Theor Appl Genet (2000) 101:511–518

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Variation in pollen viability among *Picea abies* genotypes – potential for unequal paternal success

Received: 9 December 1999 / Accepted: 22 December 1999

Abstract An in vitro germination method was used to study variation in pollen viability, that is pollen-tube growth rate together with germination percentage, among the *Picea abies* genotypes in a seed orchard. The method permits easy, rapid screening of large numbers of genotypes. Significant variation in pollen viability among the genotypes was evident, the differences among the pollen-lot means being 7–10–fold in different years. No correlation was found between the average pollen viability and the phenology, growth or growing-site characteristics of the pollen donors. However, there appeared to be pollen lots that either benefit from a higher germination temperature or else germinate faster at lower temperatures. The significant variation in pollen viability among the pollen donors indicates a potential for male gametophyte competition. This, together with the observed genotype-environment interactions in pollen performance, may contribute to the variable genetic composition of seed produced in the seed orchard.

Key words Norway spruce · Pollination · Germination of pollen · Pollen-tube growth · Functioning of seed orchard

Introduction

The fitness of male gametophytes depends both on paternal traits, which include the phenology of male flowering and the amount of pollen produced, and on pollen grain traits, such as the germination percentage, germination time, pollen-tube growth rate and selective fertilisation (Pfahler 1975). Competition among male gametophytes has been extensively studied and discussed in angiosperms (Mulcahy 1983 and references therein; Charlesworth 1988; Quesada et al. 1993). In *Hibiscus moscheutos*, for example, a faster average pollen-tube growth rate of the pollen donor has been shown to result in the siring of a larger number of seeds (Snow and Spira 1991, 1996). Also, in several gymnosperm species, e.g. *Pseudotsuga menziesii*, *Pinus radiata*, *Pinus taeda* and *Picea abies*, the application of pollen mixtures has resulted in unequal paternal success of the pollen donors. Pollen competition, including different rates of germination and tube growth, has been suggested as one of the reasons for this (Schoen and Cheliak 1987; Nakamura and Wheeler 1992; Skrøppa and Lindgren 1994). So far the only report of gymnosperms actually showing differences in the average pollen-tube growth rate among pollen donors is in *Pinus sylvestris* (Venäläinen et al. 1999), but there is no information about whether variation in this trait could also affect the genetic composition of the seed produced in seed orchards.

A seed orchard is a plantation of genetically superior trees, managed to produce frequent, abundant and easily harvested seed crops. Such an orchard is established by setting out clones or the seedling progeny of trees selected for desired characteristics (Zobel et al. 1958). In order to ensure the production of genetically diverse and physiologically high-quality seed crops, seed orchards have to fulfil certain requirements with respect to flowering and pollination in the orchard. The functioning of forest-tree seed orchards is often far from ideal. There are large differences in flowering abundance between clones and from year to year in several species (Sweet 1975; Jonsson et al. 1976; Bhumibhamon 1978), including *P. abies* (Lindgren et al. 1977; Skrøppa and Tutturen 1985). Also variation in the reproductive phenology has an effect on the genetic composition of seed produced in seed orchards (Chung 1981; Blush et al. 1993; Harju and Nikkanen 1996). Owing to the abundance of the species and effective pollen dispersal (Koski 1970; Lindgren et al. 1995), high pollen contamination has proved to be a serious problem in the functioning of seed orchards of *P. sylvestris* and *P. abies* (El-Kassaby et al. 1989; Harju and Muona 1989; Savolainen 1991; Pakkanen and

Communicated by P.M.A. Tigerstedt

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Pulkkinen 1991; Paule et al. 1993; Pakkanen et al. 2000). In addition, the genetic composition of the seed produced in seed orchards may be affected by competition among pollen grains.

The aim of the present study was to determine whether there is variation in pollen viability, that is pollen-tube growth rate together with germination percentage, among the *P. abies* genotypes in a seed orchard and, if such variation is found, whether it is connected with other characteristics of the pollen donors or any exogenous factors. The germination percentage and the pollen-tube growth rate of different pollen lots were studied using an in vitro germination method that permits easy, rapid screening of large numbers of trees. In addition, the germination conditions were varied in order to study the behaviour of pollen lots under varying environmental conditions.

