps are specified to remove about 19 W when cooling to -5°C, and deliver 18 W when heating to +60°C, assuming the base-plate temperature can be maintained at 15°C.

Control Software

A memory-resident control program for IBM PC-type microcomputers runs continuously to maintain the temperatures of all the cells, even when the computer is being used for other purposes. It uses a closed-loop proportional integral derivative (PID) algorithm which is repeated on a 10 s cycle. For each module, the first stage in the cycle is to initialize the total energy requirement, ΣE_p, to zero. ΣE_p is used to accumulate the total energy requirement for all cells, to ensure that the total demand does not overload the capacity of the power supply.

In the second stage, the computer instructs the A/D circuit to use the 16 channel multiplexer to step through the cells of each module to read the sensor voltages, one by one. After amplification, each voltage is digitized by the ADC574A, and the digital output

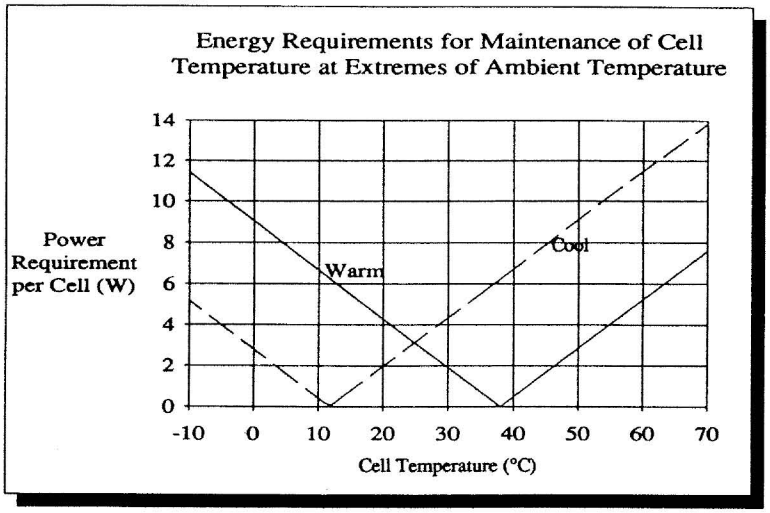

Figure 2. Summary of results of energy balance analysis for module. The two lines represent the range of energy input required to maintain the cell at the indicated temperature. "Warm" and "Cool" refer to the ambient conditions, as given by Table 1a.

Table 1a. Summary of values of variables used in energy balance analysis. This table gives the worst-case control plate temperatures. The results for these and other temperatures at 5°C intervals are shown in Figure 2.

Variable	Cooling Worst-case	Heating Worst-case
Incident short-wave radiation	500 W m^{-2}	0 W m^{-2}
Temperature of surroundings	50°C	20°C
Ambient air temperature	35°C	15°C
Temperature of lid	40°C	10°C
Temperature of base-plate	15°C	15°C
Temperature of neighbouring cells	50°C	0°C
Plate control temperature	-5°C	60°C

returned to the computer via the A-Bus, where it is converted to a temperature reading using the calibration coefficients determined for the temperature sensors. The sensor calibration coefficients are read in from an external data file when the program is loaded, so these may be changed easily. The target temperature, T_t, set by the researcher, is compared with the actual temperature just measured, T_p, and the error, e_0, is calculated as $(T_t - T_p)$. The priority for heating or cooling each cell is assessed on the basis of the magnitude of e_0. In the third stage, on completion of the measurements, the computer sorts the cells in descending order of priority (i.e. their absolute temperature errors). The error is also used to calculate the energy required (positive or negative) over the next 10 s period to bring the actual test-plate temperature to the target temperature and maintain it there, using a PID equation of the form:

$$E_p = K_p [e_0 + K_i e_i + K_d(e_0 - e_1)] \qquad (1)$$

where Ep is the energy required for the next 10 second control period in Joules; K_p, K_i and K_d are the proportional, integral and differential coefficients (derived empirically) and e_0, e_1 are the temperature error terms for the current and previous 10 s control periods, respectively. The term e_i is the integrator error, which varies depending on how close the actual temperature is to the target temperature. When the error is greater than 5.0°C, e_i is set to zero, so that the energy requirement is calculated only as a function of the error (proportional term) and of the rate of change in the temperature error (the derivative term), determined as (e_0-e_1). When the magnitude of e_0 falls below 1.0°C, the integral term is activated, and e_i is calculated as the sum of the temperature errors $(e_i = e_i + e_0)$. As the control algorithm "seeks" the required control temperature, e_0 varies between positive and negative, and the sum e_i will tend to a small value fluctuating about zero. For temperature errors in the range 1.0 to 5°C, e_i is left unchanged. This means that when the actual temperature is approaching the target, e_i is set to 0, until the magnitude of the error falls below 1.0°C. Conversely, if the temperature starts to drift away from the target, there is a broader band over which the integral term actively influences the temperature control.

Once E_p has been calculated, the energy requirement is doubled if cooling is required, (i.e. if $E_p < 0$) in recognition of the fact that the thermoelectric heat-pumps are approximately half as efficient in cooling mode. Finally, ΣE_p is accumulated by summing the absolute value of E_p for all cells in the system. When totalled, ΣE_p is compared to that available from the power supply, and if the latter is exceeded, the energy requirements for all cells are scaled down proportionately. Note that the PID control algorithm allows cooling to continue even when the temperature has fallen below the target (or heating when the temperature is above the target). The ideal situation occurs when the heat supplied or removed by the heat-pumps exactly matches the heat being lost or gained over the same period. In reality, external perturbations, the pulsed power input, and limits on temperature measurement precision, prevent a perfect balance from being achieved continuously, but the PID algorithm adjusts frequently enough to obtain satisfactory results.

For the remainder of the current cycle, and for the short period required to measure the temperatures and allocate energy for the next cycle, power is supplied to the heat-pumps in 0.05 s pulses approximately 18.2 times per second, using the IBM PC's timer interrupt. A pulse is the shortest period for which the cells may be switched on or off, with each 10 s cycle divided into 182 pulses. Each time the PC's timer issues an interrupt, the appropriate 8255A logic lines are set for the duration of the next pulse for all the connected

Table 1b. Summary of constants used in energy balance analysis. The results of the analysis are shown in Figure 2.

Variable	Value	Units	Source
Short-wave transmissivity of lid	0.48		Southwall
Albedo of plate surface	0.6		Estimate
Long-wave emissivity of plate surface	0.1		Estimate
Long-wave emissivity of walls	1		Estimate
Thermal conductivity of foam walls	0.0433	$W\ m^{-1}\ °C^{-1}$	BXL Plastics
Thermal conductivity of lid (cooling)	2.1	$W\ m^{-1}\ °C^{-1}$	Southwall
Thermal conductivity of lid (heating)	1.82	$W\ m^{-1}\ °C^{-1}$	Southwall

modules, before control is returned to the foreground task. Energy pulses from the power supply are allocated to the cells in decreasing priority order, with the highest priority cells receiving the most pulses. The computer schedules the available pulses to ensure that of the 16 cells within each thermal gradient module, no more than eight are powered simultaneously (so the total current drawn from the power supply never exceeds 20 A per module). Once the cells have reached their target temperatures, this is not normally a significant restriction, but under certain conditions (e.g. when all the cells are undergoing a step-change in temperature) more power may be required than the power supply can provide. In this event, the energy requirements for all cells are scaled down by the ratio $E_{max}/\Sigma E_p$, where E_{max} is the maximum energy that the power supply can provide. After a few 10 s cycles have elapsed, the cells with larger temperature errors will have been warmed or cooled closer to their target temperatures, so the priority order will change and other cells will then receive their power allocations.

The remaining function of the resident control program is to monitor the system clock and record 30 minute temperature averages, minima and maxima on disk, at half-hourly intervals. The 10-second temperature readings are stored for one minute, and then averaged. These one-minute averages are stored for 30 minutes, with the 30-minute averages being updated every minute. On the half-hour, the current 30-minute averages are written to the disk, together with the 30-minute maxima and minima. Data are stored sequentially in a binary file until all available records are occupied. At this point, the oldest data are overwritten, and the file becomes a ring buffer operating on a "first-in, first-out" principle. The maximum size of the data file may be easily adjusted, so that the user may decide for how long data will be retained.

A transient program provides access to the control program when the system is operating. When the user runs the transient program, it searches memory for the presence of the resident control program, and assuming the latter is located, it then "eavesdrops" on the data being collected and stored. In this way the transient program allows the user to view current temperatures, modify control parameters, and access temperature data previously stored on disk, without affecting execution of the control program. All data recorded in the binary data files can be recalled and displayed as a 24-hour or 7-day graph on the monitor screen. By default, the most recent data for a selected module are displayed, but the user may examine older data by moving backwards through the file.

The transient program also allows the researcher to set the temperature of the cells individually, or as a complete experiment involving all 16 cells within each module. For the individual cells, in addition to constant, the temperature can be made to vary as one of three simple functions of time (step, ramp or sinusoid). In the latter cases, the mean temperature, the wave amplitude and the durations of the day and night periods can all be controlled. When setting up an experiment involving all 16 cells, the researcher can set the range of temperatures, and select from one of the four time functions to be used by all the cells. One may also select an experimental design, for which four choices exist: the one-way gradient replicates four temperatures in four cells with the coldest at one side of the module and warmest at the other; the two-way gradient generates 16 temperatures with the coldest cell at one corner and the warmest at the opposite corner; the Latin square replicates four temperatures in four cells using a randomized Latin square design; the random square generates 16 randomly arranged temperatures. The randomized designs would normally be used since these help to eliminate external bias in the germination tests. (It is even possible for the technician doing the germination assessments to be kept ignorant of the temperature treatments being applied!) Systematic designs may be useful for demonstration purposes, or for routine tests when experimental rigour is not essential.

Virtually any combination of experimental design and individual cell temperature functions can be handled because the control parameters for each cell can be customized after an experimental design has been selected. In addition, further wave functions and experimental designs could be added to the control software quite easily, if the need arose. The only limitations imposed are by the rating of the power supply and the amount of cooling that can be provided. For example, it is not possible to maintain all 16 cells at 0°C simultaneously if the coolant temperature is 15°C. This should not be a problem for most purposes, since the objective of the thermal gradient system is to provide a range of biologically significant temperatures. Even this limitation could be overcome by heating or cooling the coolant and/or increasing the size of the power supply.

Performance Tests

As an initial test of the system, a simple seed germination experiment was conducted on white spruce (*Picea glauca*) (Fig. 3). Two 800-seed samples were drawn from a single seedlot, of which one sample was stratified by soaking in water for 24 h, then chilled for 3 weeks at 2°C before being placed in the thermal

gradient module. A single thermal gradient module was programmed to generate eight constant temperatures from 10° to 38°C in intervals of 4°C, each replicated twice and randomly distributed within the module. Each sample of 800 seeds was separated at random into 16 subsamples of 50 seeds. Each subsample was then placed in a transparent plastic 12 cm square seed germination tray, scattered evenly on a substrate of one layer of "Kimpak" absorbent paper covered with 2 layers of Whatman No. 1 filter paper, then moistened with 50 ml deionized water. Each tray was covered with a close-fitting lid, randomly allocated a number between 1 and 16 for easy identification and placed in the corresponding cell of the thermal gradient module. Germination assessments were made three times per week (Monday, Wednesday and Friday) for a period of two weeks. At each assessment, the number of germinated seeds was recorded, after which they were removed. Any mouldy seeds were also noted and removed. A seed was considered to be completely germinated when the radicle had extended to four seed-coat lengths. After testing the unstratified seeds, the experiment was repeated with the stratified sample.

Results and discussion

Germination Experiment

Figure 3 shows the distribution of germination percentage (during the two weeks of each test) as a function of temperature. Unfortunately, a problem in the control software caused the 30°C treatment to fail 7 days into the second test, so that the two cells operated at full power for several hours, resulting in all the chitted and germinating seeds being killed. This explains the apparently lower germination for stratified seeds at this temperature. Otherwise, the results clearly

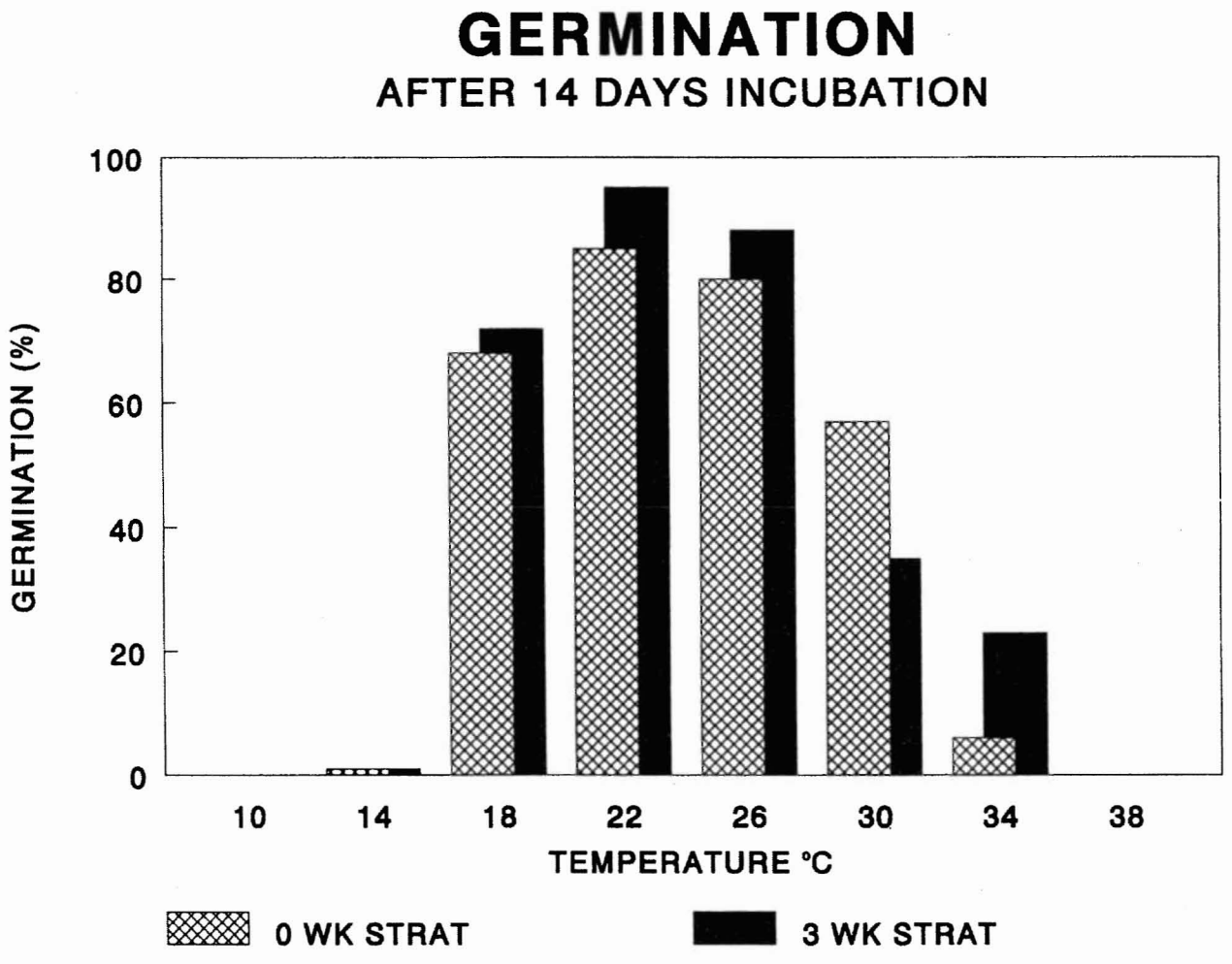

Figure 3. Germination of white spruce (*Picea glauca*) seeds after a 14-day incubation period, as a function of temperature determined using the computer-controlled solid-state thermal gradient system.

demonstrate that stratification broadens the range of temperatures optimal for germination, as well as generally increasing the germination percentage. Both germination tests indicate that the optimum temperature for germination of the white spruce seedlot we investigated lies somewhere between 18° and 26°C. A further experiment could now be carried out to investigate the range between, say, 19° and 26°C, in increments of 1°C. When properly calibrated, the thermal gradient system is certainly capable of resolving differences in germination temperature as small as 1°C.

System Performance

After fixing the obvious problems (such as the software bug mentioned above), the module electronics and system control software have been found generally to work very well. As a safety precaution, a routine has since been added to the software to prevent cells from being powered when the measured temperature falls beyond the range -20° to +70°C. Examination of large quantities of constant temperature data collected by the system show that over a 30 minute interval, the actual cell temperature recorded by the computer rarely differs from the target temperature by more than ±0.1°C. When the target temperature is itself changing with time, with the exception of an imposed step change, control is invariably kept within ±0.3°C over the specified operating range (0° to 50°C). Since it is impossible for the temperature to change immediately in response to an imposed step change, the performance specification for a step change is of interest. Complete adjustment to a large change can take more than an hour, but for changes of the order of 10°C, 90% of the adjustment usually takes place within 5 minutes when cooling, and 2.5 minutes when heating. The PID control algorithm necessarily involves some compromise, because more rapid change could be brought about at the cost of increased overshoot and slower settling time at the new target temperature. We are currently pursuing the use of an adaptive control algorithm to increase the speed of temperature adjustment, without impairing the precision of control.

Because the computer schedules the distribution of power to the cells, it allows a smaller power supply to be used more efficiently than the one used in the McLaughlin *et al.* (1985) seed germinator. In their system, a 2.1 kW (-7 to +7 VDC) unregulated power supply was constructed to allow each of 100 cells to draw about 15 W. In our system, each cell draws about 27.5 W (producing faster temperature adjustment), but since all cells do not need to be powered continuously, the computer enables 128 cells to be powered by a commercially available 2 kW (+12 VDC) regulated supply.

Calibration

After the first module was constructed, it was found that the temperature calibration coefficients determined for the individual sensors did not produce satisfactory agreement with independent measurements of the surface temperatures of the test-plate. The reason for this probably lay in the accuracy with which the transistor offset voltage and amplifier gain could be set on the analog circuit board. A technique for *in situ* calibration was required in any case, since the sensor calibrations should be checked at intervals. Of three techniques tried, the most successful used the control software to maintain constant temperatures for several hours so that the mean control temperature could be adequately compared with that recorded by an independent sensor. This approach took advantage of the extremely good temperature control capability of the module electronics and the PID algorithm. To obtain the calibration data, a calibration mode was added to the control program to generate temperatures of -5°, +15°, +35° and +55°C in a Latin square design, in four six-hour phases, on a repeating 24-hour cycle.

