NOTE

DETERMINATION OF THE STAGE AT WHICH FAILURE OCCURRED IN EMPTY CONTROL-POLLINATED SEEDS OF *PINUS RADIATA*

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ABSTRACT

In *Pinus radiata* D. Don seed orchards throughout New Zealand, controlled pollination of isolated cones produced very few full seeds in 1991–92. Standard X-ray analysis of the empty seeds showed that the female gametophyte was present but shrivelled. Excising and soaking these gametophytes and attached nucellar material in a 15% sucrose solution under vacuum for 48 hours allowed them to be dissected or sectioned. Pollination had occurred in all seeds; however, development of the pollen tube and embryo formation differed among the seeds. In 15% of the seeds either pollen grains did not germinate or pollen tube development was arrested immediately after germination. In 35% of the seeds pollen tubes penetrated only part way through the nucellar cap. Thus, for 50% of the seeds, factors leading to abortion could have occurred while the cones were still within the isolation bags. In 50% of the seeds the pollen tube penetrated through the nucellar cap. Development occurred through the proembryo stage to late embryo in 44% of the seeds. Abortion in these seeds occurred well after the removal of the isolation bags. It is not clear how the isolation bags could be associated with the collapse of these ovules some 14 months after pollination.

Keywords: controlled pollination; dissection technique; embryo abortion; histology; megagametophyte; *Pinus radiata*.

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INTRODUCTION

Recently there has been a great deal of interest in factors controlling seed production in conifers (cf. Owens *et al.* 1990, 1991). As tree improvement programmes develop, more knowledge is required on factors determining prezygotic and postzygotic losses in seed set. In open-pollinated cones of *Pseudotsuga menziesii* (Mirb.) Franco these factors may decrease seed yield by up to 40% (Owens *et al.* 1991). In *Pinus resinosa* Ait. abortion of ovules during the first year and early in the second season reduces seed production by 50–60% (Lyons 1956). Losses in seed set have also been found in seed orchards in controlled-pollination studies (Brown 1971). During the 1991–92 seed year in New Zealand *Pinus radiata* seed orchards there was a 15% yield of sound seed from controlled pollination of isolated cone primordia, compared to an 89% yield for open pollination. For controlled pollination, the cones are isolated in cellulose bags before anthesis and pollinated with select pollen. The bags are removed after cone closure, up to 40 days after the initial bagging. The process of pollination and seed development has been well documented previously (Lill 1976; Lill & Sweet 1977). Within 2–13 days of pollination, pollen reaches the nucellus where it germinates and penetrates into the nucellar cap. The pollen tube remains in this condition for the remainder of the first year. Fertilisation occurs 14–15 months after pollination, in 2–3 months tissue differentiation is evident, and seeds are mature 5 months after fertilisation.

This paper reports a technique for examining shrivelled gametophytes from empty seeds and presents information on the stage at which abortion occurred in some of the seeds produced by controlled pollination of isolated cones.

MATERIALS AND METHODS

Empty Seed Sources

Empty seeds were obtained from commercial producers in the North and South Islands of New Zealand. The seeds were rejects from normal seed separation procedures for 150 controlled pollinations of *P. radiata*. For this study the seeds were bulked and samples were selected at random.

X-ray Analysis

X-ray analysis has been used to examine the condition of seeds of a number of tree species (Dogra 1967). The methods used here were modified from Simak & Gustafson (1957). The seeds were soaked in 20% barium chloride for 16 hours and arranged, in batches of 50 seeds each, on Polaroid type 55 positive/negative film. They were then exposed under 20 Kvp at 2.8 mA for 30 seconds using a Faxitron X-ray machine, and the condition of the embryos and megagametophytes was determined. Altogether 1100 seeds were examined in this fashion.

Dissection and Microtomy of Empty Seed

Shrivelled female gametophytes and attached nucellar material excised from empty seeds could not be processed by the techniques used for living material and so they were rehydrated prior to processing. Rehydration was carried out in a solution recommended by Buchholz (1918, 1938) for use in dissections of living gymnosperm embryos. The material was soaked in a 15% sucrose solution. As the gametophytes were highly dehydrated we added 2 drops

of Tween 20 per 25 ml of sucrose solution and placed them under vacuum for 48 hours. After this, dissections were carried out on 50 seeds in a fresh 15% sucrose solution to determine the stage of embryo development (Spurr 1949) or the soaked material was fixed in FAA (40% formalin/acetic acid/95% ethanol/water, 1/1/14/1, by vol.), dehydrated through a tertiary butyl alcohol series, embedded in paraplast, sectioned at 10 μm, and stained in safranin-fast green (Johansen 1940) or toluidine blue O (Berlyn & Miksche 1976). Twenty gametophytes with nucellar caps were sectioned longitudinally to show the condition of the gametophyte. Cross-sections were made of 20 nucellar caps to determine the extent of pollen tube development.