Material and methods

The seed orchard

The variation in pollen-tube growth was studied in Norway spruce (*P. abies*) seed orchard no. 170 (Heinämäki) located at Korpilahti, southern Finland (62°13'N, 25°24'E). It consists of 67 clones originating from latitudes 64°–67°N in northern Finland (Nikkanen et al. 1999). The seed orchard, 13.2 ha in size, was established in 1968 on a hill on abandoned agricultural land (Fig. 1).

A number of the properties of this seed orchard have been studied: the variation in flowering abundance (Nikkanen and Ruotsalainen, unpublished), the phenology of flowering (Nikkanen, unpublished), and pollen contamination (Pakkanen et al. 2000). The data from progeny tests of the clones are also available (Ruotsalainen and Nikkanen 1999).

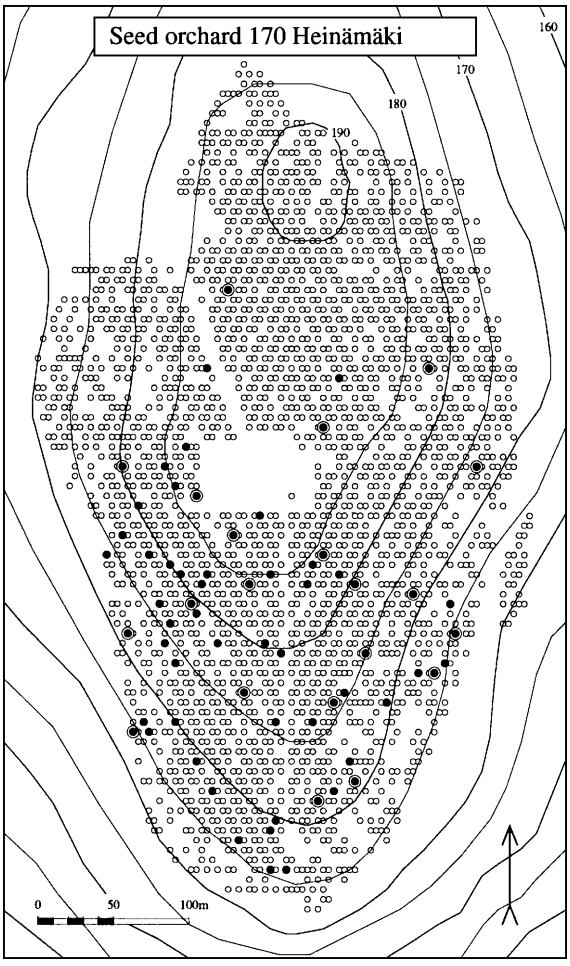

Fig. 1 A map of Norway spruce seed orchard no. 170 indicating the location of the pollen-donor genotypes (●, ◉). The 21 genotypes used in studying the effects of varying germination conditions are marked with ◉

Collection and storage of pollen

Pollen samples were collected from 66 of the 67 clones in the seed orchard in 1996 and 1998. A single graft from each clone was selected as pollen donor (Fig. 1). The same grafts were used in both years. In addition to the seed-orchard clones, five trees from surrounding areas were used as pollen donors, but these trees were not the same in 1996 and 1998.

Pollen was collected by isolating microsporangiate strobili with paper bags a few days before natural pollen shedding. Pollen collected in 1996 was stored in sealed glass bottles at –20°C. Samples from pollen collected in 1998 were taken directly from the isolation bags for in vitro germination, and the rest of the collection was stored as in 1996.

In vitro germination of pollen

Pollen lots were germinated in vitro in 24-well plates by suspending 10 mg of dry pollen in 1 ml of modified Brewbaker's and Kwack's (1963) medium, the suspensions being agitated on an orbital shaker (Infors AG, 180 rpm) as described by Häggman et al. (1997). The germination time, temperature and illumination during germination varied, but in all the experiments each pollen lot was germinated as six replications.