To measure the temperature of the cells, fine wire thermocouples were attached to the surfaces of the test-plates using adhesive tape, and connected to a datalogger, certified accurate to within ±0.5°C (Campbell Scientific model 21X). Using this technique, approximately 40 paired temperature comparisons could be obtained within 24 hours, for which a regression equation could be derived. The regression equation typically produced a standard error for the y-estimate of less than 0.3°C. Given the accuracy of the datalogger, this means that the absolute accuracy of temperature measurement is at worst, ±0.8°C, but we feel it will normally be within ±0.5°C. Further planned refinements to the temperature-sensing circuitry and better calibration techniques should improve this specification.

Other Applications

The system could potentially be used in several disciplines outside seed physiology, wherever researchers wish to determine the temperature optima for small-scale biological phenomena. These include: egg-hatching (e.g. for invertebrates and fish), embryology, genetics, tissue culture, aquatic and soil invertebrate biology, mycology, bacteriology and virology. For these purposes, various design options have been considered. For example, it would be an easy matter to replace the flat plate at the base of each

cell with a machined or cast aluminum block, designed to accommodate several small test-tubes or scintillation vials, instead of a single flat-bottomed tray or dish. This would allow temperature control to be provided all around the container and its contents, rather than just at the base.

Summary

The computer-controlled thermal gradient system allows researchers to generate virtually any conceivable combination of temperature regimes, constant or fluctuating, within the range -10° to +60°C. It should therefore enable seed researchers, producers and nursery growers to determine optimal conifer seed germination temperatures quickly and cheaply. Its modular construction should allow limited space and funding to be used more effectively, since not all applications will require large numbers of temperatures to be generated simultaneously. Other applications for the system are possible.

Acknowledgements

The Science Council of British Columbia provided an STDF-AGAR grant to support prototype development during 1990. Mr. W.T. Murrie contributed much time and effort in the design and construction of the prototype modules. Mr. M. Bellehumeur and Mr. D.R. Beames both worked long and hard for modest rewards to get the control electronics working to specifications. Dr. K.G. McNaughton first designed the temperature measurement circuit using the 2N2484 transistor. Members of the University of British Columbia, Department of Soil Science, provided much useful discussion and access to resources during development and testing of the prototype system. Burr-Brown Corporation provided free samples of their ADC574 analog-to-digital conversion chip.

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Artificial ripening of early-collected Scots pine cones and cones attached to branches

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Abstract

Scots pine cones and cones attached to branches were collected at different times early in the ripening season in northern Fennoscandia in 1988 and 1989 and subjected to artificial ripening at different temperatures, photoperiods, and cone moisture contents. Artificial ripening of cones collected in the end of July, when the seeds were anatomically very poorly developed and not germinable, was not successful, while for cones attached to branches an increased germination percentage was achieved. The percentage of filled seeds, however, showed a remarkable decrease. For cones collected later, when the anatomical development was more advanced, artificial ripening clearly increased the germination percentage. Compared to ripening in nature, the best method of artificial ripening doubled the germination percentage. A temperature of +15°C seemed to be more beneficial to ripening of early-collected and very poorly developed seeds than +6°C, but later and with more advanced anatomical development +6°C gave the best result. A long photoperiod during ripening was beneficial in most cases. The effect was most pronounced for cones attached to branches. Drying the cones from an initial level of around 50% to 38% was beneficial only for the first 2 weeks of ripening at +6°C. It was concluded that cones should not be collected before the end of August, and that artificial ripening should be performed with 24 h of light per day. For early collections, when seeds are anatomically poorly developed, temperature should be kept at +15°C for the first 2-3 weeks and should then be lowered to +6°C. Cone moisture content should be kept at the initial level during the +15°C period and later be allowed to slowly decrease; treatment time should be 4-8 weeks.

Résumé

Des cônes du pin sylvestre et des cônes fixés à des branches ont été prélevés à différentes dates, tôt au début de la saison de maturation, dans la partie nord de Fennoscandia, en 1988 et 1989. Les cônes ont été soumis à une maturation artificielle à différentes températures, photopériodes et teneurs en humidité des cônes. Les résultats de la maturation artificielle des cônes prélevés à la fin juillet, alors que les semences étaient très peu développées sur le plan anatomique et qu'elles n'avaient pas de pouvoir de germination, ont été mauvais. Le pourcentage de germination des cônes attachés aux branches a été supérieur. Toutefois, le pourcentage des graines remplies était remarquablement faible. Dans le cas des cônes collectés plus tard, alors que les graines étaient anatomiquement mieux développées, la maturation artificielle a conduit à une nette augmentation du pourcentage de germination. En comparaison de la maturation naturelle, la meilleure méthode de maturation artificielle a fait doubler le pourcentage de germination. Il semble que la température de 15°C soit plus favorable à la maturation des graines cueillies tôt et très mal développées que la température de 6°C, mais cette dernière a donné de meilleurs résultats avec les graines cueillies plus tard qui avaient un meilleur développement anatomique. Une photopériode de grande durée pendant la maturation a eu un effet positif dans la plupart des cas. Cet effet était le plus prononcé dans le cas des cônes fixés aux branches. L'assèchement des cônes, de 50 % jusqu'à 38 % d'humidité, n'a été bénéfique qu'au cours des 2 premières semaines de maturation à 6°C.Les auteurs sont parvenus à la conclusion que les cônes ne devraient pas être cueillis avant la fin d'août et que la maturation artificielle devrait se faire sous un éclairage continuel. Lorsque la cueillette est faite tôt, alors que les graines sont mal développées sur le plan anatomique, il faudrait garder la température à 15°C pendant les 2 à 3 premières semaines, pour la baisser ensuite à 6°C. La teneur en humidité des cônes devrait être conservée à sa valeur initiale au cours de la période à 15°C, pour être lentement abaissée par la suite; le traitement devrait prendre 4 à 8 semaines.

Introduction

In northern areas of Fennoscandia there is often a shortage of high-quality seeds of Scots pine (*Pinus sylvestris L.*). For these areas seed orchards do not fulfill the demand and, consequently, stand seeds must be used. In the extreme climatic conditions prevailing in northern parts of its range and at higher elevations, Scots pine often fails to produce mature cones and seeds. Several investigations have shown that for complete seed maturation a certain level of mean summer temperature or temperature sum must be reached (Wibeck 1928; Mork 1957; Kohh 1968; Sarvas 1970; Alfjorden and Remrod 1975; Henttonen *et al.* 1986). Even if the temperature is sufficient, frosts in late summer or autumn can cause damage to the seeds (Simak 1972; Kardell 1976). However, low temperature above the freezing point can be needed in the late ripening stages for breaking autumn dormancy (Sarvas 1974).

In principle, each fertilized ovule will develop into a germinable seed if the conditions for development and ripening are favourable. Even though the connection to the mother tree is important for seed development, it still should be possible to achieve improved seed quality when natural ripening conditions are unfavourable by using adequate methods for artificial ripening on early collected cones. In earlier work on this subject on Scots pine the main objective has been to prevent the seeds from being damaged by frosts occurring fairly late during the ripening period; the cones were collected and artificially ripened in September and October. Most attention has been focused on the temperature factor. Frequently, the efforts have given beneficial results, especially in comparison with collection during the winter (Nordström 1955; Kardell 1973; Nygren 1986). However, these results have not provided a proper basis for large-scale treatment, probably because critical limits for different stages of ripening have not been known and the methods for artificial ripening have not been adjusted to the actual level of ripening. Furthermore, in areas with very unfavourable temperature conditions, collection and artificial ripening should be started already in the beginning of August. At this time the ability of the cones and seeds to withstand desiccation is limited (Sahlén, K. In prep. Effects of artificial environmental conditions on anatomical and physiological ripening of *Pinus sylvestris* L. seeds), and the moisture content must therefore be kept unchanged during transport and artificial ripening. This has not been considered in earlier investigations.

Seed ripening can also be influenced by factors other than temperature. For many species it is known that the photoperiod to which the mother plant is exposed could influence the development of the seeds (Mayer and Poljakoff-Mayber 1989). During August the considerable decrease in photoperiod in northern Fennoscandia (United States Naval Observatory 1946) induces bud setting for Scots pine and should also affect seed development. Also, seed moisture content should affect the result of artificial seed ripening (cf. Bergsten 1987).

In this paper the results are presented of some introductory experiments on artificial ripening of Scots pine cones, both attached to branches and detached. The aim of the investigations was to determine the influence of photoperiod, temperature, ripening time, and moisture content on artificial ripening of cones collected at different ripening stages at localities with unfavourable temperatures.

The work was parallel to investigations on morphological, physiological and biochemical changes during ripening in nature. The parallel work was made with the intention of making guiding principles for artificial ripening, i.e. such guidelines would adapt artificial ripening conditions to the actual level of development and ripening. The guiding principles should be based on a combined development index based on morphological and physiological changes (Wiklund, Kristina. Swedish University of Agricultural Sciences, Umeå, Sweden. Personal communication) and biochemical changes (Skre, Oddvar. Norwegian Forest Research Institute, N-5047, Fana, Norway. Personal communication; Gjelsvik, Svein. Norwegian Forest Research Institute, N-5047, Fana, Norway. Personal communication). In this paper only the introductory work from artificial ripening is presented.

Material and methods

Artificial ripening of cones

Cones were collected from two localities, one in 1988 and another in 1989, situated in the north of Fennoscandia with a normally unfavourable seed-ripening climate (Table 1). Collections were made from the end of July until December. Cones from three collections in 1988 and two collections in 1989 were subjected to artificial ripening treatments.

At each time of collection cones were collected from 10 randomly chosen trees. After collection the cones were placed in insulated plastic boxes cooled by ice-pack medium and with wet towels to prevent the cones from drying out. They were then stored at +6°C for a maximum period of 1 week before cone and seed analyses were made and artificial ripening started. At

Table 1. Data on the collection localities

Type of stand	Locality	Latitude	Longitude	Altitude (m)	Age (yrs)	Year collected
Natural stand	Gällivare (Sweden)	67°5'	22°22'	420	40	1988
Natural stand	Kolari (Finland)	67°16'	23°51'	180	67	1989

the time of collection, the moisture content was determined (dry oven method +103°C, 16 h). In 1988 cones were subjected to artificial ripening for 8 weeks. Temperatures were +6°C and +15°C and light 24 h/day and 8 h/day. For two of the +15°C treatments the conditions were changed after 4 weeks. Light was changed from 24 h to 8 h/day, and for one of the treatments temperature was changed to +6°C.

In 1989 the treatment durations were 2, 4 and 8 weeks. Temperatures were +6°C and +15°C, and treatments were performed under continuous light or in darkness. For three of the treatments, cones with an initial moisture content of around 50% were subjected to repeated drying for 16 h at the beginning. The aim of the drying was to achieve a seed moisture content of about 30%, which has been found to be an optimal level for invigoration of Scots pine seed (Bergsten 1987). In a separate investigation it was found that a cone moisture content of 38% corresponded to a moisture content of about 30% in seeds. The drying was therefore stopped when the cone moisture content had decreased to this level.

Cones were placed in small glass boxes in which the air was saturated with water by means of ultrasonic humidifiers. In 1988 the cones were placed on racks while in 1989 they were treated in plastic cylinders with gables of polytetrafluorethylene membranes (™ GORE-TEX) allowing full gas exchange. The light was supplied using 400 W fluorescent tubes, Philips SON/T giving about 250 µmol m^{-2} s^{-1}. For both years, one treatment was chosen to correspond to standard cone storage in practice (SCS). In this case cones were stored in open air in darkness at +6°C and 60-80% air humidity.

Artificial ripening of cones attached to branches

Branches with attached cones were collected in 1988 in Gällivare on 26 July and 23 August, i.e. very early in the ripening season (Table 1). At each date, 2- to 4-year-old branches with a total of 60 cones were collected for each treatment (12 cones/tree from five trees). Immediately after cutting, the branches were placed in water or in a solution of mineral nutrition (Ingestad 1979) and transported to the apparatus used for artificial ripening. This apparatus was a water reservoir (about 120 x 75 x 15 cm) with the upper part (25 cm high) enclosed in plastic cloth. The apparatus was placed in a climatic chamber in which the humidity was kept high (> 90% RH) as water was pumped down close to the walls through perforated tubes. The branches with cones were kept in jars with the liquid. Temperature was +15°C and both continuous light and 8 h light per day were used.

Seed analyses

As cones and seeds were immature, and could be damaged by kilning, the cones were cut into pieces and the seeds were extracted by hand. Seeds were then dried for 16 h in dehumidified air at 20°-23°C to a moisture content of about 4-7%, and then x-rayed. After that the seeds were germinated on filter paper at +20° and continuous light for 21 days. Germinated seeds were counted after 5, 7, 10, 14 and 21 days. A seed was regarded as germinated when the length of the radicle at least equalled the length of the seed. From the x-ray radiographs, the anatomical development of the seeds and the anatomical potential (Ap), which is the maximum germination ability according to Simak (1980), and the occurrence of mechanically-damaged seeds was determined. The calculation of Ap and germination percentage (GF) was based on the number of filled (F) and undamaged seeds. Germination percentage based on pure seeds (Gp) was also calculated as a measure of the yield of germinable seeds. The number of seeds used for these analyses varied from 100-300 for each treatment.

To determine the effects of temperature, photoperiod, cone moisture content and treatment time for detached cones, and photoperiod for cones attached to branches, on seed ripening an analysis of variance was performed on the arc sine transformed percentages of F, Ap, GF, and Gp by means of GLM-ANOVA

(SAS Institute Inc. 1989). For calculation of intervals of the same confidence level for all values of GF and Gp, the number of germinated seeds which is binomially distributed was assumed to be approximately normally distributed. The standard error was consequently calculated according to the formula:

$$s.e. = \sqrt{\frac{p(1-p)}{N}} \; ; \; p = \frac{G\%}{100} \; ; \; N = \text{number of seeds}$$

The approximation resulted in an upward displacement of the confidence interval limits not exceeding 1% unit and a difference in interval width not greater than 0.3% units.

Results

Artificial ripening –1988

Following the first collection at the end of July, GF was 0. After artificial ripening, GF was still 0, while for cones remaining on the trees, GF increased markedly during the same time. At the second collection in August, GF was still 0 while Ap had increased to 7% (Table 2). Artificial ripening was now more successful and GF increased considerably. For the third collection, only 36% of the seeds germinated despite the fact that Ap was 75% (Table 2). For the best treatment of artificial ripening (+6°C, 24 h light), GF was more than doubled compared to GF at the time of collection (Fig 1).

For the second collection in August, the highest GF was achieved for treatment at +15°C and continuous light. Treatment at +15°C gave a better result than at +6°C if continuous light was used at least for the first 4-week period. For the third collection, GF was highest for treatment at +6°C and continuous light, and for treatment at +15°C and continuous light during the first 4 weeks followed by 4 weeks at +6°C and 8 h light per day. No treatment was superior to the others when Gp was compared; that is, the yield of germinable seeds was about the same for all treatments (Fig. 2). According to the analysis of variance none of the treatment factors showed a significant effect on GF or Gp.

Artificial ripening –1989

At the first collection in late July, both Ap and GF were 0 (Table 3). GF was not influenced by any treatment of artificial ripening, while the cones remaining on the trees reached 73% in GF by September 25 (Fig. 3). At the second collection late in August, GF was only 2% despite that Ap had increased up to 81% (Table 3). GF was higher after artificial ripening than for cones remaining on the trees on September 11 and 25 (2 and 4 weeks ripening), but was lower on October 23 (8 weeks ripening) (Fig. 3).

Table 2. Percentage of filled seeds (F), anatomical potential (Ap), and germination percentage (GF) at the time of collection for cones from Gällivare subjected to artificial ripening in 1988.

Date of collection	F	Ap	GF
26/7	100	0	0
23/8	84	7	0
20/9	82	75	36

Table 3. Percentage of filled seeds (F), anatomical potential (Ap), and germination percentage (GF) at the time of collection from Kolari for cones artificial ripened in 1989.

Date of collection	F	Ap	GF
31/7	68	0	0
28/8	80	81	2

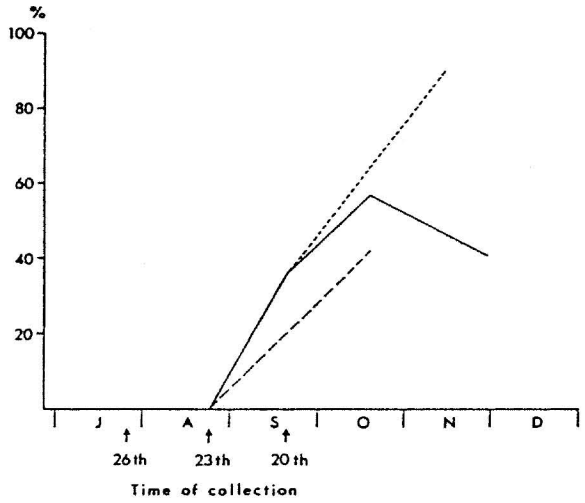

Figure 1. Germination percentage (GF) for the best method of artificial ripening of cones collected on three occasions in 1988 in Gällivare (····· +6°C, 24 h light; ------ +15°C, 24 h light). The solid line represents cones remaining on the trees.

For the second collection, the highest GF after 2 weeks of treatment was achieved for dried cones at +6°C in darkness (Fig. 3). Two weeks later, undried cones at +6°C in continuous light showed the highest GF. Eight weeks of treatment gave a higher GF than 4

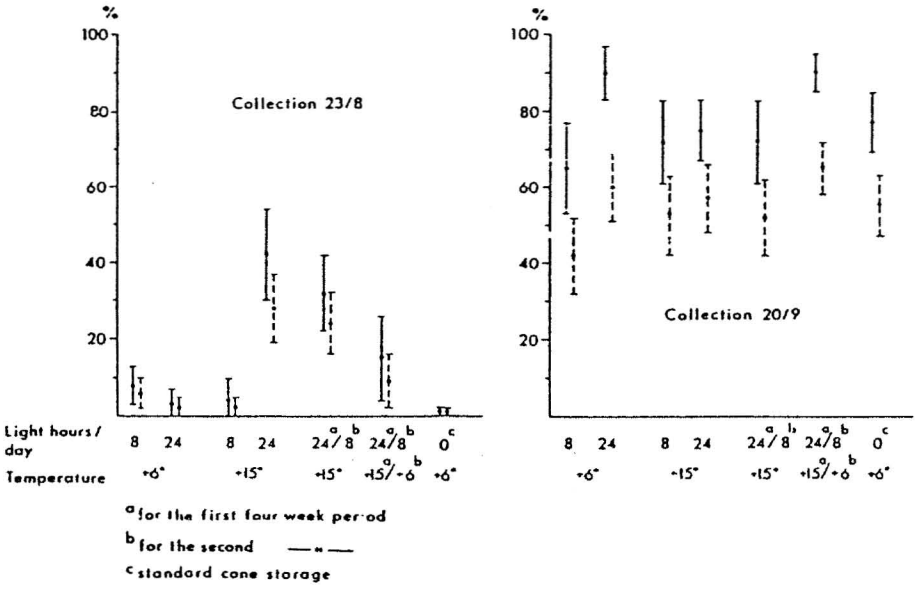

Figure 2. Germination percentage for the different treatments of artificial ripening of cones collected in Gällivare 1988. Vertical lines indicate 95% confidence intervals. Solid lines show germination percentage based on filled seeds (GF), broken lines germination percentage based on pure seeds (Gp).