RESULTS

Empty seeds did not differ in external appearance from full seeds. X-ray analysis showed that the empty seeds were uniform in condition; each of the 1100 seed examined contained a shrivelled female gametophyte (cf. Fig. 1A) with no indication of the presence of embryos.

Sections of the gametophytes revealed that pollination had occurred; however, the number of pollen grains was not determined because some were removed in excising the tissues from the seeds. Longitudinal sections showed evidence of a corrosion cavity in all the gametophytes (Fig. 2). Sections of the nucellar cap revealed that not all the pollen grains germinated and penetrated the nucellar material. No development of pollen tubes was seen in 15% (3 of 20) of the caps (Fig. 3). In 35% (7 of 20) the pollen tube developed only half way through the cap (Fig. 4). In the remaining 50% (10 of 20) pollen tubes completely traversed the nucellar cap (Fig. 5). Evidence of embryos was seen in some of the sectioned gametophytes (Fig. 6); however, accurate counts could not be made with this material.

Dissections of 50 partially rehydrated gametophytes showed that 44% (22 of 50) of them contained embryos (Fig. 7) in early stages, prior to tissue differentiation, of late embryogeny. These embryos were well into the corrosion cavity. No proembryo stages were detected in the dissections or the sections.

DISCUSSION

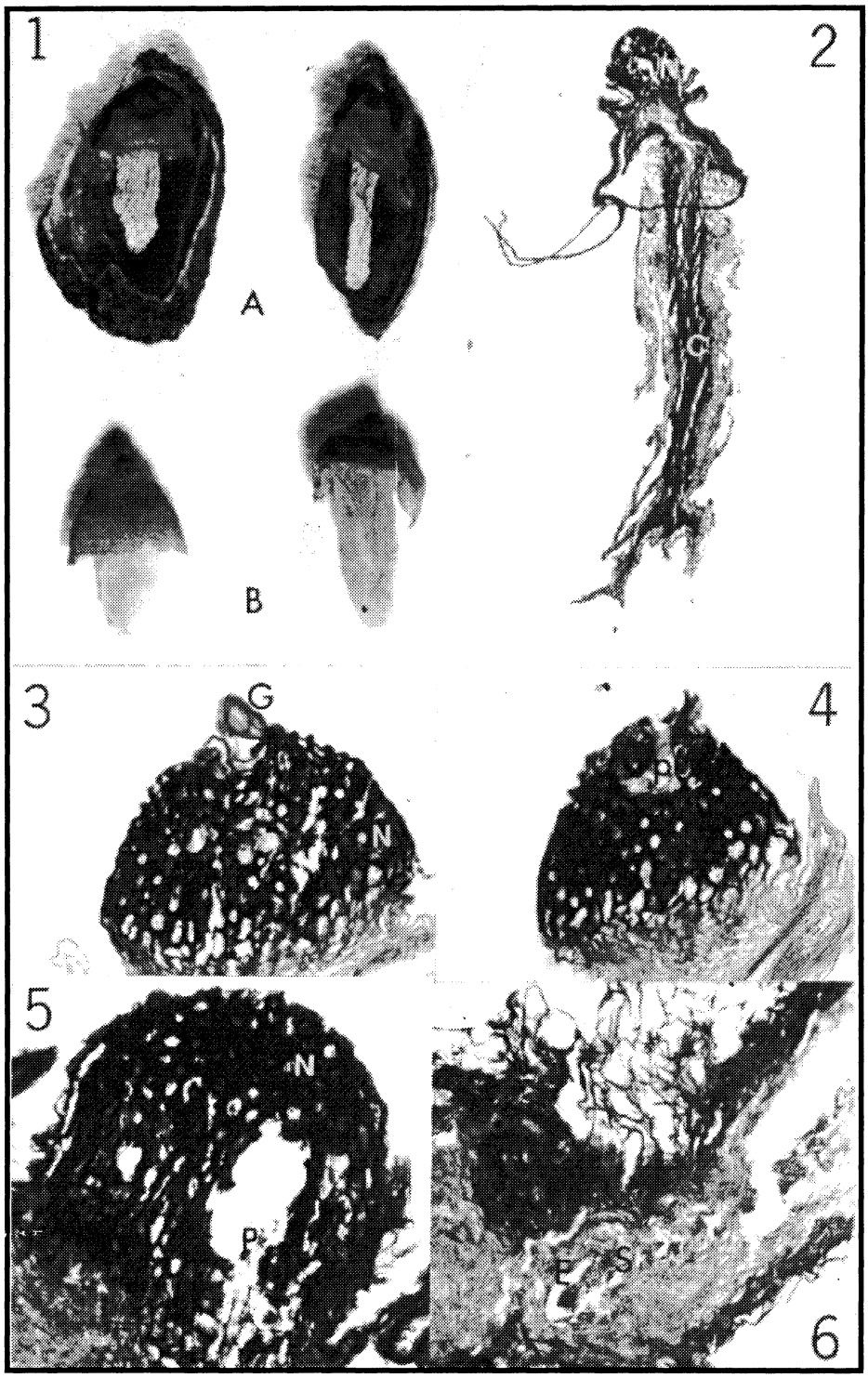
Partial rehydration of the megagametophytes allowed dissections without destroying the developing embryos. The osmotic potential of the tissue, which varies with developmental stage (Gates & Greenwood 1991), should be balanced with that of the rehydration fluid. A lower sucrose concentration allowed more complete hydration but the tissue was more