The experimental design included three series of germinations. (1) All the pollen lots were germinated under routinely used in vitro conditions, i.e. for 27 h at +28°C in the dark (Häggman et al. 1997; Aronen et al. 1998; Venäläinen et al. 1999), in order to study the variation in pollen viability. In the 1998 collection germination was performed with fresh pollen, while in the 1996 collection pollen stored for 18 months at –20°C was used.
(2) In the 1998 germinations the behaviour of 21 pollen lots selected from the seed-orchard genotypes and the five pollen lots originating from the surrounding forests was studied under varying germination conditions, either outdoors or indoors at different constant temperatures. In the outdoor experiment with fresh pollen, the pollen suspensions on the orbital shaker were placed outside under a light shelter to protect the samples from direct sunlight and rain, but subjected to diffuse illumination and natural temperature variations. During the experiment the highest daily temperatures in the shelter were around +15°C and the lowest night temperatures around +2°C, the daily mean temperature being +8°C. Germination was started in the late afternoon and lasted for 92 h, i.e. until the mixture of pollen samples used for monitoring the progress of germination appeared to be well-germinated as evaluated under a microscope. The dependence of pollen viability on the germination temperature was studied using the same 1998 pollen lots stored for 9 months at –20°C. Germination of the stored pollen was performed in the dark at constant temperatures of +28°C, +18°C, and +8°C. The germination times were adjusted so that the pollen lots received the same effective temperature sum in all treatments: germination lasted for 27 h at +28°C, for 42 h at +18°C, and for 95 h at +8°C.
(3) An experiment was also performed to investigate how long pollen keeps its viability unchanged after shedding. For this pur-

pose, the 1998 pollen of four genotypes (P375, P491, P498 and P695) was kept in open isolation bags in a greenhouse and germinated for 27 h at +28°C in the dark 1, 4, 7, 14, 19 and 26 days after pollen collection.

Measurements of pollen-tube length

Random samples of germinated suspensions of pollen were photographed under an Olympus CK2 microscope (magnification 13.2 ×) using an attached SC35 camera. The negatives of the black and white films were enlarged (10 ×) with a photographic enlarger. The lengths of the pollen-tubes were measured from these enlargements using a calliper rule connected to a computer. About 50 pollen grains were measured per replication, the total number of measurements being about 73 800. The possible appearance of a second pollen-tube was also recorded for each pollen grain. The pollen-tube length of non-germinated pollen grains was recorded as zero. This procedure was adopted because the aim was to determine the actual competitive ability of the pollen lot under in vivo conditions in which a non-germinating pollen grain also occupies space in the pollen chamber. Moreover, very slowly germinating and non-viable pollen grains could not yet be distinguished after a 27-h germination. In addition, the diameter of the pollen grains including sacci was measured on a sample of about 300 pollen grains per pollen lot collected in 1998 and stored for 9 months at –20°C.

Statistical analyses

Statistical analyses of the measurements of pollen-tube length were carried out using the replication means as observations. Single-tube lengths were not used because the tubes growing in the same well could have been affected by the same environmental disturbances, e.g. growth of microbial contaminants. The experimental design thus provides six observations for each lot, i.e. pollen collected from one genotype and treatment combination. The analysis of variance and post-hoc tests for the treatment means were performed using the GLM General Factorial procedure, and the calculation of correlation coefficients using the Bivariate Correlations procedure of SPSS Base 8.0 statistical software (SPSS Inc. 1998).