Table 4. Percentage of filled seeds (F), anatomical potential (Ap), and germination percentage (GF) after 8 weeks of artificial ripening at different photoperiods (3 x 50 seeds). The cones were attached to branches and collected in Gällivare in 1988. Regression coefficient (R2) and F-value for the effect of photoperiod according to analysis of variance of arc sine transformed values (* $P < 0.05$, *** $P < 0.001$).

Light	Date of collection					
	26/7			23/8		
(h per day)	F	Ap	GF	F	Ap	GF
24	18	46	33	62	53	31
8	10	4	0	60	1	0
R2	0.24	0.54	0.30	0.00	0.91	0.59
F-value	1.89	6.99*	2.62	0.00	57.41***	8.80*

weeks of treatment at +6°C in darkness (Fig. 4). The poorest result was achieved by standard cone storage. None of the treatment factors showed a significant effect on GF or Gp.

Artificial ripening of branches with attached cones

Ap and GF were higher after long-day than after short-day treatment (Table 4). For both collections, the short-day treatment seemed to arrest seed development almost completely. The percentage of filled seeds (F) decreased considerably for cones from the first collection (cf. Table 2), indicating that a great number of filled seeds decayed during the treatment period. Water and mineral nutrition gave the same effect and the values are therefore pooled.

Discussion

Artificial ripening of cones

Collection time and anatomical potential

It was possible to improve GF for seeds from cones collected near the end of August by using artificial

ripening even if the seeds were very poorly developed (Ap and GF about zero).

In investigations from more southerly areas, the earliest collection time for successful artificial ripening of Scots pine cones was at the turn of August-September and Ap was always above zero (Nordström 1955; Blomqvist 1973; Kardell 1973; Nygren 1986). Successful results of artificial ripening for slash pine (*Pinus elliottii* Engelm. (Bevege 1965)) and sugar pine (*Pinus lambertiana* Dougl. (Krugman 1966)) were not achieved until anatomical development had advanced considerably.

The best result from artificial ripening was achieved for cones collected on 22 July 1988 and 20 August 1989. In earlier investigations on Scots pine, the end of September (Kardell 1973) and the whole of September (Nordström 1955) were concluded to be the best collection times for artificial ripening. However, these conclusions are based on investigations in more southerly areas. The suitable collection time for artificial ripening is related to the climatic conditions in nature. Cones must always be collected before the first severe frosts appear in the autumn even if they occur before seeds have ripened. This means that collection should be made earlier in northerly areas than in the more southerly ones. However, as a consequence of the more slowly advancing seed development in the north, this will not always be feasible. In the north, seeds might be anatomically very poorly developed and, consequently, the possibilities of achieving high germination capacities are low.

From the above it can be concluded that a prerequisite for achieving an improved seed quality by artificial ripening is that the anatomical potential at the time of collection is above zero, i.e. the embryo must have reached a certain length before the seeds can be artificially ripened. Even if the seeds have reached this anatomical development stage, artificial ripening can still be unsuccessful if cones are collected too early and/or improper ripening conditions are used. Thus, to determine the earliest collection time a combination of

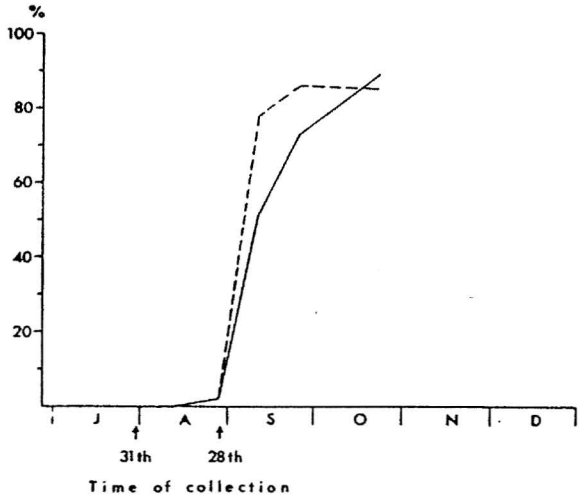

Figure 3. Germination percentage (GF) for the best method of artificial ripening of cones collected on two occasions in Kolari in 1989 (------ +6°C, 0 h light, cones dried, and +6°C, 24 h light, cones not dried). The solid line represents cones remaining on the trees.

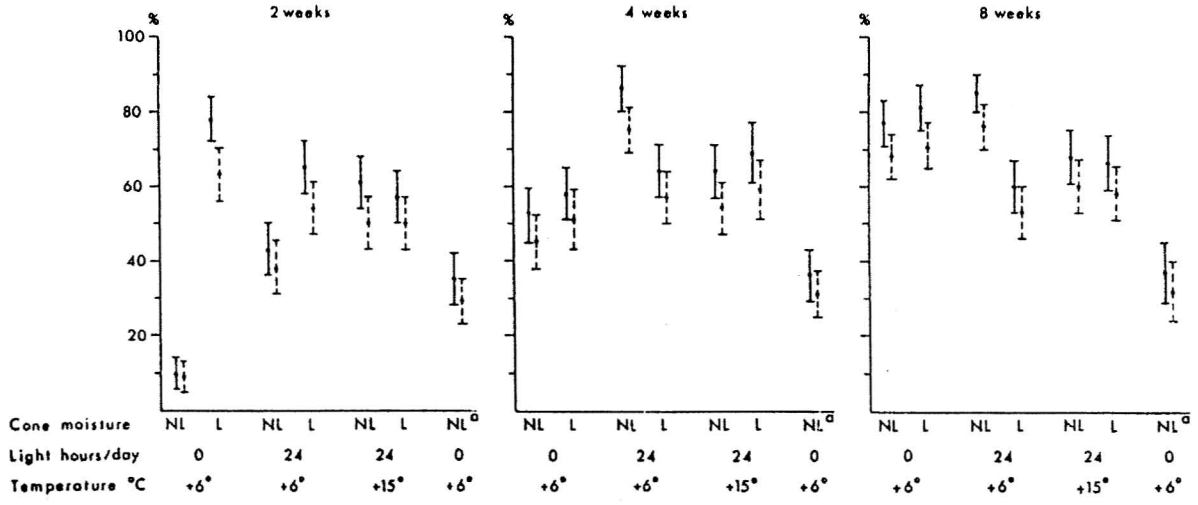

Figure 4. Germination percentage for the different treatments of artificial ripening of cones collected in Kolari on 28 August 1989. Vertical lines show 95% confidence intervals. Solid lines show germination percentage based on filled seeds (GF) and broken lines show germination percentage based on pure seeds (Gp).

morphological, physiological, and biochemical measures of seed ripeness has to be used.

Temperature

In the early stage of ripening, +15°C seemed to be more beneficial for artificial ripening than +6°C. For later collections when the seeds were anatomically more developed, the +6°C treatments gave a slightly better result than +15°C, especially after long treatment time (Figs. 2 and 4). It can be assumed, therefore, that a higher temperature is needed for the early ripening processes, but for complete ripening a period of low temperature is also needed as suggested by Sarvas (1974).

Cone moisture

Drying the cones to a moisture content of 38% seemed to be beneficial for the first 2 weeks of ripening at +6°C, but with increasing ripening time GF for undried cones reached the same level (cf. Fig. 4). In most other investigations on artificial ripening, cone moisture content was relatively uncontrolled.

Ripening with free air circulation proved to be better than ripening in moist peat moss for *Abies grandis* (Dougl.) Lindl. (Pfister 1966), *Abies procera* Rehd. (Franklin 1965), loblolly pine (Waldrip 1970), and red pine (*Pinus resinosa* Ait.) (Winston and Haddon 1981). In these investigations the anatomical development of the seeds was highly advanced at the time of collection. In contrast, for Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) cones collected when the seeds were anatomically poorly developed moist treatment was better than treatment in free air (Silen 1958). It therefore seems that the early-collected cones should be stored in a way that allows a slow lowering of the moisture content, similar to that which occurs in nature.

Light

In several treatments a long photoperiod gave better seed ripening than a short one (Figs. 2 and 4). It seems probable therefore that seed ripening for Scots pine is influenced by photoperiod as it is for other species (Mayer and Poljakoff-Mayber 1989). Another effect of light could be that the respiration losses were limited due to carbon dioxide refixation (Linder and Troeng 1981).

Artificial ripening of branches with attached cones

It is evident that it was beneficial to seed ripening if cones were attached to the branches when collection was made very early. After artificial ripening, GF for cones collected on 26 July was zero (Fig. 2) while GF for cones attached to branches had increased up to 50%. However, the percentage of filled seeds decreased considerably because of the fact that anatomically very-poorly-developed seeds decayed during the ripening time. The positive effect of long photoperiod was far more pronounced for cones attached to branches than for detached cones. Whether this was a photoperiodic or a photosynthetic effect is not possible to determine from these experiments.

Conclusions and suggestions

Based on the results reported here, some preliminary conclusions for artificial ripening of northerly collected Scots pine cones can be drawn.

1. Time of collection: collections should not be made before the end of August.
2. Light: artificial ripening of early-collected cones should be performed with 24 h of light per day.
3. Temperature: for cones collected very early when the seeds are anatomically poorly developed, the temperature should be kept high (+15°C) but only for the first 2-3 weeks. After that, the temperature should be lowered to 5-6°C. For later collections, only low temperature should be used.
4. Cone moisture content: For cones collected very early, the cone moisture content should be kept at the initial level for the +15°C period. After that the moisture should be allowed to decrease slowly as it does in nature. Cones collected later should be allowed to dry from the beginning of the artificial ripening.
5. Time of treatment: The time of treatment should be at least 4 weeks but should not exceed 8 weeks.
6. Analyses during treatment: To decide what ripening conditions are suitable, it is necessary to determine the anatomical development and cone moisture content at the time of collection. During treatment, cone moisture content as well as anatomical development and germination percentage should be regularly tested to determine when to interrupt artificial ripening.

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Dynamics of buried seed population of four annual weeds in potato fields under slash and burn agriculture (Jhum) and terrace cultivation in north-east India

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Abstract

The dynamics of germinable, dormant, and non-viable buried seeds of four annual weeds, *Spergula arvensis* L., *Galinsoga ciliata* (Rafin) Blake, *Polygonum alatum* Ham. and *Ambrosia artemisiifolia* L., were studied in relation to soil depth and season in potato fields under slash and burn agriculture (Jhum) and terrace cultivation in north-east India. An attempt was made to relate variations in seed populations with various soil conditions such as soil temperature, soil moisture and CO_2 evolution. Soil temperature and moisture content decreased with increasing soil depth, while CO_2 evolution increased with soil depth. Temperature and moisture content were lower during winter season compared to other seasons, while CO_2 evolution was higher, presumably due to better aeration. Germinable, dormant, and non-viable fractions of buried seeds fluctuated seasonally, the proportion of germinable seeds being much greater during the rainy season than in other seasons in both fields, and was higher in the terrace field in all seasons. The proportions of enforced-dormant, induced-dormant and dead fractions of buried seeds were higher during winter in all species except *S. arvensis*. The proportion of germinable seeds declined with increasing soil depth, while enforced-dormant seeds increased with soil depth down to 15 cm, beyond which the proportion of this fraction also declined.

Résumé

La dynamique des graines enfouies qui ont la capacité de germer, dormantes et non viables de 4 mauvaises herbes annuelles, *Spergula arvensis* L., *Galinsoga ciliata* (Rafin) Blake, *Polygonum alatum* Ham. et *Ambrosia artemisiifolia* a été étudiée en fonction de la profondeur du sol et de la saison, dans des champs de pommes de terre faisant l'objet d'une culture sur brûlis (Jhum) et faisant l'objet d'une culture en terrasses, dans le nord-est de l'Inde. On a tenté d'établir un rapport entre les variations dans les populations de graines et différentes conditions du sol telles que la température, l'humidité et le dégagement de CO2. La température et la teneur en humidité du sol diminuent avec l'augmentation de la profondeur, tandis que le dégagement de CO2 augmente. La température et la teneur en humidité sont moins élevées en hiver que durant les autres saisons alors que le dégagement de CO2 est supérieur, présumément à cause d'une meilleure aération. Dans les graines enfouies, la proportion de celles qui ont la capacité de germer, qui sont dormantes ou non viables varie d'une saison à l'autre; sur les deux terrains, la proportion des graines qui avaient la capacité de germer était nettement supérieure durant la saison des pluies à ce qu'elle était durant les autres saisons, et, sur le terrain en terrasses, elle l'était en toutes saisons. La proportion de graines en dormance provoquée, en dormance induite et mortes, par rapport au total des graines enfouies, était le plus élevé durant l'hiver chez toutes les espèces sauf *S. arvensis*. Plus le sol était profond, moins il y avait de graines ayant la capacité de germer; la proportion des graines en dormance forcée augmentait avec la profondeur du sol jusqu'à 15 cm, après quoi cette proportion se mettait à diminuer aussi.

Introduction

Buried seeds of annual weeds show a cyclic change in their dormancy behaviour (Baskin and Baskin 1985) which is regulated by a number of soil conditions such as light (Black 1969), temperature (Mukherjee *et al.* 1980, Baskin and Baskin 1984), moisture (Schafter and Chilcote 1970), 0_2 supply (Russell 1961), pH (Ellenberg 1950), CO_2 level (Popay and Roberts 1970) and texture (Hay 1962). The objective of this study was to examine the effects of some of these factors on the behaviour of buried-seed populations of four annual weed species *viz.*, *Spergula arvensis* L., *Galinsoga ciliata* (Rafin) Blake, *Polygonum alatum* Ham. and *Ambrosia artemisiifolia* L. in potato crop fields under contrasting types of cultivation, *viz.* slash and burn agriculture (locally called "Jhum") and terrace cultivation widely practiced in north-east India.

Materials and methods

The study was conducted on Jhum and terrace potato fields at Upper Shillong (latitude 25°34'N, longitude

91°56'E, altitude 1825 m), about 12 km south of Shillong, the capital of the state of Meghalaya in north-east India. The Jhum field was abandoned for 4 years before it was brought under cultivation in 1988, while the terrace field had been cultivated continuously for the previous 8 years with regular use of herbicides, chemical fertilizers, and farmyard manure.

The area receives an average of 2616 mm rainfall (based on rainfall data during 1985-1989), 90% of which occurs during the rainy season (May to September) which is generally warm. October (autumn) represents a transition between the rainy and winter seasons. November to February (winter), are generally dry and cold. March and April are relatively warmer and represent spring. The soil is lateritic, sandy loam, pale brown in colour and acidic (pH 5.1-5.7).

Twenty soil cores (5.327 x 20 cm depth) were collected from each field at monthly intervals during October 1988 to September 1989. They were brought to the laboratory and cut horizontally corresponding to 0-5, 5-10, 10-15 and 15-20 cm soil depths, air-dried and sieved through a 2 mm metallic sieve to discard the stone, root and large litter particles. For each soil depth, the twenty samples were pooled and divided into 6 replications of 100 g each. Three replications were used to determine the total viable buried seeds (V_t) following the method outlined by Roberts and Ricketts (1979). Viability was determined by using 0.1% tetrazolium salt (Malone 1967). The other three replications were spread uniformly in plastic Petri dishes (10.5 x 1.5 cm), watered and kept in a seed germinator at an alternating temperature of 7-30°C, with 12 h at each temperature; light was provided during the higher temperature. Seedlings emerging in the Petri dishes were identified and counted daily. After cessation of germination, soil was again assessed to determine the remaining viable seeds in the sample. The number of seedlings was considered equivalent to the number of seeds in a state of enforced-dormancy (ED) at the time of recovery, while ungerminated viable seeds were grouped into the induced-dormant (ID) fraction and the rest were regarded as non-viable and empty seeds, and were put into the category of dead (D) seeds. The germinable (G) fraction was estimated by subtracting the number of enforced- and induced-dormant seeds from the total viable seeds in soil. At each sampling date soil temperature, soil moisture and CO_2 evolution were also determined at different depths. CO_2 evolution was measured according to MacFadyen (1970).

Table 1. Seasonal variation in soil temperature, soil moisture and CO_2 evolution at different soil depths in Jhum and terrace fields at Upper Shillong, India.

		Jhum			Terrace		
Season	Depth cm	Temperature (°C)	Moisture (%)	CO_2 evolution (mg kg^{-1} 24 h^{-1})	Temperature (°C)	Moisture (%)	CO_2 evolution (mg kg^{-1} 24 h^{-1})
Winter	0-5	8.2	16.3	34.0	7.8	23.6	30.0
	5-10	7.2	16.0	38.2	7.2	23.2	32.6
	10-15	5.4	16.0	40.8	6.8	23.0	38.0
	15-20	4.4	16.2	46.0	6.0	23.2	40.4
Spring	0-5	19.0	20.6	28.0	18.5	24.6	25.0
	5-10	18.0	20.0	32.0	17.5	24.8	28.0
	10-15	16.0	20.4	34.0	16.0	24.0	31.0
	15-20	15.2	20.0	36.0	15.5	23.8	32.0
Rainy	0-5	21.0	28.7	30.2	20.5	32.4	28.0
	5-10	20.4	28.4	34.0	19.0	32.0	30.0
	10-15	18.6	28.4	36.0	18.0	31.8	32.0
	15-20	16.8	23.6	40.2	17.0	31.6	34.2
Autumn	0-5	18.2	26.4	21.0	18.4	27.9	15.0
	5-10	16.4	26.4	23.0	16.4	27.9	16.0
	10-15	14.5	25.8	25.0	14.8	26.8	18.0
	15-20	12.6	25.4	28.6	14.0	26.6	22.0

Results

Spatial and Temporal Variation in Soil Conditions

Soil temperature and moisture were higher in the surface soil declining gradually with increasing soil depth (Table 1). Soil moisture and soil temperature decreased during the winter season and increased during the warm rainy season under both types of cultivation. Soil temperature and CO_2 evolution were relatively higher in the Jhum field compared to the terrace field while soil moisture was higher in the terrace field (Table 1). CO_2 evolution in both fields increased with increasing soil depth and was always higher during the winter season.

Vertical distribution of seeds in the soil

The population of germinable seeds in all four species declined with increasing soil depth in both Jhum and terrace fields (Fig. 1 and 2), but their number was always greater in the terrace field. The proportion of seeds under enforced-dormancy, however, increased down to 15 cm and then declined beyond this depth (Fig. 1 and 2). This was true for all the species irrespective of the fields. The population of seeds that were in the state of induced-dormancy gradually increased with increasing soil depth, but significant differences due to the depth ($P < 0.1$) was found only in *P. alatum* and *A. artemisiifolia* (Fig. 2). The proportion of non-viable seeds was also high in lower layers of soil in both Jhum and terrace fields.