FIG. 1–Empty seeds of *Pinus radiata* (×6). A = Dissections showing the shrivelled female gametophyte. B = Gametophytes and attached nucellar material after soaking in a 15% sucrose solution.

FIG. 2–Longitudinal section of a rehydrated female gametophyte and nucellar cap (×21). C = corrosion cavity.

FIG. 3 to 5–Longitudinal sections of the nucellar cap (x130): Fig. 3 = Pollen grain with no obvious penetration of the pollen tube into the nucellar cap; Fig. 4 = Pollen tube developed half way through the nucellar cap; Fig. 5 = Cavity in the base of a nucellar cap indicating where the pollen tube developed—a portion of a pollen tube is evident.
G = pollen grain, N = nucellar cap, P = pollen tube.

FIG. 6–Section of female gametophyte with evidence of an embryo in the corrosion cavity (×130). E = embryo, S = suspensor.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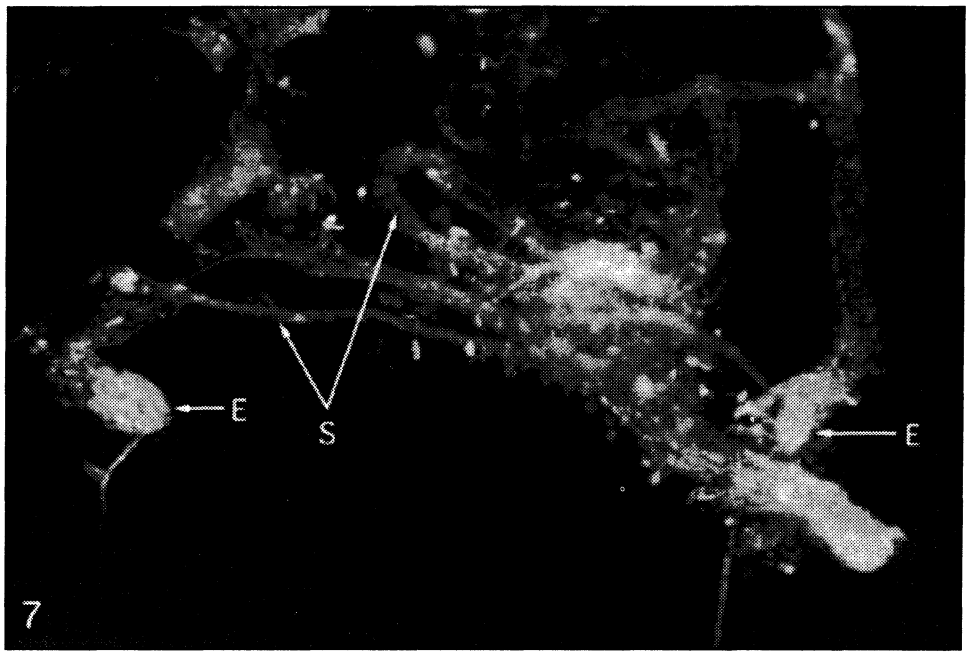

FIG. 7–Content of corrosion cavity showing embryos and suspensors (×400).
E = embryo, S = suspensor.

friable. The sucrose level used was one which supported the growth of embryos of *P. resinosa in vitro* (Gates & Greenwood 1991) and has been useful in dissections of a number of conifer embryos (Buchholz 1938; Dogra 1967).