Results

Differences in pollen viability among genotypes

Considerable variation was observed in both the germination percentage and the average tube growth rate of the pollen lots. After 27-h in vitro –germination, the percentage of germinated pollen grains in the pollen lots collected in 1998 and germinated immediately after harvesting varied from 62 to 98, the grand mean being 91 (± SE 0.8). The average pollen-tube length in these pollen lots ranged from 37 to 252 µm, the grand mean being 160 (± 6.0) µm. In the pollen lots collected in 1996 and stored at –20°C for 18 months before germination, the average germination percentage was 72 (± 1.9), varying from 31 to 94%. The pollen-lot means for tube length in the 1996 material varied from 26 to 249 µm, the grand mean being 131 (± 7.9) µm. When only the germinated pollen grains are considered, the grand means for pollen-tube length in the 1998 and 1996 materials were the same, 175 (± 6.2) and 172 (± 7.8) µm, respectively. There was, however, a significant correlation between the germination percentage and the average tube length of the germinated pollen grains in both years, the Pearson *r* value being 0.270 ($P = 0.028$) in 1998 and 0.670 ($P = 0.000$) in 1996.

Table 1 Analysis of variance for average pollen-tube length after in vitro germination of 1996 and 1998 pollen lots originating from Norway spruce seed orchard no. 170. The 1996 material was stored at –20°C before germination, while the 1998 pollen was fresh

Source	*df*	MS	F-value	*P*-value
Pollen donor	65	25 085	34.1	0.000
Collection year	1	89 360	121.4	0.000
Interaction	64	12 506	17.0	0.000
Error	654	736		

The significance of the factors affecting pollen viability (length measurements including non-germinated pollen grains as zero values), i.e. pollen donor and collection year, was studied using analysis of variance. Both the effects of the pollen donor and collection year, as well as their interaction, were found to be significant ($P = 0.000$) (Table 1). The Student-Newman-Keuls test for a multiple comparison of means also indicated significant ($P<0.05$) differences among the pollen donors. When the collection years were analysed separately, the pollen donors from the area surrounding the seed orchard, i.e. the trees representing background pollinators, did not form a group of their own. Their lot-means fell evenly within the total range of the tube-length values. The Pearson correlation coefficient for the pollen-lot means in tube length between the years 1998 and 1996 was 0.324 ($P = 0.008$).

There was also variation in the average pollen-grain diameter and in the appearance of a second pollen-tube among the pollen lots. The pollen-lot means for the grain diameter varied from 95 to 116 µm, the grand mean being 106 (± 0.98) µm. In 1998, the average percentage of pollen grains with a second tube varied from 0 to 49%, the grand mean being 14 (± 1.3). In the 1996 material, the corresponding values were 2–54% and 21 (± 1.5). The Pearson *r* value for the pollen-lot means in the ap-

Table 2 Analysis of variance for average pollen-tube length of 1998 pollen lots germinated under varying germination conditions, (a) fresh pollen germinated at a constant temperature of +28°C and outdoors, and (b) stored pollen germinated at +28°C, +18°C, and +8°C

Source	*df*	MS	F-value	*P*-value
a				
Pollen donor	24	11 357	13.9	0.000
Germination condition	1	459 686	562.9	0.000
Interaction	24	6303	7.7	0.000
Error	250	817		
b				
Pollen donor	25	31 241	108.4	0.000
Germination condition	2	299 773	1040.8	0.000
Interaction	50	7352	25.5	0.000
Error	390	288		