Seasonal variation in soil seed bank

The number of germinable seeds showed three peaks (October, April, and July) in *S. arvensis*, two (November and June) in *G. ciliata* and only one (June) in *P. alatum* and *A. artemisiifolia*. This trend was broadly similar in both types of crop fields. *S. arvensis* seeds which were in the state of enforced-dormancy did not show marked seasonal variation in the Jhum field, but their proportion was higher during winter months in the terrace field (Fig. 1). In the other three species, seeds in enforced-dormancy were proportionally higher during the rainy season in both crop fields. Seeds of *S. arvensis* under induced-dormancy were recorded throughout the study period while in other species, such seeds were found only in the winter months. Seed mortality was almost constant throughout the year for *S. arvensis*, but other species suffered higher mortality during winter (Fig. 1 and 2).

Discussion

The climatic conditions which regulate germination of weed seeds in the soil by altering temperature and moisture conditions in the soil-seed environment seem to have played a key role in bringing about seasonal variation in the viable seeds observed in this study. Suppression of germination due to low soil temperature and moisture appeared to be the chief cause of accumulation of larger number of viable seeds in the soil during winter. Conversely, higher temperature and moisture during the rainy season, which favoured seedling emergence in large numbers, were responsible for a reduction of viable seeds. However, different germination peaks for different species could be attributed to their specific germination requirements. In this respect *S. arvensis* had a wider ecological amplitude than the winter- and rainy- season-annual, *G. ciliata*, and the rainy-season-annuals, *P. alatum* and *A. artemisiifolia*. Seeds of all four species showed better germination during the rainy season before they entered enforced-dormancy during the late part of this season. As winter season approached, many of them become induced-dormant. In all species except *S. arvensis*, seeds held in enforced-dormancy during the rainy season encountered conditions that caused induced-dormancy to develop.

Lack of light and low temperature in the lower soil layers prevent germination by enforcing a higher proportion of seeds to enter into a dormant state (Wesson and Wareing 1967; Black 1969). This also appeared to be true in the present study as when the soil from the lower depths were kept under favourable conditions in an incubator, dormant seeds started germinating. The higher concentration of CO_2 in the lowest soil depth possibly converted some of the enforced-dormant seeds into an induced-dormant state, as indicated by a lower proportion of seeds under enforced-dormancy at this soil depth. Low moisture content during the winter months was probably responsible for damaging a greater proportion of the viable seeds in the soil. This view is supported by the work of Schafter and Chilcote (1970).

Restricted gas exchange during the time when seeds are buried in the soil plays an important role in inducing seed dormancy. Higher soil-CO_2 evolution during winter might have prevented germination of many seeds by inducing dormancy. The lower proportion of induced-dormant seeds in the terrace field compared to the Jhum field could also be related to a lower rate of CO_2 evolution in the terrace field. This may be attributed to a greater soil disturbance in the terrace field due to frequent tillage and hoeing. Conversely, less soil disturbance in the Jhum field resulted in the accumulation of induced-dormant seeds in deeper soil layers. The significantly ($P < 0.05$) higher proportion of viable seeds in the less-disturbed Jhum field indicates that disturbance also plays an

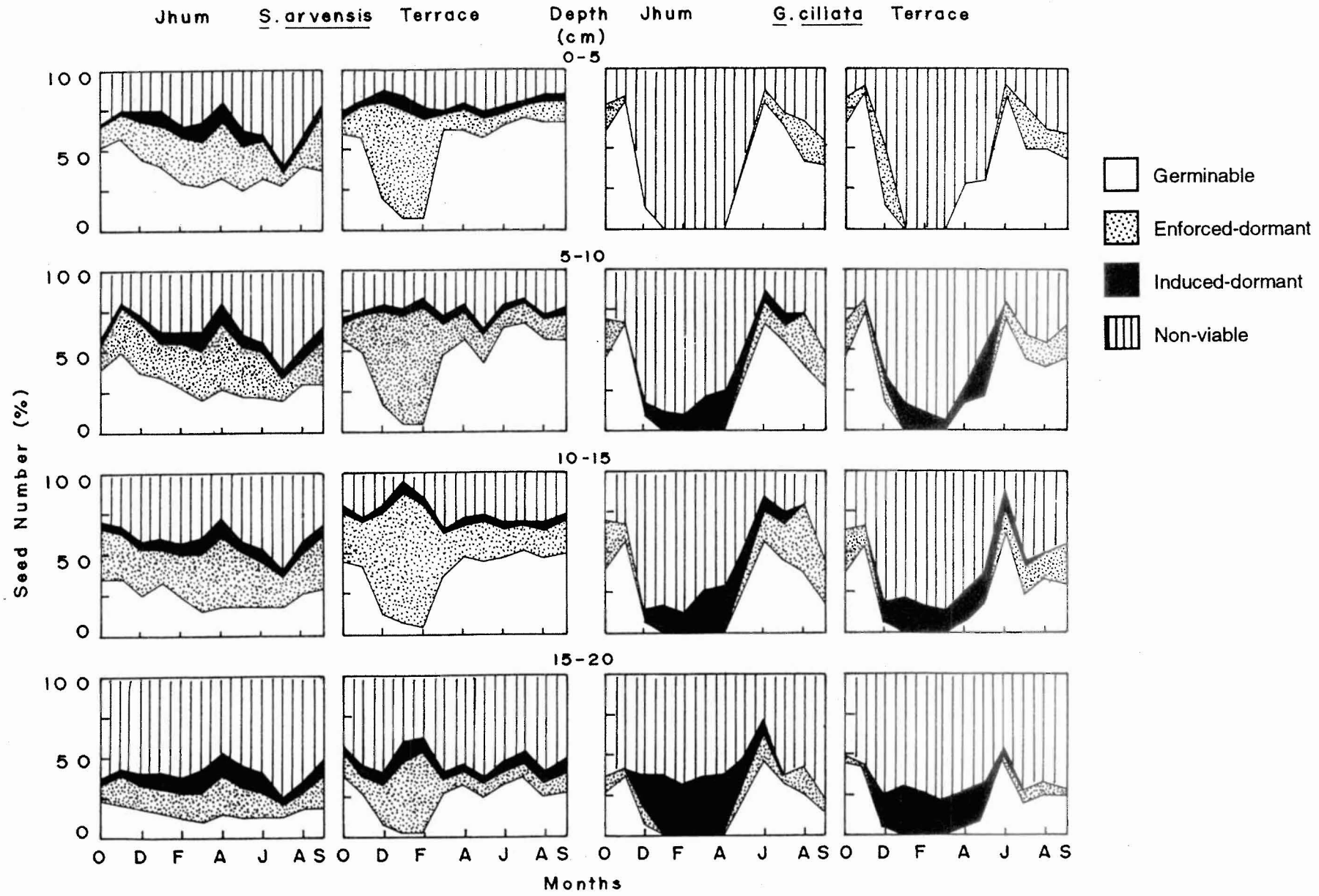

Figure 1. Seasonal variation in the size of different fractions of buried seed population of *S. arvensis* and *G. ciliata* in the potato crop field under Jhum and terrace cultivation.

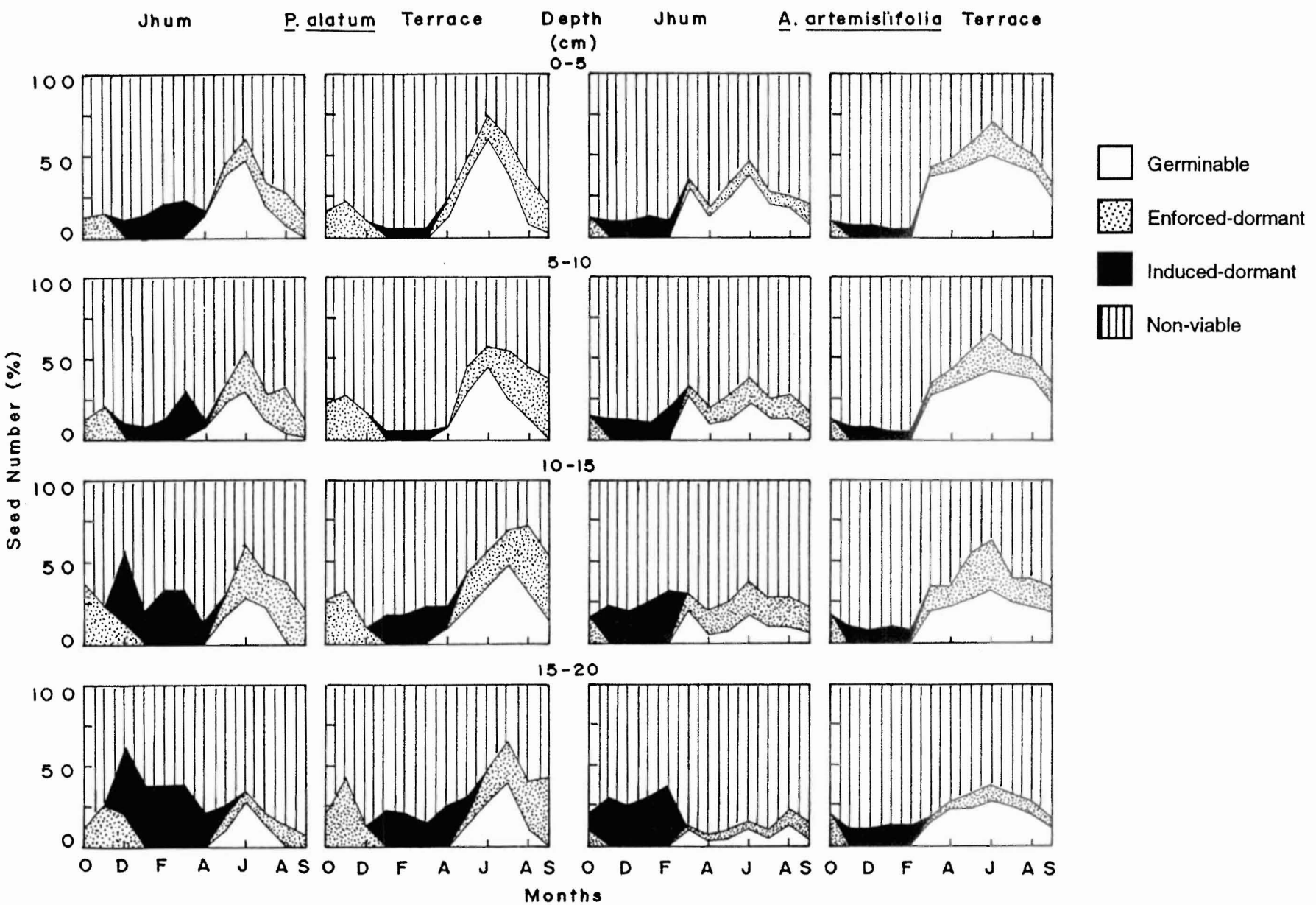

Figure 2. Seasonal variation in the size of different fractions of buried seed population of *P. alatum* and *A. artemisiifolia* in the potato crop field under Jhum and terrace cultivation.

important role in regulating the size of viable weed-seed population. The larger population of viable seeds at deeper soil layers in the terrace field may be attributed to the greater depth and higher frequency of ploughing.

Acknowledgement

We wish to thank the University Grants Commission for providing financial assistance.

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Barriers to germination of *Larix occidentalis* and *Larix lyallii* seeds

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Abstract

Larix occidentalis Nutt. (western larch) and *Larix lyallii* Parl. (alpine larch) grow in the upper Columbia River Basin of North America. Western larch often is a major component at low to moderate elevation forests while alpine larch is found only at timberline in some of the high mountains. Both species are monoecious, and flower buds develop throughout the crown. Western larch seed cone buds open throughout April to early May and the cones mature in August; and in May and mid-September for alpine larch. Developing strobili can survive spring frosts as low as -4°C in western larch and -10°C in alpine larch. Major barriers to quality western larch seeds occur during cone development. Mature dominant and codominant trees produce megasporangia every year, but frost or insects or both often reduce the potential by 75% soon after bud burst. Then, mature cones usually have less than 20% filled seeds; the remainder are mostly empty or damaged by insects. Seed dormancy was easily overcome in 38 tests by stratification for 30 days at 1°C in the dark. Germinative energy averaged 75% at 7 days and germinative capacity averaged 94% at 13 days. Previous attempts to germinate alpine larch seeds under laboratory conditions were mostly unsuccessful. But about 95% of the seeds with embryos germinated after stratifying in moist peat in leach tubes for 30 days in the dark at 2°C and then incubating them at 22°C with 16 hours of fluorescent light and 8 hours of dark. Germinative energy was 70% at 10 days and germinative capacity was 90% at 16 days. Soil chemistry may be important in preparing alpine larch seeds for germination.

Résumé

Le *Larix occidentalis* Nutt. (mélèze occidental) et le *Larix lyallii* Parl. (mélèze subalpin) poussent dans le bassin supérieur du fleuve Columbia en Amérique du Nord. Le mélèze occidental constitue souvent une essence importante dans les forêts situées à faible ou moyenne altitude, alors que le mélèze subalpin n'est trouvé qu'à la limite de la végétation arborescente dans les hautes montagnes. Les deux espèces sont monoïques et les bourgeons floraux se développent partout dans la cime. Les bourgeons reproducteurs du mélèze occidental débourrent en avril et au début de mai, et les cônes ont leur maturation en août; les dates correspondantes chez le mélèze subalpin sont mai et la mi-septembre. Les strobiles en développement peuvent survivre à des gelées printanières de -4°C dans le cas du mélèze occidental, et de -10°C dans le cas du mélèze subalpin. Les principaux obstacles à l'obtention de graines du mélèze occidental qui soient de bonne qualité apparaissent durant le développement des cônes. Les arbres à maturité dominants et codominants produisent des mégasporanges chaque année, mais le gel, les insectes ou ces deux facteurs réduisent souvent le potentiel de 75 % peu après l'éclosion des bourgeons. Par surcroît, les cônes à maturité ont habituellement moins de 20 % de graines remplies; les autres sont surtout vides ou endommagées par des insectes. La dormance des graines a été facilement levée, lors de 38 tests, par une stratification de 30 jours à 1°C dans l'obscurité. La vigueur germinative était d'en moyenne 75 % à 7 jours et la capacité germinative était d'en moyenne 94 % à 13 jours. Les tentatives antérieures de faire germer des graines de mélèze subalpin au laboratoire ont eu peu de succès. Mais environ 95 % des graines porteuses d'embryons ont germé après une stratification dans la tourbe humectée, disposée dans des tubes de lessivage pendant 30 jours, dans l'obscurité et à 2°C; ce traitement était suivi d'une incubation à 22°C avec éclairement au fluorescent de 16 heures suivi de 8 heures d'obscurité. La vigueur germinative était de 70 % à 10 jours et la capacité germinative était de 90 % à 16 jours. La chimie du sol peut constituer, pour les graines du mélèze subalpin, un important facteur de préparation à la germination.

Introduction

Western larch (*Larix occidentalis*), sometimes called tamarack or western tamarack, grows in the mountains (610 to 2130 m) of the Upper Columbia River Basin in southeast British Columbia, Canada, in northwestern Montana, northern and west-central Idaho, east of the crest of the Cascade Mountains in Washington, and in north-central and northeastern Oregon (Little 1979). Pollen and seed buds open in April and early May (Schmidt and Shearer 1990). Although vigorous larch as young as 8 years occasionally produce cones, heavy cone production occurs on trees 50 to 400 years of age (Schmidt *et al.* 1976).

Alpine larch (*Larix lyallii*) is found only at timberline in the high mountains (1520 to 3010 m) of southeastern British Columbia and adjacent southwestern Alberta, north-central Washington, northern Idaho, and western Montana (Arno and Habeck 1972; Little 1979). Seed and pollen cone buds open in late May and pollination occurs in June (Arno 1990). Cones occur on 100-year-old trees; abundant cone crops were produced on trees from about 200 years until death (Arno and Habeck 1972). For alpine larch, an average lifespan of 500 years, and a 1000-year maximum age has been estimated (Arno and Habeck 1972). But Worrall (1990) speculates that alpine larch in Manning Park, British Columbia, attain much greater age.

Western larch begins growth earlier than its associated conifers (Shearer and Theroux 1986). As temperature and daylight increase in late March and April, the flower and vegetative buds quickly swell, then open. Even before the leaves elongate in the spring to mix their light-green hue with darker green needles of the other conifer species, seed- and pollen-cone development begin. Much of the viable seeds have dispersed when the needles of western larch change to yellow from mid to late October.

Alpine larch, in contrast, compacts its major growth functions into a 3-month period. Floral bud development starts in late May, a few days before the needles begin to elongate (Arno and Habeck 1972). When alpine larch foliage turns golden in mid-September, heralding the advent of autumn, much of the seed crop has dispersed. Virtually all viable seeds drop from the cones by the end of September.

Alpine larch seeds reportedly are hard to germinate (Richards 1981; Shearer 1961) and seedlings are difficult to grow (Arno 1990). Shearer (1961) suggested embryo dormancy as a possible mechanism for germination failure; he overcame dormancy by soaking seeds in hydrogen peroxide for 24 hours at room temperature instead of stratification. He did not test the seedlings resulting from the hydrogen peroxide treatment for growth and development following germination.

The purpose of this paper is to discuss barriers to maturation, extraction, and germination of western and alpine larch seed. Our conclusions are based mostly on the results of current studies on cone and seed production of western and alpine larch.

Methods

This paper reports interim results of two studies. The first study was initiated to determine why western larch has low cone and seed yields in the Northwestern United States. The second study was designed to artificially cross alpine and western larch and to germinate the seeds and grow seedlings of each species and the hybrid.

Western larch

A study of western larch cone and seed production in Idaho, Montana, Oregon, and Washington began in 1985 and included 14 stands ranging in age from 46 to 100 years. At each location 10 open-growing trees, except 5 at one location, were selected using the following criteria: crown length at least 40% of the total tree height, accessibility for climbing, and evidence of prior cone production. After snowmelt each spring, we visited the study areas and estimated the number of new seed cones on each sample tree using binoculars. The five trees with the greatest seed cone counts were climbed and the number of developing cones was accurately estimated by counting the number of (1) branches with seed cones and (2) new seed cones (living and dead) on six random branches (two from each third of the crown). We estimated the number of potential seed cones by multiplying the average number of cones per sample branch by the number of cone-bearing branches. We estimated seed cone survival in August by counting the number of cones that matured on the six branches selected in the spring. Seed cone mortality was determined by subtracting surviving cones from the total cones counted at the first visit of the year.

We documented cone development and time and cause of cone damage for the two trees at each site with the most cones, at recurring observations on 25 cones marked at the first examination. Cone length and damage were noted at each visit. Dead cones were removed and probable cause of death identified.