The presence of pollen in all seeds was expected as pollination is required for development of the female gametophyte in pines (Owens & Blake 1985; Sweet 1973). Pollen grains may reach the nucellar material within 2 days of pollination (Lill & Sweet 1977). In many pines, pollen germination occurs as soon as the pollen grain lands on the nucellus. Penetration of the pollen tube into the nucellar material has been shown within the first few days for *P. strobus* L. (Ferguson 1904), *P. roxburghii* Sar., *P. wallichiana* A.B.Jackson (Konar 1960), and *P. gerardiana* Wall. (Konar 1962). This contrasts with the situation in *P. contorta* Loudon where germination does not occur until 2 months after pollination (Owens *et al.* 1981). Pollen tubes may develop half way through the nucellar cap before meiosis of the megaspore mother cell in *P. radiata* (Fig. 1 in Lill 1976). The limited development of the pollen tubes in 50% of the seeds could be due to conditions in the pollination bags. Elevated temperatures in the isolation bags during pollination and pollen germination could alter both "germinability" and "fertility" of the pollen (Stanley & Linskens 1974).

Bagging megasporangiate cones for pollination frequently decreases seed set (Bramlett & O'Gwynn 1981; Dorman 1976; Nienstaedt & Kriebel 1955) unless large amounts of pollen are provided (Bramlett 1997). The decrease in yield has been attributed to elevated temperature within the pollination bags and some workers have recommended that only cones on the "north" (shady) side of trees be bagged (Orr-Ewing 1956). Temperatures of

megasporangiate strobili inside cellulosic sausage-casing isolation bags were 10–16°C higher than shade temperatures in the same tree. Temperatures of 41°C were lethal to pollen grains of *P. nigra* Arnold (McWilliam 1959). Elevated temperatures would also influence metabolism within the pollen grain. Unlike angiosperms (Taylor & Hepler 1997), germination and early development of pollen in pine are not supported by stored mRNA, rRNA, and proteins but require RNA and protein synthesis (Frankis 1990). During pollen tube development there is a shift in protein synthesis. Thus, active transcription and translation are required for pollen tube development. Both the tube nucleus and generative nucleus are active during this period and would be subject to DNA degradation at high temperatures. There is no "heat shock" response in germinating pollen (McCormick 1993). High temperatures during this period could definitely alter later development and the ability of pollen to effect full seed production. Brown (1971) found that in isolation bags, fresh pollen was more effective than 1-year-old pollen and that increased time in isolation bags decreased the number of full seeds. Orr-Ewing (1956) also found that fresh pollen was more effective than 2-year-old pollen in producing full seed despite good germination in pollen of both ages. This could be explained by enzyme activity in storage decreasing the respiratory substrate in the pollen grain (Stanley & Linskens 1974). Elevated temperatures in pollination bags would also accelerate respiration rates, decreasing the substrate found within the pollen tube (Owens & Morris 1990).

Development of a corrosion cavity in the gametophytes indicates that their development proceeded through the formation of archegonia even where the pollen tube did not complete development through the nucellar cap. Development of the corrosion cavity in *P. radiata* is typical (Lill 1976), starting below the archegonia and progressing as the embryo develops. However, corrosion cavities have been found in a number of pine species when there are no embryos (Buchholz 1918; Dogra 1967).

In 44% of the seeds, embryo development had proceeded to the point where the embryos were in contact with tissue of the female gametophyte. During this early stage of late embryogeny there is competition between embryos from the same archegonia and neighbouring archegonia. Selection at this time has been described for several conifers (Buchholz 1926). The rate of abortion frequently increases (Owens *et al.* 1991) indicating that genetic selection may be occurring within the ovule. Thus, breakdown may occur because of the presence of lethal and semi-lethal genes which might be expressed at this time. This is quite evident with self-pollination (Sorensen 1969) but may also occur in outcrossing situations (cf. Griffin & Lindgren 1985; Owens *et al.* 1991). Nutritional and environmental factors are also very important at this stage of development (Anderson 1965; Sarvas 1962; Sweet 1973; Sweet & Bollmann 1970).