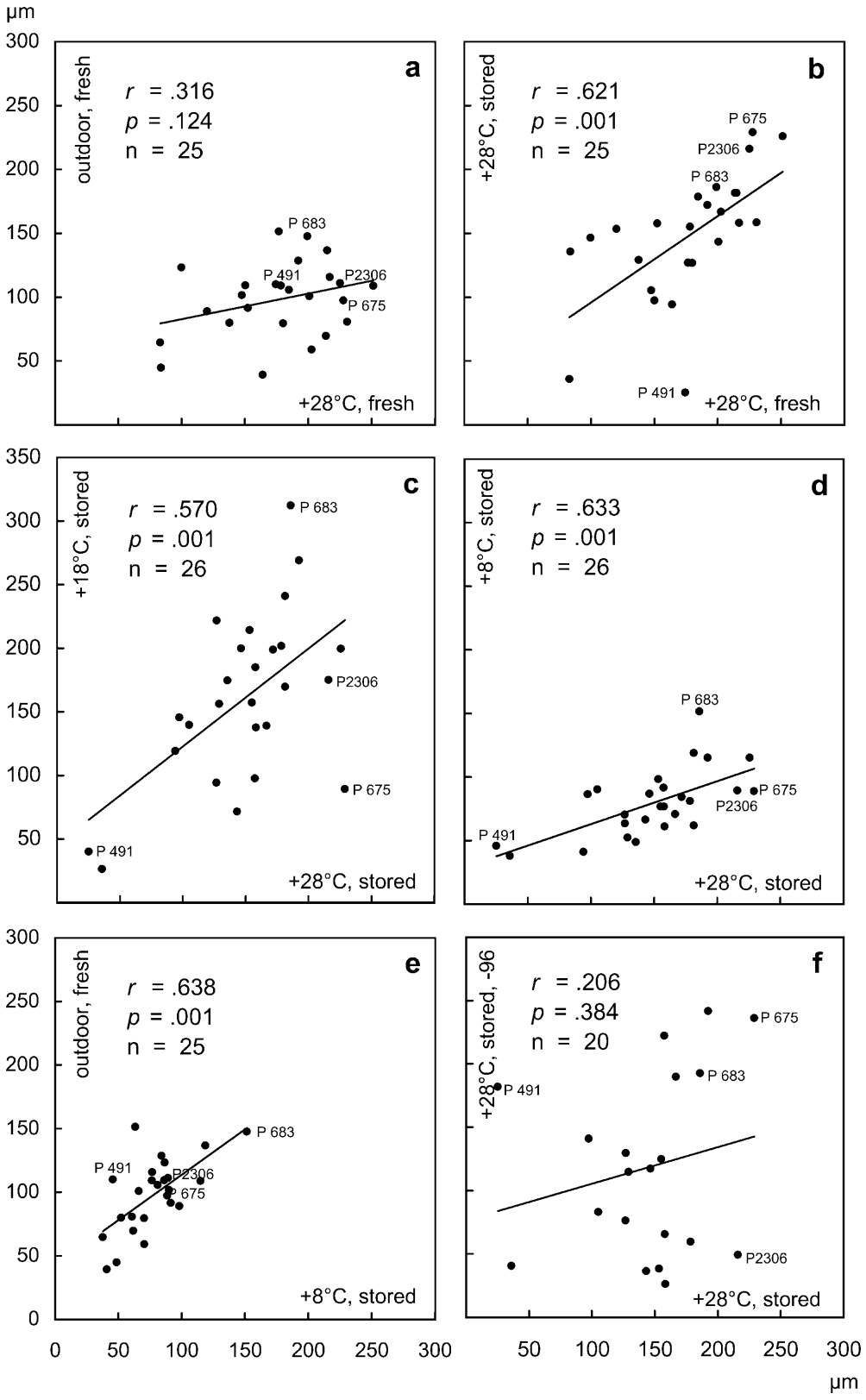

Fig. 2a–f The average pollen-tube lengths after in vitro germination under varying conditions. The Pearson correlation coefficient (*r*) together with its significance (*P*) and the number of pollen lots (*n*) in each comparison are shown. Some of the pollen lots are marked with their clone number. Comparison between **a** germination at a constant temperature of +28°C and outdoors using fresh 1998 pollen, **b** fresh and stored 1998 pollen at +28°C, **c** stored pollen at +28°C and +18°C, **d** stored pollen at +28°C and +8°C, **e** stored pollen at +8°C and fresh pollen germinated outdoors, and **f** stored 1998- and 1996-pollen at +28°C

pearance of a second tube between the years 1998 and 1996 was 0.555 (P = 0.000). Also the correlation between the appearance of the second tube and the germination percentage or the average pollen-tube length was positive and significant in both years: with Pearson correlation coefficients of 0.403 and 0.398 (P = 0.001 for both) in 1998, and 0.470 (P = 0.000) and 0.277 (P = 0.026) in 1996, respectively.