When available, 50 mature cones were picked at each study area in late August for analysis. Ten of the 50 cones were randomly selected for detailed study, then placed in separate paper bags and allowed to dry

and open. Before analysis, these cones were heated to 43°C for 24 hours in a forced-air oven. Cone analysis consisted of dissecting the cones and counting the number of scales: lower infertile, upper infertile, and fertile. After accounting for the seeds from each cone that were eaten by insects, the undamaged seeds were x-rayed and categorized as abnormal, damaged, empty, malformed, or filled. The seeds were then placed on pads in germination boxes, moistened with tap water and stratified at 1°C for 30 days. Germination was tested by incubating for 3 weeks using 12 hours at 24°C under incandescent and fluorescent light, and 12 hours at 19°C in the dark.

Alpine larch

We are currently studying hybridization between western and alpine larch in western Montana. This research includes controlled reciprocal crosses between the two species. We selected a park-like stand of nearly pure alpine larch on Carlton Ridge, about 30 km southwest of Missoula, Montana. It grows on a well-developed mantle of volcanic ash that overlays schist and gneiss and is regarded as a climatic climax on zonal soils (Arno and Habeck 1972). Six alpine larch with straight boles, about 20 m tall and ranging in age from 100 to 450 years old, were chosen in 1990. All selected trees had prolific seed-cone potential, at least 1000 megasporangia per tree. Developing female strobili were isolated with pollination bags about mid-May, well before any natural pollen was dispersed. Western larch pollen was injected into the bags, onto the females, when the females were judged receptive. Pollination bags were removed once the female cones closed, about 10 days after pollination, and were replaced with nylon mesh bags to protect the cones from insect depredation and to retain early maturing seeds. By mid-September, when cones were mature, we collected cones with hybrid and open-pollinated seeds from the sample trees. Cones were air-dried until they opened completely (about 2 weeks) and the seeds were easily shaken out. Seeds were dewinged, cleaned, and stored in sealed glass vials until viability and germination tests could be done.

Viability of alpine and hybrid seeds was tested by cutting the seeds and treating intact embryos with tetrazolium chloride (Moore 1976). Percent filled seeds, and percent seeds with indicated viable embryos, were recorded. Germination was tested by a procedure designed to approximate natural conditions. Leach tubes (25 mm diameter by 160 mm length) used in standard greenhouse operations were filled to within 2 cm of the top with Terra-Lite Forestry Mix[1], a peat mix blended for seedling production. This medium was thoroughly saturated with tap water. An aliquot of seeds was placed on the soil and covered with about 1 cm of soil mix and wetted. Tubes were placed on racks and covered with clear polyethylene to retain moisture, and refrigerated at 2°C. After 30 days in the cold room, tubes were placed under fluorescent lights at 30°C. Germination was counted weekly.

Results and discussion

Western larch

Seed-cone buds were produced on all sample trees each year, except within the youngest stand. An earlier study (Shearer 1986) also showed the consistency of western larch to produce cones each year. The number of potential seed cones varied widely each year (1) by location and (2) among sample trees at each of the 14 locations. Seed cone buds opened throughout April and early May, depending on year and elevation; earlier in 1986, 1987, and 1990 than in 1985, 1988, and 1989. Flower buds usually opened from early to mid-April on trees at elevations < 1000 m; from mid- to late-April at elevations between 1000 and 1400 m; in late-April at elevations between 1400 and 1600 m; and about May 1 at higher elevations. These data refine more general information available in current source books (Rudolf 1974; Schmidt and Shearer 1990).

Seed cones were located throughout the live crowns of sample trees. An average of 46% (a range of 28% to 67%) was produced within the upper third of the crown, 41% (29% to 66%) within the middle third, and 13% (3% to 24%) in the lower third. An average of 1393 potential cones were produced per tree per year ranging from 163 to 1270 in 1985, 24 to 2839 in 1986, 2 to 2108 in 1987, 44 to 3029 in 1988 (Shearer 1990), 0 to 15 169 in 1989, and 1 to 6843 in 1990.

Cone development proceeded rapidly after the buds opened. Cones reached about 80% of their total length by the end of April (2 to 4 weeks after bud burst), 90% by the end of May, and completed their growth by the end of June. Cones attained their maximum length on low-elevation trees about 2 weeks sooner than cones growing on trees at higher elevations.

Less than one-fourth of the potential cones matured during this study. Seed cone survival ranged from 0% to 35% at the nine Idaho sites, 25% to 30% at the two Montana sites, 9% to 62% at the Oregon sites, and 67% at the Washington site. Cone survival was greatest in 1987 (45%) and 1989 (39%), much lower in 1988

[1]The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

Table 1. Composition of western larch seeds extracted from mature cones collected at 14 study sites, 1985–1990

	Mean		s.d.	Range
Seed composition	no.	%	no.	no.
Potentially viable	19	16	20	5-66
Empty	54	47	30	22-83
Insect damaged	28	25	28	6-53
Abnormally developed	12	11	18	7-32
Malformed embryos	1	1	3	< 1-3
TOTAL	114	100	18	18-164

(8%), 1985 (6%), 1990 (5%), and least in 1986 (< 1%). Most seed cones died in April or early May after flower buds opened, usually the result of frost or insects.

Low temperatures often froze all or most of the cones, except at low-elevation sites. From 1985 through 1990, only 1987 had a low incidence of frost at all locations. We are not sure how long any given temperature below freezing must be maintained to kill conelets, but overnight low air temperatures of -4°C or colder were usually associated with frozen seed cones.

Four insect species damaged western larch seed cones. (1) The larch cone maggot (*Strobilomyia laricis* Michelsen Diptera: Anthomyiidae) was the most serious pest. It killed some cones, but more typically destroyed most developing seeds in cones as it fed spirally around the axis.

(2) The western spruce budworm (*Choristoneura occidentalis* Freeman; Lepidoptera: Tortricidae) can destroy all larch cones during moderate or high infestations (Fellin and Shearer 1968; Dewey and Jenkins 1982). Fortunately, populations of this insect were low and it caused relatively little damage to cones and seeds at only 5 of the 14 study areas.

(3) A woolly adelgid (*Adelges viridis* = *A. strobilobius* Ratzeburg; Homoptera: Phylloxeridae) was found associated with larch cones most years at all study sites. Cone development decreased when large numbers of this sucking insect covered the surface. Although this adelgid occasionally kills cones and seeds, the greatest problem is associated with the failure of scales on mature cones, damaged by the woolly adelgid, to fully open and release their seeds.

(4) Cone midges (Resseliella) often infested scales of western larch seed cones, but rarely caused direct damage to either the cone or the seeds. Pitch from scales damaged by this insect frequently impaired the extraction of seeds from mature cones.

Analysis of mature cones collected from each study area showed low potential seed viability and high and damaged seeds (Table 1).

Only 16% of the seeds were potentially viable. This ranged from 5% to 28% for 13 sites in Idaho, Montana, and Oregon, but was 57% at the Washington site (for 1989, the only year that cones matured there). One-fourth of all seeds were damaged by insects, mostly by the larch cone maggot and on some sites by the western spruce budworm. Analysis of cones without evidence of insect damage showed an increase of 10% (to 26%) for potentially viable seeds, 7% (to 54%) for empty seeds, and 1% (to 2%) for seeds with malformed embryos; and a decrease of 4 percent (to 7%) for abnormally developed seeds.

Most of the seeds were hollow, which is common among other species of the genus *Larix* (Rudolph 1974). For example, European larch (*L. decidua)* always has a high proportion of empty seeds caused by, in one study, lack of pollination (17%), disturbances in morphogenesis (9%), failure of pollen (17%), and embryo degeneration (30%) (Kosinski 1987).

Western larch seeds germinate quickly after stratification at 0°C for 30 days in the dark (Schmidt 1962; Shearer 1977). Germinative energy (GE) for 38 tests (14 stands spanning 6 years) averaged 75% after 7 days of incubation. GE ranged from a high of 88% in 5 days to a low of 49% in 8 days. For these same tests, germinative capacity averaged 94% in 13 days and ranged from 99% in 13 days to 87% in 15 days. The average total germination for all of the tests was 98%, ranging from a high of nearly 100% in 1987 to a low of 79% in 1990.

Alpine Larch

Viability tests showed a reasonably high success rate for seed production in the open-pollinated cones. About 50% of the seeds tested were filled, and 99% of

the embryos stained positive for viability. But only about 1% of the hybrid seeds, except for tree no. 6, were filled; all of the hybrid embryos tested with TZC were viable. Tree no. 6 had about 30% of the seeds filled with viable embryos.

Germination in the Leach tubes paralleled the cutting/staining tests. This is noteworthy because of the previous poor germination found by Shearer (1961) and Richards (1981). GE was 70% 7 days after stratification, GC was 90% in 16 days, and total germination reached 95% in 22 days. We have about 800 alpine larch seedlings and about 200 hybrid larch growing at this time, and most of them appear healthy. We consider this highly successful in contrast to Richards (1981) who obtained only two living seedlings from 5000 seeds planted; those seeds had an indicated viability (by the TZC test) of 40% to 60%.

The poor seed set of alpine larch cones pollinated with western larch pollen probably was caused by placing pollination bags over the cones too early. Development of open-pollinated cones appeared normal, despite variable and adverse weather conditions on Carlton Ridge. During the first 10 days of May 1990, temperatures were very warm, up to 28°C, and alpine larch strobili began to develop quickly, even though the snowpack was still 3-4 m deep. We bagged cones at this time because it looked as if pollen would be dispersed within a week. But a cold period followed and temperatures fell to -8° to -14°C; cone development virtually stopped. This cold period lasted about 10 days; temperatures finally rose to 18°-22°C and non-bagged cones resumed development. They appeared to be uninjured by the cold. The bagged cones responded differently. The bags protected the cones from the severe weather and the cones continued to develop slowly, but their scales did not fully reflex as they should when they are receptive to pollen. But we pollinated the bagged cones, hoping for some success. Indeed, as the temperatures warmed, the bagged cones resumed growth and appeared to develop normally. At the end of the cold period, but before any pollen was shed, we put isolation bags on one additional tree (no. 6) because of the abnormal development we noticed for cones bagged 2 weeks previously. Tree no. 6 had about 30% filled, viable seeds, compared to 1% for the cones bagged earlier. Therefore we believe that the premature bagging, and not genetic incompatibility, caused the poor set of hybrid seeds.

Conclusions

Western larch

Vigorous, open-grown western larch over 50 years of age usually produce seed-cone buds each year over its range. But cone maturity is uncertain because low temperatures at, or soon after, bud burst can kill most or all of the succulent, rapidly developing strobili. The larch cone maggot and western spruce budworm larvae can kill cones outright or damage most of the viable seeds. Woolly adelgids and scale midges may damage a few seeds, but their damage to the cone scales hinders complete seed extraction. This 6-year study showed that for every 1000 potential western larch seeds at the time of bud burst only an average of 39 matured as filled seeds. Because this study shows the uncertainty of adequate cone production in many natural stands, some agencies now consider potted seeds orchards the safest and probably the most economical way to produce high quality seeds for their regeneration needs. Establishment of western larch seed orchards and seed production areas are recommended for low-elevation sites with a low probability of frost in April.

Alpine larch

Our work with alpine larch seed germination and subsequent seedling growth is a significant breakthrough that can aid others who work with this species. Stratifying seeds *in situ* in a peat mix (Terra-Lite) overcame barriers to germination experienced by Richards (1981) and Shearer (1961). In the future, public and professional attention will focus more on multiple outputs from forest ecosystems. High-elevation watersheds are important for water yield and esthetics. Research dealing with the tree species indigenous to these ecosystems, such as alpine larch, will be needed to fully understand their functional relationships there. This research undoubtedly will at times deal with cultured seedlings, and we now have a method to germinate and grow alpine larch.

Acknowledgements

Thanks to Leon J. Theroux, Biological Technician, for assistance in data acquisition and summary of the western larch data and to Jack A. Schmidt, Forester, for his help in germination and culture of alpine and hybrid larch seeds.

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Effects of seed treatment methods on germination of *Simarouba glauca* var. *latifolia* Cronq.

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Abstract

The seeds of *Simarouba glauca* var. *latifolia* Cronq. exhibit dormancy problems during storage and subsequent germination. Five pre-germination treatments were selected to investigate methods to break post-harvest dormancy and increase both rate and total germination of seed. Complete removal of the kernel from the seed coat and seed coat cracking improved germination in both the nursery and the laboratory for SECID 2290, a seedlot that was handled by conventional procedures. PADF 945, a seedlot that differed by an additional step of tumbling the seeds in an abrasive drum, exhibited no differences between the manual scarification methods and the control. Water soaks did not improve germination for either seedlot. Correlation between laboratory and nursery germination totals was poor, mainly due to differences in germination substrate. Germination in the laboratory was lower than germination under nursery conditions. Seedling heights measured 2 months after sowing in the nursery showed poorest growth when the kernel was planted without its seedcoat.

Résumé

Il y a des problèmes avec les graines de *Simarouba glauca* var. *latifolia* Cronq. en dormance qui sont entreposées, ainsi qu'avec la germination subséquente. Cinq traitements ont été retenus dans un plan de recherche sur les moyens de lever la dormance qui suit la récolte et d'augmenter le taux de germination des graines et la germination totale. La séparation de l'amande et du tégument ainsi que le cassage de ce dernier ont amélioré la germination du lot de semences SECID 2290 en laboratoire et en pépinière; ce lot a été traité au moyen des méthodes classiques. Le lot PADF 945, un lot de semences qui différait du précédent en ce qu'on avait ajouté une étape de culbutage dans un culbuteur à abrasion, n'a pas permis de montrer de différence entre les méthodes de scarification manuelles et les témoins. Le trempage dans l'eau n'a amélioré la germination d'aucun des deux lots. Il y a avait une faible corrélation entre la germination totale obtenue en laboratoire et en pépinière; cela était principalement attribuable à des différences dans le substrat de germination. La germination au laboratoire a été moins efficace que celle obtenue en pépinière. Deux mois après le semis en pépinière, l'amande mise en terre sans le tégument avait donné les plus petits plants.

Introduction

Simarouba glauca var. *latifolia*, known in Haiti as frenn or bois blanc, and in English as simarouba or princess tree, is a common tree species of the subtropical moist forest of Hispaniola. Simarouba is native to the Greater Antilles, Mexico and Central America (Liogier, 1985). It grows well on both basalt and limestone derived soils from sea level to 800 meters.

In Haiti, S. *glauca* is much more common than the endemic *Simarouba berteroana*, though the latter is appreciated for its higher wood quality. Both species are dioecious and may hybridize, though the extent of hybridization is unknown. Due to its relatively fast growth, *S. glauca* is an important source of utility wood in house construction and medium grade furniture; other products include fuel wood, oil extracted from the seeds, and a source of ingredients in folk remedies (Rath, 1987; Jenkins, 1989).

The fruiting of this species peaks during the summer period between May and July. The problem with simarouba seeds is that germination and emergence rates are not uniform due to dormancy factors. Stored seeds that tested 70% viable by the tetrazolium test failed to germinate (pers. comm., C. Hughes, Oxford For. Inst., Mar. 1990). This requires sowing only fresh seeds in the nursery, and limits such sowing to a single season.

Simarouba seeds are generally sown without any pre-germination treatment. However, methods have evolved in several containerized nurseries in Haiti that seem to increase germination rate and uniformity. Several methods are currently used to break dormancy: 1) slightly cracking the endocarp to allow water imbibition, but not so much as to allow the kernel to rot; 2) extracting the kernel from the endocarp; 3) immersing the seeds first in 80°C water and letting them soak for 24 hours and 4) soaking the seeds in cold water for 24 hours. This study investigates whether these methods are effective, particularly since the mechanical scarification methods are labor intensive and costly. The major objectives of the study were i) to determine the pre-treatment methods that maximize total germination and germination rate of simarouba in the nursery, and ii) to compare laboratory germination with nursery emergence.

Materials and methods

Study Sites

The study was conducted at two sites. Laboratory analyses were conducted at the tree seed center of Proje Pyebwa (Pan American Development Foundation (PADF)) located at Delmas #31 in Port-au-Prince. All nursery trials were completed at the Operation Double Harvest (ODH) nursery in Roche Blanche near Croix des Bouquets.

Seed Collection and Handling

Seeds were harvested during May 1990, corresponding to the peak harvest period of simarouba in most areas of Haiti. Two bulked seed lots were harvested and are referred to in this study as SECID 2290 and PADF 945. SECID 2290 was harvested during the period of May 12-18 from elite tree candidates located in two separate regions of Haiti (Table 1). PADF 945 was harvested from a single location near Grand Goâve on May 30. Both seedlots were cleaned by removing the pulp with water and air dried in the shade for several days. PADF 945 was further processed, after drying, by being rotated in a drum lined with sandpaper for 1.5 hours.

Seed Moisture Determinations

Seed moisture determinations were performed for each of the seed lots prior to conducting the pre-germination treatments. Methods followed ISTA (International Seed Testing Association 1985) rules. A comparison of seed moisture contents was made between ground seeds, conforming to ISTA standards, and unground seeds. Seed moisture contents were compared to kernel moisture contents.

Pre-germination Treatments

Each seedlot was divided into five parts and received the following pre-germination treatments:

Treatment	Description
Hot soak	Complete seed immersed in hot water at 80°C and soaked for 24 hours
Kernel	Kernel extracted completely from the endocarp
Cold soak	Complete seed soaked in cold water for 24 hours
Crack	Endocarp cracked longitudinally
Control	No treatment applied

Germination

The germination tests were initiated June 20, 1990. The pre-germination treatments were tested in the laboratory and the nursery. Germination in the

Table 1. Summary of seeds harvested for this study.

Seed lot no.	Family no	Harvest Date	Locality	Commune
SECID 2290	201	28/05/90	Moussignac	Miragoane
	203	28/05/90	Moussignac	Miragoane
	228	12/05/90	Felician	Lascahobas
	257	18/05/90	Chabanne	Petit Goave
	281	28/05/90	Savanne Longue	Lascahobas
	284	17/05/90	Savanne Laraine	Lascahobas
PADF 945		30/05/90	Carrefour Fauche	Grand Goave

laboratory was conducted under ambient temperature (daily minimum 22°C and maximum 28°C) and light conditions utilizing plastic Lamarre trays. Four replications of 100 seeds per treatment were placed on top of Kimpak germination paper saturated with distilled water.

The nursery trials utilized the large Winstrip container, consisting of 15 cm-high zig-zag sheets of plastic fitting together to form cells approximately 5 cm x 5 cm (375 cc volume). Fafard Growmix was selected as the potting medium to minimize the effect of the potting medium on germination and growth. Four replications of 100 seeds per treatment were sown in 8 Winstrip cases (50 seeds / case) on June 21, 1990. Daily counts of germinated seeds in both laboratory and nursery were conducted beginning on the 7th day and continued until the 30th day.