It is not clear how conditions in the isolation bags would affect ovule abortion after fertilisation when embryo development has commenced, 14 to 15 months after pollination. Earlier work suggests that embryos arising from healthy pollen are at an advantage over those from less vigorous pollen. Thus, the first embryo into the gametophyte is usually the one that survives (cf. Sweet 1973). However, in the present situation it is not just some of the embryos that are aborting but all of them. With the high rate of abortion due to lack of fertilisation, the remaining ovules should have had enough nutrients to complete development. There is a possibility that high temperatures during pollen germination and early development of the pollen tube could lead to shifts in protein synthesis and a reduction in carbohydrate reserves

(Frankis 1990; Stanley & Linskens 1974). In pines there is paternal cytoplasmic inheritance (cf. Mogensen 1996; Owens & Morris 1990, 1991), and so changes in the pollen tubes could lead to embryo abortion, especially in the early stages of late embryogenesis which were seen in the present study.

There are severe limitations to retrospective determination of seed loss. The lack of proembryo stages indicates that we may well have missed some embryos. It might be possible to use more specific stains for pollen tubes. However, the results of the present investigation are supported by findings of a developmental study carried out by Dale Smith and his associates at the New Zealand Forest Research Institute in Rotorua, based on dissections of seeds collected at the time of and immediately after fertilisation. They reported a 60% fertilisation rate, based on the presence of embryos beyond the proembryo stage (cf. Setiawati 1994). However, sound seed production was only 24%. Thus, for the empty seed (76% of the total seeds) approximately half (47%, or 36% of the total seeds) would contain embryos and half (53%, or 40% of the total seeds) would show no evidence of fertilisation. We looked at only the empty seed and found that 50% did not show penetration of pollen tube completely through the nucellar cap—thus fertilisation could not have occurred—and 44% had evidence of fertilisation. We would predict, at the 95% confidence interval, that 46 ±6% of the empty seeds would have embryos. It appears that rehydration of shrivelled seeds can yield valuable information on timing of seed abortion.

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REFERENCES

ANDERSON, E. 1965: Cone and seed studies in Norway spruce *Picea abies* (L.) Karst. *Studia Forestalia Suecica 23*: 1–214.

BERLYN, G.P.; MIKSCHE, J.P. 1976: "Botanical Microtechnique and Cytochemistry". Iowa State University Press, Ames, Iowa.

BRAMLETT, D.L. 1997: Genetic gain from mass controlled pollination and topworking. *Journal of Forestry 95(3)*: 15–19.

BRAMLETT, D.L.; O'GWYNN, C.H. 1981: Controlled pollination. Pp. 44–51 *in* Franklin, E.C. (Ed.) Pollen Management Handbook. *USDA Forest Service, Agriculture Handbook No. 587.*

BROWN, I.R. 1971: Flowering and seed production in grafted clones of Scots pine. *Silvae Genetica 20*: 121–132.

BUCHHOLZ, J.T. 1918: Suspensor and early embryo of *Pinus*. *Botanical Gazette 66*: 185–228.

———1926: Origin of cleavage polyembryony in conifers. *Botanical Gazette 81*: 55–71.

———1938: Dissection, staining, and mounting of the embryos of conifers. *Stain Technology 13*: 53–64.

DOGRA, P.D. 1967: Seed sterility and disturbances in embryogeny in conifers with particular reference to seed testing and tree breeding in Pinaceae. *Studia Forestalia Suecica 45*: 1–96.

DORMAN, K.W. 1976: The Genetics and Breeding of Southern Pines. *USDA Forest Service, Agriculture Handbook No. 471.*

FERGUSON, M.C. 1904: Contributions to the knowledge of the life history of *Pinus* with special reference to sporogenesis, the development of the gametophytes and fertilization. *Proceedings Washington Academy of Sciences 6*: 1–202.

FRANKIS, R.C. Jr 1990: RNA and protein synthesis in germinating pine pollen. *Journal of Experimental Botany 41*: 1469–1473.

GATES, J.C.; GREENWOOD, M.S. 1991: The physical and chemical environment of the developing embryo of *Pinus resinosa*. *American Journal of Botany 78*: 1002–1009.

GRIFFIN, A.R.; LINDGREN, D. 1985: Effect of inbreeding on production of filled seed in *Pinus radiata*—experimental results and a model of gene action. *Theoretical and Applied Genetics 71*: 334–343.

JOHANSEN, D.A. 1940: "Plant Microtechnique". McGraw Hill Book Co. Inc., New York.