Potential connections between the variation observed in pollen viability and other characteristics of the pollen

donors or specific exogenous factors were studied using correlation analysis. No correlation was found with the geographical origin, the abundance of male flowering, or the average performance of the progenies of the pollen-donor genotype, nor with the growth characteristics or the phenology of male flowering of the particular graft from which the pollen was collected. No correlation was found with the average pollen-grain diameter, and none with graft spacing or soil factors, such as the pH or exchangeable Ca, K, P, or Mg concentrations of the growing site of each particular pollen-donor graft. Also when the appearance of the second pollen-tube was examined, no correlations with any genotype, graft, or soil characteristics studied were found.

Effect of germination conditions on pollen viability

The behaviour of the pollen lots under varying environmental conditions was studied by changing the in vitro germination conditions of the 1998 material. The significance of the pollen donor and germination conditions, as well as their interaction, was studied using analysis of variance. The effects of the pollen donor, germination conditions and their interaction were found to be significant (P = 0.000) in the case of both fresh pollen germinated at a constant temperature of +28°C in the dark and outdoors (Table 2a), and for pollen stored at –20°C and germinated at temperatures of +28°C, +18°C and +8°C (Table 2b).

The pollen-lot means for tube length in different germination conditions were compared using correlation analysis. The pollen-lot means of the most important comparisons are plotted against each other in Fig. 2. No significant correlation was found between the pollen-lot means when fresh pollen was germinated either under routinely used in vitro conditions, i.e. for 27 h at +28°C in the dark, or outdoors (Fig. 2a).

Based on the finding that the pollen lots, either fresh or stored at –20°C, behaved in a rather similar fashion under the same germination conditions, with the exception of lot P491 (Fig. 2b), the effect of germination temperature on pollen viability was studied more closely using pollen stored at –20°C. As can be seen from Fig. 2c and d, there was a significant positive correlation between germination at either +18°C or +8°C and at +28°C. When the pollen was germinated at +18°C the differences among the pollen lots were larger than at +28°C, although the grand means for these germinations were similar, 161 (± 13) µm and 149 (± 9.7) µm. At +8°C, all the pollen lots germinated slowly, the grand mean being 80 (± 5.2) µm. The differences among the lots were subsequently smaller. There were a few pollen lots that behaved differently under varying germination conditions. For example, lots P675 and P2306 benefited from a higher germination temperature, while lot P683 germinated relatively faster at lower temperatures. The comparison between germination outdoors under temperatures varying from +2 to 15°C and germination at +8°C showed a significant positive correlation (Fig. 2e) that was also present between germination outdoors and at +18°C, the Pearson r value for the latter case being 0.529 (P = 0.006). No correlation was found between the means for pollen-tube length in the lots collected in 1998 and 1996 and stored at –20°C for either 9 or 18 months and germinated under the same conditions (Fig. 2f).

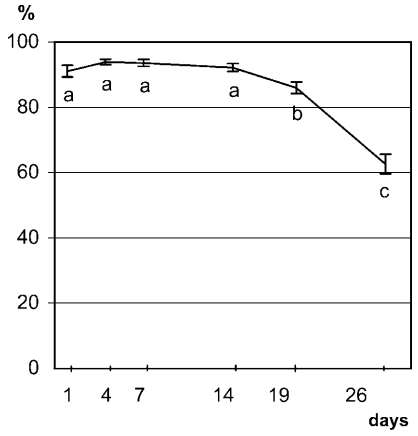

Fig. 3 The mean germination percentage of pollen lots P375, P491, P498, and P695 at 1–26 days after pollen collection. The daily means marked with differing letters differ significantly from each other, P<0.05 in the Student-Newman-Keuls test for a multiple comparison of means

Persistence of pollen-germination ability

The persistence of pollen viability was studied by measuring the germination percentage and tube length of the germinated pollen grains of lots P375, P491, P498, and P695. The germination percentage remained unchanged for 2 weeks after pollen shedding (Fig. 3). The tube length of the germinated pollen grains varied between 164 (± 15) and 214 (± 11) µm during days 1–19, and was significantly lower (138 (± 18) µm, P<0.05 in a Student-Newman-Keuls test for a multiple comparison of means) on day 26 after shedding.