Seedling Heights

Two months after treated seeds were sown in the nursery, seedling height was measured to determine the effect of pre-germination treatment on early seedling growth. Forty seedlings were randomly selected from the inner portion of the Winstrip case (i.e., edge seedlings were eliminated from the analysis) for each combination of seed lot and pre-germination treatment. These were measured to the nearest 0.1 cm. Sample sizes for treatments exhibiting poor germination in the nursery were less than 40 seedlings for the cold soak treatments of both seedlots; 10 seedlings for PADF 945 and 23 seedlings for SECID 2290.

Statistical Analyses

All statistical analyses were performed using SAS Version 6.04 software (SAS Institute, Inc., Box 8000, Cary, NC 27512-8000) installed on a NEC Powermate 286 Plus. ANOVAs were computed for a 2 X 2 X 5 factorial experiment (Snedecor and Cochran 1980). The factors were seed lot (2 levels), germination method (2 levels) and pre-germination treatment (5 levels). Comparison of means were conducted by the Duncan-Waller and Studentized t-test procedures.

Results and discussion

Seed moisture content

No differences in moisture content were found between unground and ground seeds. However, moisture content of the kernel was half that of the whole seed, which included the endocarp. Seed moisture content (fresh weight basis) for PADF 945 and SECID 2290 were 10% and 9.5%, respectively, while kernel moisture contents were 6% and 5%, respectively. It is not likely that these differences are significant, though insufficient replications were conducted to analyze for differences. It is not known what the interaction effect of seed moisture content had on the seed treatments.

Analysis of Variance

The ANOVA (Table 2) supports rejecting the null hypothesis that germination totals are equal 1) between seed lots, 2) between laboratory and nursery, and 3)

Table 2. ANOVA of *Simarouba glauca* germination percentages after 30 days.

SOURCE	DF	SS	MSE	F	PR > F
Model	19	42880.7375	2256.8809	21.51	0.0001
Error	60	6295.7500	104.9292		
Corrected Total	79	49176.4875			
R-Square	C.V.	Root MSE		GERM Mean	
0.871976	29.46708	10.24349		34.762500	
SOURCE	DF	TYPE I SS	MSE	F	Pr > F
LOT	1	9923.5125	9923.5125	94.57	0.0001
METHOD	1	15318.1125	15318.1125	145.99	0.0001
TREATMENT(TRT)	4	8286.0500	2071.5125	19.74	0.0001
LOT*METHOD	1	667.0125	667.0125	6.36	0.0144
LOT*TRT	4	2587.3000	646.8250	6.16	0.0003
METHOD*TRT	4	3009.7000	752.4250	7.17	0.0001
LOT*METHOD*TRT	4	3089.0500	772.2625	7.36	0.0001

between pre-germination treatments. All differences were significant P = 0.0001. All interaction effects were significant. The general linear model could explain 87% of the variation observed in the germination experiments.

Comparison of Pre-germination Treatments

Seed treatments applied prior to sowing in the nursery attempt to increase uniformity, rate and germination totals of a given seed lot. These three variables were analyzed for both seed lots, since it would be natural that the interaction between seed quality and seed treatment would effect germination differently.

Table 3 shows the comparison of total germination means using the Duncan's Multiple Range Test. PADF 945 showed significant differences between treatments in the nursery; no differences were exhibited in the laboratory. SECID 2290 showed differences both in the laboratory and the nursery. The best treatments for PADF 945 were not the best for SECID 2290. Overall, the cold soak treatment exhibited poor germination for the two seed lots in both the laboratory and the nursery.

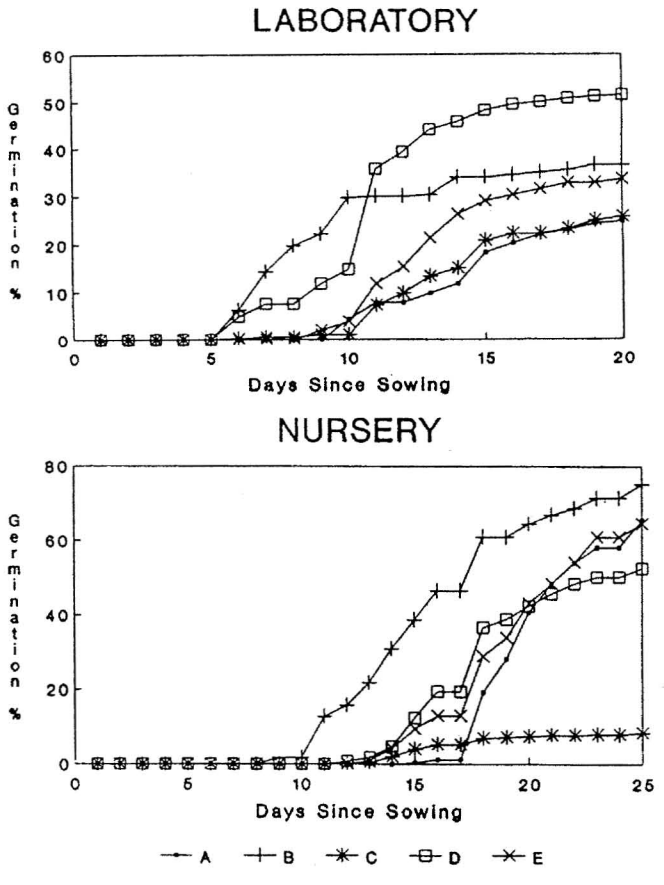

Figure 1. Cumulative germination of pre-sowing seed treatments for *Simarouba glauca*, PADF lot no. 945. A - Hot water at 80°C; B - Kernel only; C - Cold water for 24 h; D - Split endocarp; E - Control (no treatment).

It is logical that pre-germination treatments would affect not only total germination percentages, but also the rate of germination. Rate of germination is important to insure that the seedlings develop uniformly in the nursery and that seedling quality be maintained. In general, the same trends in germination rate that were shown in the laboratory were exhibited in the nursery.

Figure 1 compares germination for PADF 945. Changes in germination rates occurred in both the laboratory and the nursery. In general, the treatments that exhibited the highest total germination were also the treatments that produced rapid germination. As mentioned previously, these treatments showed interaction effects (i.e., laboratory results were not comparable to nursery results). Figure 2 compares germination rates for SECID 2290. Emergence in the nursery falls into two groups: the rapid and high germination of the kernel and cracking treatments versus the slower rate and lower germination totals of the control and water soaks.

The final result of any difference in pre-germination treatment would be the effect that changes in germination rate may have on seedling growth in the nursery. A comparison of the mean heights for the five seed treatments is shown in Table 4.

It is interesting to note that the poorest growth for both seedlots was exhibited by seedlings germinated from the kernel without the seedcoat. However, from a nursery management perspective, these differences are not as important as the differences in germination vigor (i.e., germination rate + germination total) due to pre-germination treatment.

Comparison of Laboratory and Nursery Germination

Germination in the laboratory is tested at the PADF seed center prior to distribution to nurseries throughout Haiti. In general, it is assumed that test results in the laboratory are indicative of what is to be expected in the nursery. This may be true for many species. However, for the simarouba seed lots in this study, the data show that correlations between laboratory and nursery results were poor.

Total germination was higher in all treatments in the nursery than in the

laboratory for PADF 945, except for the cold soak treatment which resulted in the poorest germination (Figure 3). Highest germination in the nursery was produced by hot soaking and extracting the kernels, and these were significantly different from the laboratory results (Table 5).

Figure 4 shows the laboratory/nursery comparison for SECID 2290. The difference in germination totals between the laboratory and the nursery were greater for SECID 2290 than PADF 945. This may be a natural variation in which case this observation is of no consequence. However, if the additional handling procedure of PADF 945 had an effect on germination aside from the fact that no two seedlots are the same, then the data suggests that some pre-germination treatment is necessary to optimize germination performance. In all cases, the nursery totals were significantly greater than the laboratory results (Table 5), indicating that in-laboratory germination is not necessarily indicative of performance in the nursery.

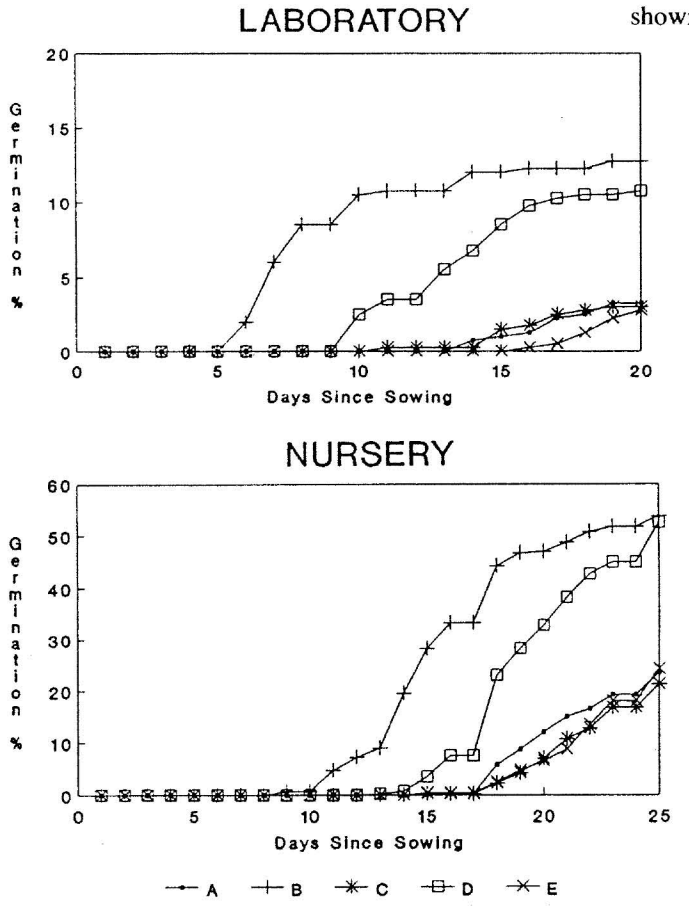

Figure 2. Cumulative germination of pre-sowing seed treatments for *Simarouba glauca*, SECID lot no. 2290. A - Hot water at 80°C; B - Kernel only; C - Cold water for 24 h; D - Split endocarp; E - Control (no treatment).

Seed Lot Comparisons

The two seed lots differed considerably in their performance, both in the laboratory and the nursery. PADF 945 performed better overall than SECID 2290, particularly in the laboratory. Many factors not tested in this study could have contributed to the difference. The PADF seed lot was collected at one location and time, minimizing the spatial and temporal factors that affect seed viability. PADF 945 also underwent an additional handling treatment in the rotating abrasive drum. The SECID seed lot was collected from individual trees located in two regions of Haiti during an 18 day period. No doubt air drying times and handling conditions were different as well.

Significant differences existed between the seed lots for every pre-germination treatment in the laboratory (Table 6). PADF 940 exhibited higher germination than SECID 2290 for all treatments. This was not the case in the nursery where germination differences between the two seed lots were not as great. In fact, SECID 2290 germinated significantly higher than PADF 940 for the cold soak treatment; no significant difference was shown for the cracking treatment (Table 6).

Conclusions and recommendations

The results obtained from this study on seed treatments and the various germination methods for *Simarouba glauca* led us to draw the following conclusions:

1. Dormancy factors that inhibit the germination of simarouba seed can be partially overcome by cracking or extracting the kernel from the endocarp. Soaking treatments do not improve germination.
2. The germination of PADF 945, a seed lot tumbled in an abrasive drum, was not improved by scarification or soaking. The cold soak had a negative effect on emergence in the nursery.
3. Tumbling simarouba seed in an abrasive drum merits further testing as a cost effective method for conditioning large quantities of seed and improving germination in the nursery.

Table 3. Comparison of germination means for *Simarouba glauca* var. *latifolia* seed lots by pre-treatment method in the laboratory and the nursery. Means followed by the same letter are not significantly different at the 95% probability level.

Seed Lot	Treatment	Nursery Germination (%)	Laboratory Germination (%)
PADF 945	Kernel	76.75 a	36.50 a
	Control	70.50 a	34.25 a
	Hot soak	67.50 ab	25.25 a
	Cracking	59.50 b	51.75 a
	Cold soak	10.00 c	27.00 a
SECID 2290	Cracking	57.75 a	11.25 a
	Kernel	54.00 a	12.75 ab
	Control	31.50 b	3.75 b
	Cold soak	30.00 b	3.50 b
	Hot soak	28.50 b	3.25 b

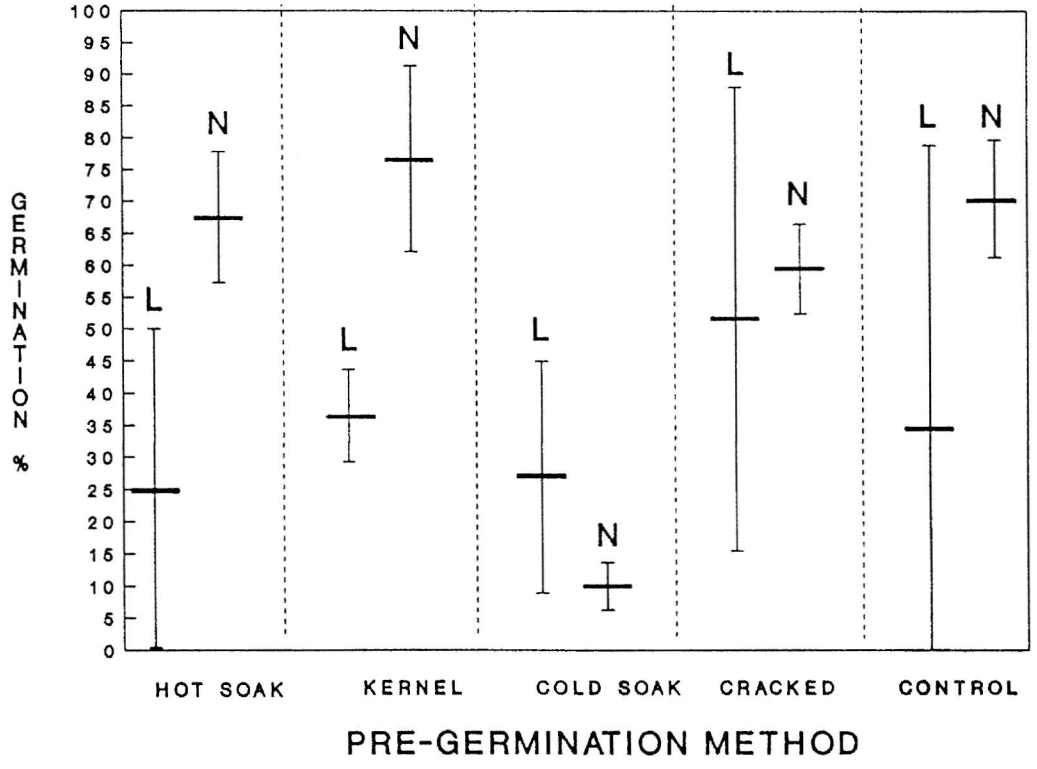

Figure 3. Comparison of laboratory and nursery germination means for PADF 945 by pre-germination treatment.

Table 4. Average height of *Simarouba glauca* after two months in nursery. Means followed by the same letter are not significantly different.

TREATMENTS	N	PADF 945 HEIGHT (cm)	N	SECID 2290 HEIGHT (cm)
Control	40	11.915 a	40	11.091 a
Hot soak	40	11.820 a	34	11.160 ab
Cracking	40	11.103 ab	40	10.463 ab
Cold soak	10	10.350 b	23	11.587 ab
Kernel	40	10.012 b	40	10.163 b

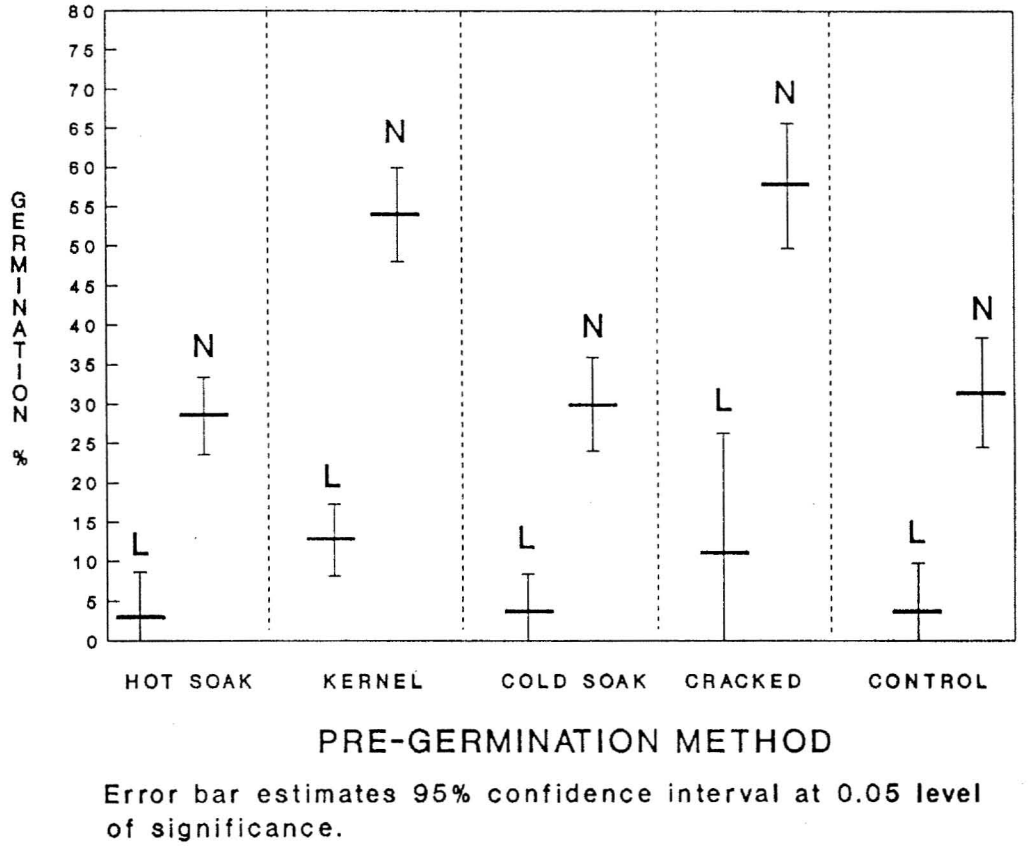

Figure 4. Comparison of laboratory and nursery germination means for SECID 2290 by pre-germination treatment.

4. Removal of the endocarp decreases seedling height growth in the nursery. However, this is not as important as the factors that control germination vigor and uniformity in the nursery.

Further studies should be directed toward proper handling and testing of simarouba seeds from seed harvest to country-wide distribution in Haiti. These include optimal tumbling procedures to condition bulk seeds and germination tests that correlate better with performance in the nursery.

Table 5. Results of paired comparisons (t-test) between laboratory and nursery germination.