KONAR, R.N. 1960: The morphology and embryology of *Pinus roxburghii* Sar. with a comparison with *Pinus wallichiana* Jack. *Phytomorphology 10*: 305–319.

———1962: Some observations on the life history of *Pinus gerardiana* Wall. *Phytomorphology 12*: 196–201.

LILL, B.S. 1976: Ovule and seed development in *Pinus radiata*: postmeiotic development, fertilization, and embryogeny. *Canadian Journal of Botany 54*: 2141–2154.

LILL, B.S.; SWEET, G.B. 1977: Pollination in *Pinus radiata*. *New Zealand Journal of Forestry Science 7*: 23–34.

LYONS, L.A. 1956: The seed production capacity and efficiency of red pine cones (*Pinus resinosa* Ait.). *Canadian Journal of Botany 34*: 27–36.

McCORMICK, S. 1993: Male gametophyte development. *The Plant Cell 5*: 1265–1275.

McWILLIAM, J.R. 1959: Effect of temperature on pollen germination of *Pinus* and its bearing on controlled pollination. *Forest Science 5*: 10–17.

MOGENSEN, H.L. 1996: The hows and whys of cytoplasmic inheritance in seed plants. *American Journal of Botany 83*: 383–404.

NIENSTAEDT, H.; KRIEBEL, H.B. 1955: Controlled pollination of eastern hemlock. *Forest Science 1*: 115–120.

ORR-EWING, A.L. 1956: Controlled pollination techniques for the Douglas fir. *Journal of Forestry 2*: 251–257.

OWENS, J.N.; BLAKE, M.D. 1985: Forest tree seed production. A review of literature and recommendations for future research. *Canadian Forest Service, Petawawa National Forest Institute, Information Report PI-X-53*.

OWENS J.N.; MORRIS, S.J. 1990: Cytological basis for cytoplasmic inheritance in *Pseudotsuga menziesii*. I. Pollen tube and archegonial development. *American Journal of Botany 77*: 433–445.

———1991: Cytological basis for cytoplasmic inheritance in *Pseudotsuga menziesii*. II. Fertilization and proembryo development. *American Journal of Botany 78*: 1515–1527.

OWENS, J.N.; COLANGELI, A.M.; MORRIS, S.J. 1990: The effect of self-, cross-, and no pollination on ovule, embryo, seed, and cone development in western red cedar (*Thuja plicata*). *Canadian Journal of Forest Research 20*: 66–75.

———1991: Factors affecting seed set in Douglas-fir (*Pseudotsuga menziesii*). *Canadian Journal of Botany 69*: 229–238.

OWENS, J.N.; SIMPSON, S.J.; MOLDER, M. 1981: Sexual reproduction of *Pinus contorta*. I. Pollen development, the pollination mechanism, and early ovule development. *Canadian Journal of Botany 59*: 1828–1843.

SARVAS, R. 1962: Investigation on the flowering and seed crop of *Pinus sylvestris*. *Communications Instituti Forestalis Fenniae 53(4)*: 1–198.

SETIAWATI, Y.G.B. 1994: Study of seed yield and physical quality in *Pinus radiata* D. Don. seed orchards. M. For. Sci. Thesis, University of Canterbury, Christchurch, New Zealand.

SIMAK, M.; GUSTAFSON, A. 1957: X-ray photography and sensitivity in forest tree sp. *Hereditas 39*: 458–468.

SORENSEN, F. 1969: Embryonic genetic load in coastal Douglas-fir, *Pseudotsuga menziesii* var. *menziesii*. *American Naturalist 103*: 389–398.

SPURR, A.R. 1949: Histogenesis and organization of the embryo in *Pinus strobus* L. *American Journal of Botany 36*: 629–641.

STANLEY, R.G.; LINSKENS, H.F. 1974: "Pollen: Biology, Biochemistry, Management". Springer-Verlag, New York Heidelberg Berlin.

SWEET, G.B. 1973: Shedding of reproductive structures in forest trees. Pp. 341–382 *in* Kozlowski, T.T. (Ed.) "Shedding of Plant Parts". Academic Press Inc., New York.

SWEET, G.B.; BOLLMANN, M.P. 1970: Investigations into the causes of conelet drop in *Pinus radiata* in New Zealand. *In* "Sexual Reproduction in Forest Trees", Vol. 2. Finnish Forest Research Institute, Helsinki.

TAYLOR, L.P.; HEPLER, P.K. 1997: Pollen germination and tube growth. *Annual Review of Plant Physiology and Plant Molecular Biology 48*: 461–491.