Discussion

There was significant variation in pollen viability among the *P. abies* genotypes in the seed orchard, the differences among the pollen-lot means being 7–10–fold in different years. These results confirm the earlier report of considerable variation in pollen-tube growth rate among individuals selected from natural populations of *P. sylvestris* (Venäläinen et al. 1999). There is good evidence that the pollen-tube growth rate of angiosperms is phenotypically variable, and that this variation affects fitness (see Havens 1994). It has been suggested that pollen competition might also be one of the reasons for unequal reproductive success in gymnosperms (Schoen and Cheliak 1987; Nakamura and Wheeler 1992 and ref-

erences therein; Skrøppa and Lindgren 1994). Ours, however, is the first report showing variation in pollen viability in a seed orchard of an industrially important tree species.

In the present work, differences in pollen viability were studied in vitro. Germination and the early stages of the tube growth of spruce pollen have been found to be similar both in vitro and in vivo, although they occur faster in the former (Dawkins and Owens 1993; Martinussen 1994; Lazzaro 1996). After 27-h in vitro germination in the present study, the pollen-tubes in the fastest lots had elongated on the average to 250 µm. This is approximately 1/3 of the distance they have to grow in a female flower to reach the archegonium and fertilise the egg cell (Sarvas 1968; Christiansen 1972). In the slowest pollen lots the tubes had elongated on the average to only about 30 µm, i.e. approximately 1/25 of the total distance in the female flower. In nature, tube formation in *P. abies* pollen takes place during two growth periods, interrupted by a resting period, during which both male gametes and archegonia with egg cells develop. In the first growth period, which occurs soon after pollination, the tube attains a length of about 240–400 µm and then continues to grow 3–4 weeks later, i.e. immediately before fertilisation (Sarvas 1968; Christiansen 1972).

The reproductive biology of *P. abies* provides an opportunity for male gametophyte competition through differential germination ability and tube growth rate. The pollen chambers of the species can accommodate more than ten pollen grains, five being the average number (Sarvas 1968). Fertilisation takes place relatively soon after pollination and, under in vitro conditions at least, elongation of the tubes continues in a linear fashion up to lengths comparable with the final distance to the archegonia (Martinussen 1994; Martinussen et al. 1994). This suggests that the resting period reported during in vivo germination may not change the order of competing tubes. Moreover, no pre-zygotic incompatibility mechanisms have been reported. However, there are also features that might affect the results of gametophyte competition. In *P. abies*, there is usually more than one archegonium per ovule, and in most cases two competing embryos are formed to ensure the formation of full seed. Thus the genotypes homozygous for lethal, sublethal or defective genes are eliminated either by early abortion of the zygotes or, later on, through embryo competition (Sarvas 1968).

According to the results of the present study, the fitness of the *P. abies* pollen donors with respect to the germination ability and tube length of their pollen varies, as has also been reported in many angiosperm species. According to Havens (1994), however, it is unclear whether the variation found in pollen performance is really heritable or is caused by environmental effects. Her results obtained with pollen from cuttings of *Oenothera organensis* suggest that the condition of the plant, flowering shoot and / or flower may be more important than genotype in determining the pollen-tube growth rate. In the present study, only a single graft per genotype was used as pollen donor but, on the other hand, potential connections between pollen viability and several exogenous factors and other characteristics of the pollen donors were also investigated. Since no correlation was found between the average pollen-tube length and, e.g., the phenology, growth or site characteristics of the pollen donors, the variation found is expected to reflect the genetic potential of the genotypes. Unlike several studies on angiosperms (Mulcahy 1983 and references therein; Quesada et al. 1993), no connection was, however, found between the pollen-tube growth rate and the performance of the progenies. This is in accordance with the results obtained with another conifer, *P. sylvestris* (Venäläinen et al. 1999).