Seed Treatment	N	Laboratory	Nursery	t	Pr>|t|
		Seed lot PADF 945			
Hot water	4	25.25	67.50	5.00	0.0024
Kernel	4	36.50	76.75	7.87	0.0002
Cold water	4	27.00	10.00	2.94	0.0260
Crack	4	51.75	59.50	0.67	0.5295
Control	4	34.25	70.50	2.53	0.0445
		Seed lot SECID 2290			
Hot water	4	3.25	28.50	10.96	0.0001
Kernel	4	12.75	54.00	17.49	0.0001
Cold water	4	3.50	30.00	10.89	0.0001
Crack	4	11.25	57.75	8.67	0.0001
Control	4	3.75	31.50	9.62	0.0001

Table 6. Comparison of the germination totals (t-test) of PADF 945 and SECID 2290 by treatment.

Treatment		Seed lot PADF	Seed lot SECID	t	Pr > t
LAB	HOT SOAK	25.25	3.25	2.75	0.0331
LAB	KERNEL	36.50	12.75	8.88	0.0001
LAB	COLD SOAK	27.00	3.50	4.00	0.0072
LAB	CRACKING	51.75	11.25	3.28	0.0169
LAB	CONTROL	34.25	3.75	2.16	0.0744
NURS	HOT SOAK	67.50	28.50	10.89	0.0001
NURS	KERNEL	76.75	54.00	4.59	0.0037
NURS	COLD SOAK	10.00	30.00	9.10	0.0001
NURS	CRACKING	59.50	57.75	0.52	0.6189
NURS	CONTROL	70.50	31.50	10.75	0.0001

Acknowledgements

The authors would like to extend their appreciation to USAID for funding the research, and to Raynold Orilien and Georges Marcellus for helping set up the laboratory research at the PADF seed center, St. John Dorsanville for supervising the propagation of seedlings at the ODH nursery, Duverger Vernus for collecting laboratory and nursery data, and Sigrid d'Aquin for data analyses.

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Incubation, drying, and separation method to separate viable from non-viable, artificially-aged seeds of *Pinus roxburghii* Sarg.

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Abstract

A study was carried out with *Pinus roxburghii* Sarg. seeds to determine the usefulness of the incubation, drying, and separation (IDS) method to remove filled-dead seeds from a seed lot subject to different artificial ageing periods. Seeds were aged at 41°C and 100 percent relative humidity (RH) for 0, 24, 48, 72, 96, 120, 144, 168, and 192 hours. The germination of seeds after ageing varied from 15-69 percent. Seeds were incubated at 15°C, 100 percent RH, and 1000 lux illuminations for 72 hours, dried for 4 hours, and separated using 1.04 specific gravity sucrose solution. This study shows that the IDS method is effective in removing filled-dead and empty seeds from seed lots during deterioration, but not from lots of completely aged seeds that have very poor viability. The IDS method enhances germination because dead seeds are separated from seed lots. However, further studies with naturally-aged seeds are needed to confirm these results. Radiographic interpretation confirmed the accuracy of the IDS method in separating the filled, dead, and empty seeds.

Résumé

Les auteurs ont tenté de déterminer l'utilité de la méthode d'incubation, séchage et séparation pour l'extraction des graines mortes et remplies comprises dans un lot de graines de *Pinus roxburghii* Sarg., lot soumis à différentes périodes de vieillissement artificiel. Le vieillissement des graines s'est fait à 41°C et 100 % d'humidité relative (HR) pendant 0, 24, 48, 72, 96, 120, 144, 168 et 192 heures. La germination des graines après le vieillissement a varié entre 15 et 69 %. Les graines ont été incubées à 15°C et 100 % d'HR, avec éclairement à 1000 lux pendant 72 h; elles ont été mises à sécher pendant 4 heures et ont été séparées dans une solution de sucrose dont la densité était de 1,04. Cette étude montre que la méthode est efficace lorsqu'il s'agit d'enlever les graines mortes et remplies ainsi que les graines vides des lots pendant la détérioration, mais pas des lots de graines complètement vieillies qui ont une très mauvaise viabilité. La méthode permet d'améliorer la germination du fait que les graines mortes sont extraites du lot de graines. Toutefois, il faudra poursuivre les travaux sur des graines qui vieillissent naturellement afin de confirmer ces résultats. L'interprétation radiographique a confirmé qu'il s'agit d'une méthode exacte de séparation des graines remplies, mortes et vides.

Introduction

Incubation, drying, and separation (IDS) techniques have been shown effective in removing filled-dead and empty seeds of conifers to enhance the germination percentage of a seed lot. This technique conserves genetically improved seed-orchard seeds and reduces the labour costs involved in seedling production. According to Simak (1984), the IDS method is based on the principle that after incubation and subsequent drying, viable seeds lose absorbed water at a much lower rate than dead seeds. This principle makes it possible to distinguish filled-dead seeds because of weight and density differences using separation methods based on physical principles such as flotation and gravity separation.

Singh and Vozzo (1990) have reported that the IDS method for *Pinus roxburghii* Sarg. using 72 hours incubation, 4 hours drying, and separation using 1.04 specific gravity sucrose solution, removed 28% of the dead seeds, increasing seed lot germination to 94%. The objective of this study was to show the applicability of this method for removing filled-dead seeds from seed lots that had different viability status influenced by accelerated ageing.

Materials and methods

Seeds of *Pinus roxburghii* obtained from Mistletoe Sales-S & S Seeds, 5690 Casifas Pass Road, Carpinteria, California, were used for this investigation, which was conducted at the Forestry Sciences Laboratory, USDA Forest Service, Starkville, Mississippi.

About 50 g of seeds were surface-sterilized with 2.7% sodium hypochlorite and rinsed thoroughly with distilled water. Accelerated ageing was performed according to the Seed Vigor Testing Handbook (AOSA 1983). Seeds were placed in 5.5 x 2-inch plastic boxes with covers. Each box contained a bronze wire mesh suspended over 100 ml of distilled water. Seeds were then placed in an accelerated ageing chamber maintained at 41°C and 100 percent RH. Seeds were withdrawn from the chamber after 24, 48, 72, 96, 120, 144, 168, and 192 hours of ageing. Un-aged seeds served as controls. The seed samples drawn from each treatment were tested for moisture content (Bonner 1979) and germination. The seed quality of aged seeds is shown in Table 1.

Filled-dead and empty seeds were separated from the seed samples by incubation, drying, and separation (IDS).

Incubation

200 seeds were incubated between two moistened blotter paper sheets and kept in a germinator for 72 hours at 15°C, 100 percent RH, and 1000 lux illumination.

Drying

After incubation, the seeds were surface-dried and spread on a layer of blotter paper for 4 hours at room temperature.

Separation

The dried seeds were floated in a beaker containing 1.04 specific gravity sucrose solution. After 5 minutes, the buoyant seeds on the surface were removed. They represented the dead seed fraction (floaters). The sunken seeds on the bottom represented the viable seed fraction (sinkers).

The sinkers and floaters were counted and expressed in percentage. All seeds were radiographed at 3 mas, 25 kVp, and 65 cm focus-film distance for 30 seconds with Kodak Industrial Type M film (processed manually). Moisture content of seeds after incubation and drying was determined according to Bonner (1979).

Sinkers and floaters were germinated on moistened cellulose sheets (Kimpak) in germinators set at alternating night and day temperatures of 20°C and 30°C with dark and light regimes of 16 and 8 hours, respectively. Germination counts were taken after 7, 14, 21, and 28 days. At the end of the germination test, all ungerminated seeds were examined by a cutting test. The following categories were established:

Good, ungerminated seeds	= full, live
Abnormal germinated seeds	= full, live
Rotten seeds	= full, dead
Empty seeds	= empty, dead

Table 1. Seed quality of *Pinus roxburghii* during accelerated ageing

Accelerated ageing (hours)	Germinated seeds %)	Good ungerminated seeds (%)	Abnormal germinated seeds (%)	Rotten seeds (%)	Empty seeds (%)
0	51	7	5	28	9
24	52	9	3	26	10
48	69	0	0	22	9
72	66	0	1	25	8
96	56	0	0	32	12
120	52	0	0	37	11
144	54	1	1	29	15
168	29	0	0	58	13
192	15	0	0	68	17
Least significant difference (LSD) (P=0.05)	6.0	-	-	4.3	3.1

Results and discussion

Moisture content of acceleratedly aged seeds varied from 8.4% (0 hours' ageing) to 28.7% (168 hours' ageing). This result is similar to the results for other aged tree seeds (Blanche *et al.* 1988). Moisture content of seeds after incubation remained the same for treatments from 0 to 144 hours' ageing (32.4%) but was slightly higher for 168 and 192 hours' ageing (37.3%) (Table 2). The moisture content of seeds after drying showed little variation among the treatments, ranging only from 23.6% to 24.8%.

Sinkers decreased from 75.5% (0 hours) to 46.5% (192 hours) as the ageing increased, while the percentage of floaters increased from 24.5% to 53.5% (Table 2).

There was no difference in the germination of sinkers for treatments up to 120 hours' ageing (90.1% to 93.1% compared with 51% to 69% before IDS) (Table 3). The seeds aged for 144 hours had 81.9% germination (compared with 54% before IDS). IDS did not improve germination percentage of seeds aged for 168 and 192 hours. These results show that IDS enhanced the germination percentage of deteriorating seed lots during the ageing processes, but did not influence germination for completely deteriorated seed lots or seed lots with poor viability. In floating fractions, germination up to 23.9% was recorded (Table 3).

In general, sinkers and floaters contained a mixture of live and dead seeds. Singh and Vozzo (1990) reported similar results. Simak (1984) found that some germinable seeds have a low viability, their ability to retain water is low when they are dried, and they are subsequently removed with dead seeds during separation. Mechanically-damaged and insect-damaged seeds absorb water easily and sink to the bottom with viable seeds during separation.

Few abnormal germinated seeds were observed in sinker fractions of the seed lots aged up to 120 hours, and none were seen in the floaters (Table 3).

The percentage of rotten seeds was low (5.2%–9.1%) in the seed lots aged up to 120 hours but increased to 62.4% at 192 hours' ageing. This finding suggests that the IDS method was not effective in separating dead seeds from completely aged or deteriorated seed lots. Rotten seeds in the floating fraction varied from 29.4%-71.1%, increasing with ageing periods (Table 3). No empty seeds were observed in radiographs of sinkers, but empty seeds ranged from 27.9%-59.2% in floating fractions (Table 3).

Radiography of seeds aged 0, 72, and 192 hours revealed complete separation of empty seeds from full seeds (Figs. 1, 2 and 3). Empty seeds were observed in floaters but not in sinkers. Radiography did not distinguish between filled-live and filled-dead seeds in either fraction. However, IDS did distinguish them. The cutting test revealed that some of the filled seeds from floater fraction had germinated. Although a small number of filled-live seeds are lost with empty seeds in the floater fraction, the sinker fraction had better germination in the nursery. Thus, using the sinker fraction would reduce the cost of seed and seedling production.

The IDS method is effective in removing filled-dead and empty seeds of seed lots for artificial ageing only during their deterioration. Using this method with completely deteriorated or fully-aged seed lots possessing low germinability is not advisable because the separation of filled-dead seeds is poor. However, additional studies with naturally deteriorating seed lots are required to confirm these results.

Table 2. Moisture content of *Pinus roxburghii* seeds after accelerated ageing followed by IDS method and percentage of sinkers and floaters after separation.

Accelerated ageing (hours)	Seed moisture content (%)			Seed fraction (%)	
	after ageing	after incubation	after drying	sinkers	floaters
0	8.4	32.4	23.6	75.5	24.5
24	19.5	33.2	24.6	74.5	25.5
48	22.0	33.3	24.5	72.0	28.0
72	24.3	33.8	24.8	72.5	27.5
96	25.9	33.7	24.0	66.5	33.5
120	26.0	33.9	24.1	60.5	39.5
144	28.3	33.8	24.0	58.0	42.0
168	28.7	37.3	23.6	50.5	49.5
192	28.6	36.6	24.2	46.5	53.5
LSD (P=0.05)	1.04	0.88	0.69	3.30	1.32

Table 3. Data of germination test conducted with sinkers and floaters of *Pinus roxburghii* seeds after accelerated ageing followed by IDS method.

Accelerated ageing (hours)	Germinated seeds (%)		Abnormal germinated seeds (%)		Rotten seeds (%)		Empty seeds (%)	
	Sink	Float	Sink	Float	Sink	Float	Sink	Float
0	90.7	8.2	2.7	0.0	6.6	32.6	0.0	59.2
24	93.1	10.7	1.4	0.0	5.5	39.3	0.0	50.0
48	90.6	15.7	0.0	0.0	9.4	29.4	0.0	54.9
72	92.4	9.1	2.1	0.0	5.5	40.9	0.0	50.0
96	92.5	23.9	2.3	0.0	5.2	34.3	0.0	41.8
120	90.1	15.2	0.8	0.0	9.1	51.9	0.0	32.9
144	81.9	2.4	0.0	0.0	18.1	47.6	0.0	50.0
168	52.5	3.0	0.0	0.0	47.5	62.7	0.0	34.3
192	37.6	1.0	0.0	0.0	62.4	71.1	0.0	27.9
LSD (P=0.05)	3.80	0.71	-	-	1.03	1.16	-	4.23

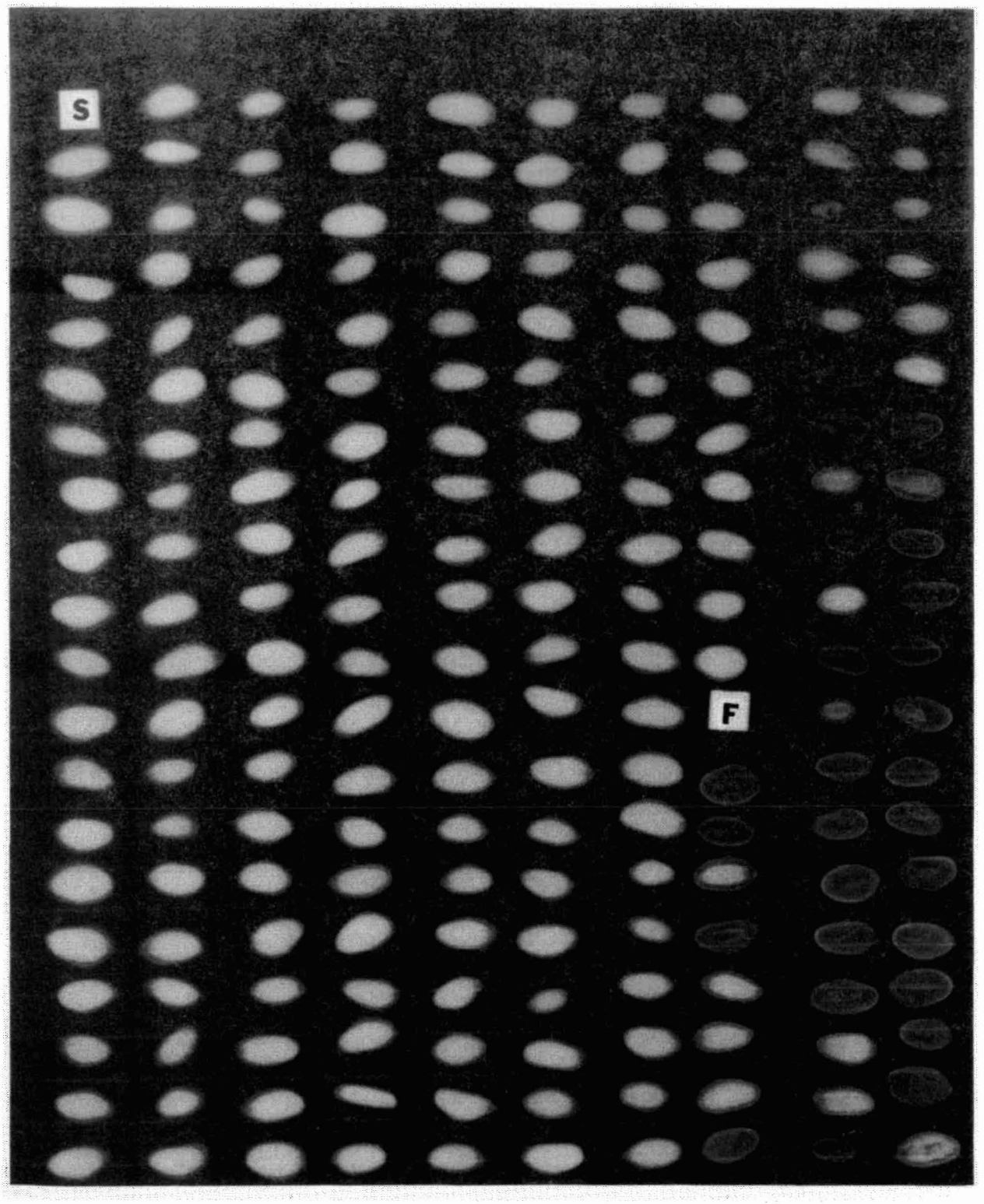

Figure 1. Radiographs of *Pinus roxburghii* seeds separated by IDS method into sinkers (S) and floaters (F). Control seeds (no artificial ageing).

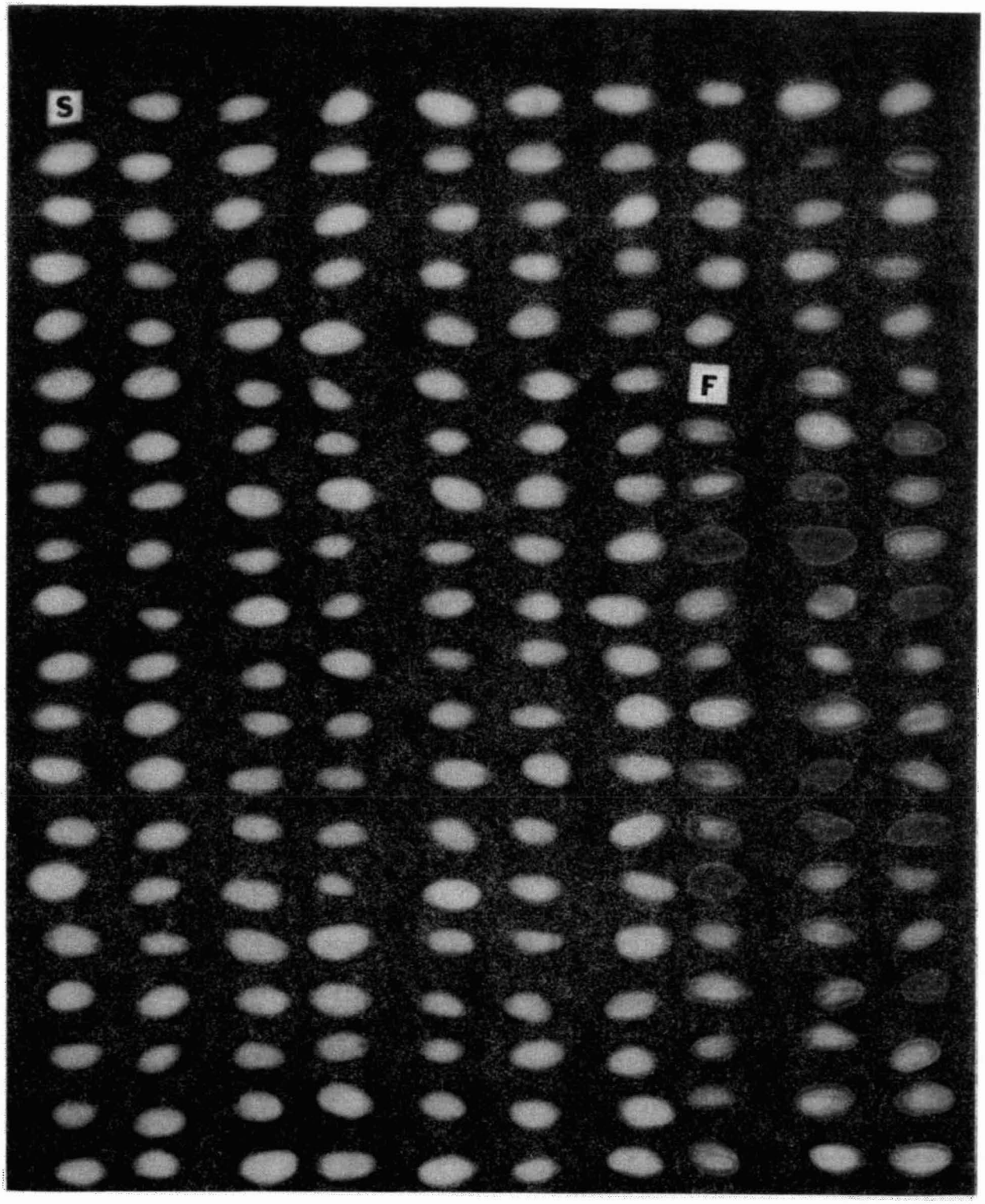

Figure 2. Radiographs of *Pinus roxburghii* seeds separated by IDS method into sinkers (S) and floaters (F). After 72 hours of artificial ageing.

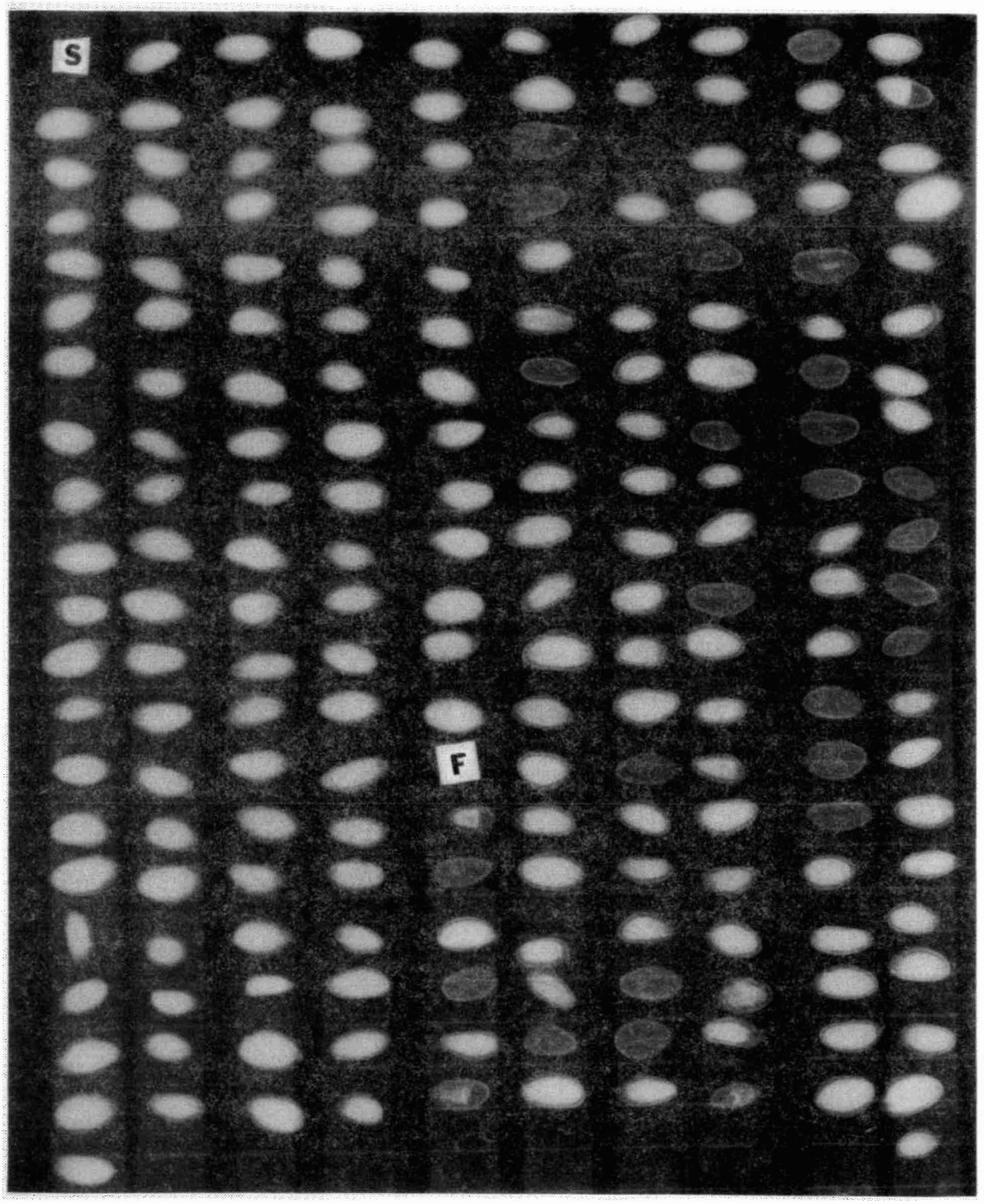

Figure 3. Radiographs of *Pinus roxburghii* seeds separated by IDS method into sinkers (S) and floaters (F). After 192 hours of artificial ageing.

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Seeds are the biological potential in Argentine's forestry — germination and dormancy in forest-seeds

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Abstract

Germination in forest tree seeds, together with many of the factors involved, is reviewed. Attention is given to the causes of seed dormancy and the means of overcoming this condition. The Argentinian government's sweeping reforestation program is based upon control and coordinated production of high quality seeds, especially of fast-growing species, that are contributing to the global needs for pulp and paper.

Résumé

La germination des semences d'essences forestières ainsi que de nombreux facteurs de ce processus sont passés en revue. Une attention particulière est portée aux causes de la dormance des semences et aux façons de la lever. Le programme de reboisement général du gouvernement de l'Argentine est fondé sur le contrôle et la production coordonnée de semences de grande qualité, notamment des semences d'espèces à croissance rapide susceptibles de contribuer à la production mondiale de pâtes et papiers.

Introduction

The term "seed", as applied by specialists in vegetable science, means the dispersion of the germinating unit of the fertilized ovule. This definition is quite distinct from the strict botanical meaning which defines a seed as the result of one fertilized, ripe ovule containing an embryonic plant, stocked reserve elements, and layers of protective coverings.

If we want to follow the changes that result in the transmission of life in the tree, we must start from the study of its structural basic units, the seeds. Not all of them survive in the struggle for existence since any notable change in the environmental conditions (humidity, temperature, light, soil composition, etc.) may create adverse conditions for germination. The struggle for survival is reflected in the incredible varieties of shapes, structures, and sizes of the seeds that different plant species produce.

The proverb "Man will harvest what he sows" is an indubitable truth. To sow seeds that will not sprout or that are of low viability is a waste of time and money. That is why it is essential to guarantee the quality of the seeds, especially for forest seeds, because of the cost of industrial seedling production. For this reason, qualitative and quantitative laboratory analyses of tree seeds should be imposed on all private and public forest enterprises.

As a result of genetic research involving cultivated trees many types, shapes and races that have, or potentially have, valuable economic properties have been produced. Selection from these types and races has led to the economic development of our forests by preserving and improving their hereditary nature. The principle reason for cultivation of forest seeds is the preservation and dissemination of woody species whose germinability, genuiness and technological characteristics to be obtained in the future emphasize the basic economic properties required within the country.

Vegetative growth and fruit formation are lengthy processes that are determined by the general nutritional conditions of the parent plant. Although seed development is the normal result of the pollination process, it does not always reach a happy end. When fertilization takes place, the embryo starts to grow but completion can be delayed. The inability of the embryo to develop and mature is due to inherent physiological conditions that may be related to the surrounding endosperm. In general, fruit and seed development occur simultaneously and synchronously. Some fruits may develop seeds without pollination/fertilization taking place, a condition known as parthenocarpy. There are also parthenocarpic fruits that contain mature ovules that are mostly without embryos.

Fruit growth involves cell division, elongation, and differentiation. Water, carbohydrates, nitrogenous compounds, mineral salts, and growth substances are all required, and a shortage of one or more of these elements reduces or curtails growth rate.

Germination

This is the process of reactivation of growth in the embryo that results in the breakage of the seed integuments and the appearance of the new plant. Mature seeds germinate when environmental conditions are favorable. In nature, germination usually takes place on or near the soil surface. In the natural forest, many seeds fall into the interstices of detritus that constitute the upper layer of many forestry soils. When they germinate, the forest is said to be naturally regenerated.

Germination starts with water imbibition by the integuments or seedcoat. As the internal tissues are hydrated, enzymes are activated, promoting growth of the embryo which leads to a splitting of the integuments so that the new plant can establish itself in the soil.

The basic conditions required for germination are a) a supply of water, b) oxygen, c) favourable temperatures and, in many cases, d) light.

Water

The physiological processes of living cells take place in a watery medium and there will be no germination if the seed does not absorb water from the surrounding substrate. The amount of water absorbed by different species, or by varieties of the same species, fluctuates widely. Water vapor in the environment may be sufficient to imbibe the seeds of some species so the first steps of germination may occur in a saturated or near-saturated atmosphere. If atmospheric vapor pressure falls below the saturation level, germination may be damaged or inhibited.

Temperature

There are temperature limits above and below which germination does not occur. In the case of seeds of temperate forest trees, germination can occur in environments that vary from subtropical temperatures to those of very cold regions, but the optimum temperature lies between these values.

Oxygen

During the early phases of germination, respiration is fully, or almost fully, anaerobic due to the relative imperviousness of the seedcoat to oxygen even when hydrated. Not until the integuments are split by the swelling embryo does aerobic respiration takes over.

Light

Light exerts specific action in germination of the seeds of some tree species. Seeds of certain species do not germinate unless they are exposed to light, others germinate better when exposed to light, but light is not essential, while others are delayed or inhibited by light.

Ewart (1908) categorised seeds in three biological groups according to their ability to withstand optimal storage conditions:

1) microbiotic, in which the maximum storage duration is no longer than 3 years;
2) mesobiotic, whose maximum storage duration is 3-15 years; and
3) macrobiotic, whose maximum storage extends from 15-100 years.

Baldwin (1942) considered the majority of tree seeds to be mesobiotic, and that, with exceptions such as some Leguminosae and Rosaceae, microbiotic examples are rare. It is generally thought that longevity is related to the texture of the pericarp as in, for example, *Robinia pseudoacacia* L., the seedcoat of which is hard and impervious.

The seeds of many temperate zone broad-leaved trees could be graded less than microbiotic since their storability is limited to a few months; broadleaved examples include *Acer saccharinum* L., *Aesculus hippocastanum* L., *Castanea sativa* Mill., *Juglans regia* L., *Populus alba* L., *Salix alba* L., and *Ulmus pumila* L., while the coniferous species of *Araucaria angustifolia* (Bert.) O. Kuntze, *Araucaria araucana* L., and *Tabebuia ipe* L. should also be added.

Clements (1910) found viable seeds in *Pinus contorta* Dougl. cones that had remained on the tree for 75 years. In contrast, seeds of *Salix* (willow) and *Populus* (poplar) species lose their germinablility rapidly. Nakajima (1921) showed that viability of willow seeds could be prolonged if they were dried and cold stored, extreme care being required in the control of seed moisture content during storage. *Cedrus* and *Libocedrus* species are very sensitive to environmental conditions and their germinablility seldom last for more than 1 year even under dry cold conditions. Likewise, the storability of *Araucaria angustifolia* and *Araucaria araucana* (both native trees of Argentina) seeds is limited to no more than 3 months.

The seeds of the hard pines such as *Pinus contorta*, *Pinus banksiana* Lamb., *Pinus radiata* D. Don, *Pinus ponderosa* Laws., *Pinus murrayana*, and *Pinus halepensis* Mill. have greater longevity if they are stored at 8°C, and germinability will still be 60% after 2 years if stored under proper conditions. For *Pinus elliottii* Engelm. and *Pinus taeda* L., if seed moisture

content is kept between 2-9%, and storage temperature is between 2-10°C, they can be preserved up to 3 years.

Dormancy

Frequently, germination does not occur in mature seeds even in a favorable environment due to internal causes within the seeds. Seeds in which growth is inhibited as a result of internal causes are said to be dormant, to be in lethargy or in a rest period. Forest seed dormancy may be caused by a combination of factors:

a) When the seeds are mature, the integuments are completely impervious to water and oxygen. This frequently happens in Leguminosae seeds such as the acacia blanca (*Robinia pseudoacacia*) and acacia negra (*Gleditsia triacanthos* L.). Germination cannot occur until water penetrates the integuments and hydrates the tissues, which increases their perviousness. Such dormancy can be shortened through the action of soil bacteria and fungi in the forest floor.

b) Some seeds maintain their dormancy by means of the mechanical resistance of the integuments which are strong enough to resist the expansion and development of the embryo. In some seeds such dormancy may occur for 30 years or more.

High temperatures can interrupt dormancy and stimulate germination. In some species, as the seeds develop the minimum temperature for germination decreases and their embryos do not enter dormancy, and they may germinate rapidly. In other cases, the embryos are not developed when the seed separates from the mother plant and germination occurs only after a post-maturity, or after-ripening, period which occurs while seeds lie on the forest floor over winter. Dormancy is then broken the next spring. In species in which two or more seeds develop in the fruit, both seeds may not be equally dormant.

Germination is sometimes prevented or delayed by the presence of inhibitors such as coumarin and para-sorbic acid.

Some seeds are incapable of germinating under favorable conditions if they have been improperly stored. This condition is often referred to as secondary dormancy. Seeds that are light-sensitive progress into secondary dormancy if kept in darkness; conversely, seeds that normally germinate in darkness may become secondarily dormant if exposed to light.

Methods for breaking dormancy

Dormancy is a practical problem with considerable economic relevance, but it can be overcome by means of proper methods, the most commonly-used ones including:

1) scarification, which involves mechanical treatments that weaken or soften the integuments, or that split or crack them, allowing water penetration;
2) a similar effect sometimes can be achieved with the use of strong mineral acids;
3) post-maturity is produced more rapidly when moist conifer seeds are stratified at a temperature between 5-10°C for several weeks, or up to 2-3 months, depending on the species. The most important effect in the breaking of dormancy is related to increased respiration through the absorption of oxygen and expiration of carbon dioxide;
4) in some species dormancy can be broken by alternate freezing and thawing;
5) light can be used to interrupt dormancy in species that produce light-sensitive seeds;
6) the action of hydraulic pressure, for example 2.000 atm. at 18°C for 5-20 minutes, has been observed to produce an increase of 50-100% in germination. The pressure effect remains after drying and storage since it causes increased water permeability in the integuments;
7) in some species dormancy decreases during seed storage.

To break dormancy in seeds of *Robinia pseudoacacia*, and to promote embryo activity that has been inhibited by the impervious integuments, several tests were carried out using mineral acids, boiling water, acetone, potassium nitrate, xylol, citric acid, etc. Results showed that germination could be accelerated following treatment with mineral acids, but that dipping time and acid concentration had to be carefully controlled. In some cases the embryo would not tolerate the presence of acids at all.

In *Pinus taeda* seeds, soaking in water for 3 days is normally sufficient to overcome dormancy. This method is simple enough to be commonly used by nurseries for seedling production. Dormancy can be maintained in many forest seeds until required, but it can be removed by the careful application of different techniques that avoid embryo damage.

Summary

During the development of agriculture, silviculture has emerged as a specialized science. Many silvicultural problems have a physiological basis and can be resolved by researchers acquainted with the physiological processes of the trees with which they are working.

Through studies of the factors affecting the resumption of embryo growth, dormancy processes, methods of breaking dormancy, seed longevity, storage methods, and other evidence to determine viability, many seed germination problems have been solved. This has led to increased and improved plant production, which has given greater homogeneity of plantations and improved yields of wood quality and quantity. Forest seed dormancy can be maintained until germination is required, but it can be interrupted in most species by means of different techniques chosen to avoid damage to the embryo. The Argentine government has approved a sweeping reforestation program that provides the controls for, and that coordinates the production of, forest tree seeds to insure their quality, genuiness and preservation. This will permit the country to achieve, in a short period, self-sufficiency in the supply of forest seeds, especially of rapid-growing species, for the production of pulp and paper the shortage of which is felt world-wide. South America, and especially the Argentina Republic with its special conditions of climate and soils, can meet the world's deficit of lumber and other forest products.

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Temperature control of germination in Norway spruce

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Abstract

The effect of constant temperature on the germination of Norway spruce (*Picea abies* (L.) Karst.) seeds collected from three parent trees was tested in darkness using a temperature-gradient incubator (GRADIPLATE*). Logistic regression models were applied to describe germination capacities. There were significant variations between trees in germination rate and capacity in high constant-temperature conditions. Between +10 and +23°C, the mean thermal time requirement of germination was almost constant (≈ 82 $d.d._{+6}$) and variation between trees was small. Seeds that remained dormant in low and high temperatures germinated when removed to a constant-temperature chamber (+22°C), and germination depended on the previous treatment, the effect of the mother tree and their interaction. Seed coat mass and seed mass appeared to be associated with germination under constant-temperature conditions.

Résumé

Les effets de températures constantes sur la germination des graines d'épinettes de Norvège (*Picea abies* (L.) Karst.) récoltées chez trois lignées parentales ont été vérifiés dans l'obscurité à l'aide d'un incubateur à gradient de température (GRADIPLATE*). Des modèles de régression logistique ont été utilisés pour décrire les facultés germinatives. Dans des conditions de température élevée constante, le rythme de germination et la faculté germinative présentaient des variations significatives d'une essence à l'autre. Entre 10 et 23 °C, le temps moyen d'exposition à la chaleur nécessaire à la germination était presque constant (-82 $d.d._{+6}$) et la variation entre les lignées était faible. Les graines qui sont restées dans un état de dormance à des températures faibles et élevées ont germé lorsqu'elles ont été placées dans une chambre à température constante (+22 °C) et la germination était fonction du traitement antérieur, des effets de la lignée parentale et de leurs interactions. Dans des conditions de température constante, la masse du tégument et de la graine semblait être associée à la germination.