Significant genotype-year interaction was found when the pollen viability of all the genotypes of the seed orchard were examined in different collection years. The varying behaviour of the pollen lots in the 1996 and 1998 collections may be caused by a number of factors. The weather conditions in 1996 and 1998 during flowering were different. The fact that the 1996 material was stored at –20°C before germination complicates the results because some of the pollen lots may have suffered from freezing, as was found for the 26 lots in the 1998 material that were germinated under varying conditions.

Our study suggests that there are pollen lots that either benefit from a higher germination temperature or germinate faster at lower temperatures. This may indicate the adaptation of different *P. abies* genotypes to produce fast-germinating pollen for different environmental conditions. As pointed out by Delph et al. (1997), genotype-environment interactions in pollen performance will promote the maintenance of genetic variation within populations even if pollen performance is related to fitness. Johnsen et al. (1996) had earlier suggested that some environmental signals during the reproductive process taking place in the female flowers, for instance pollen-tube growth, may cause variation in the phenology traits of the progeny. In seed orchards, these phenomena may contribute to the variable genetic composition of seed produced in different years.

As a consequence of the genotype-environment interactions in pollen performance, a single in vitro germination under routinely used conditions cannot give a complete picture of the variation among genotypes. According to the literature, the recommended conditions for the in vitro germination of spruce pollen include a relatively high constant temperature, from +25 to +30°C, and no illumination (Christiansen 1972; Lanteri et al. 1993; Martinussen 1994; Lazzaro 1996; Häggman et al. 1997; Aronen et al. 1998). In the present study the lot-means for pollen viability at a constant temperature of +28°C did not correlate with those in outdoor conditions, while the correlation between pollen viability at a constant temperature of +8°C and outdoor conditions was positive and significant. On the other hand, a significant positive correlation for pollen viability was found when the germination temperature alone was changed. In future studies, however, it would be reasonable to use lower in vitro

germination temperatures than the recommended temperature of +25 to +30°C in order to achieve the results that best correspond to the variation occurring in nature. The weather conditions in Finland during the flowering period of spruce differ considerably from year to year, the daily mean temperatures varying from +8 to +21°C during 1984–95, with a mean of +13°C (Nikkanen and Ruotsalainen, unpublished). The outdoor conditions in 1998 were colder than the average but still favourable for successful pollination in *P. abies*.

Under the experimental conditions employed in this study, i.e. dry pollen in open isolation bags in the greenhouse, both the germination percentage and the tube growth of *P. abies* pollen remained unchanged for 2 weeks after pollen shedding. Hak (1996) has earlier shown that vacuum-processed *Picea mariana* pollen stored for 1 year at +18°C can retain a high germinability of 78%. In the case of *P. abies* and *P. sylvestris*, it has been estimated that the share of pollen that has migrated from another population located over hundreds of kilometres away is small on the average, but can account for a significant proportion of the total pollination in some years (Koski 1970; Lindgren et al. 1995). Moreover, long-distance pollen of *P. sylvestris* has been shown to maintain high germinability (Lindgren et al. 1995). In spruce seed orchards, the high persistence of the germination ability of pollen enables fertilisation by pollen that has travelled over long distances. Air-borne pollen is exposed to direct ultraviolet radiation, and this has been shown to reduce pollen-tube growth in 19 of 34 taxa representing both monocotyledons and dicotyledons. In coniferous pollen both stimulating and inhibiting effects have been reported (Torabinejad et al. 1998 and references therein).

The significant variation in pollen viability found among the *P. abies* genotypes in the present seed orchard study indicates a potential for male gametophyte competition. Together with the observed genotype-environment interactions in pollen performance, this may contribute to the variable genetic composition of the seed produced in the orchard. The magnitude of the effects of pollen viability can, however, be assumed to be smaller than the effects of variation in flowering abundance and phenology within the seed orchard and of pollination by non-orchard sources. Pollen-tube competition in *P. abies* will be further studied by making controlled crossings with pollen-lot mixtures including fast and slowly elongating pollen-tubes, and by carrying out paternity analysis on the progenies.

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