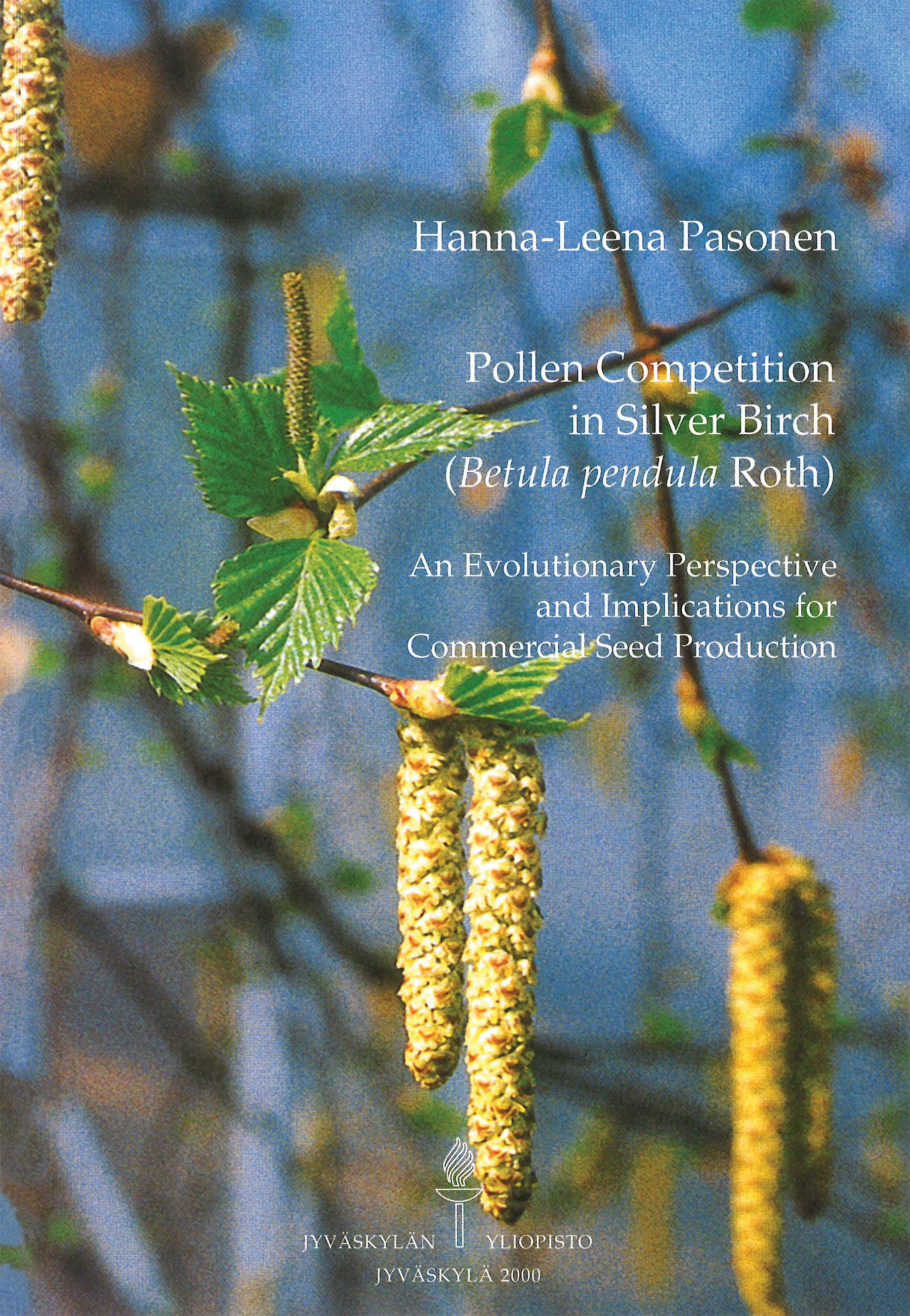

Hanna-Leena Pasonen

Pollen Competition in Silver Birch (*Betula pendula* Roth)

An Evolutionary Perspective and Implications for Commercial Seed Production

JYVÄSKYLÄN YLIOPISTO
JYVÄSKYLÄ 2000

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ABSTRACT

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Pollen competition arises from differences among pollen donors in pollen-tube growth rate. According to the pollen competition hypothesis, only the fastest-growing pollen tubes are assumed to achieve fertilization. By this thesis, I wanted to obtain new information about pollen competition and nonrandom fertilization and the relationship between pollen and progeny performance in *Betula pendula* Roth. I also considered the possible consequences of pollen competition for sexual selection, and for commercial production of genetically-improved *B. pendula* seed. The results of this thesis revealed that pollen competition is a real phenomenon in *B. pendula* seed orchards. There were significant differences among pollen donors in pollen-tube growth rate and a positive relationship between pollen-tube growth rate and seed siring success. These findings indicate that selection among pollen donors can occur on the basis of the differences in pollen performance. The role of pollen-tube growth rate as a predictor of progeny performance remained obscure. In natural birch stands, the relationship between pollen-tube growth rate and seed siring success might not be as straightforward as in controlled greenhouse conditions. Microclimatic variability and maternal environmental effects have random effects on pollen-tube growth rates and the outcome of pollen competition. Possibilities for pollen competition to lead to ongoing sexual selection and evolutionary consequences are likely to be diminished in nature. Microsite variability and genotype-environment interactions can partly explain the maintenance of variation in pollen-tube growth rates.

Key words: Genotype-environment interactions; maternal effects; paternal effects; pollen competition; pollen-pollen interactions; pollen population effect; pollen-tube growth rate.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles which are referred to in the text by their Roman numerals. I have personally written each paper and performed large part of the work.

I Pasonen, H.L., Pulkkinen, P., Käpylä, M. & Blom, A. 1999. Pollen-tube growth rate and seed-siring success among *Betula pendula* clones. New Phytologist 143: 243-251.

II Pasonen, H.L., Pulkkinen, P. & Käpylä, M. 2000. Do pollen donors with fastest-growing pollen tubes sire the best offspring in an anemophilous tree, *Betula pendula* (Betulaceae)? American Journal of Botany. In press.

III Pasonen, H.L., Käpylä, M. & Pulkkinen, P. 2000. Effects of temperature and pollination site on pollen performance in *Betula pendula* Roth – evidence for genotype-environment interactions. Theoretical and Applied Genetics 100: 1108-1112.

IV Pasonen, H.L., Pulkkinen, P. & Kärkkäinen, K. 2000. Genotype-environment interactions in pollen competitive ability. Submitted.

V Pasonen H.L. & Käpylä, M. 1998. Pollen-pollen interactions in *Betula pendula in vitro*. New Phytologist 138: 481-487.

1 INTRODUCTION

1.1 Theoretical background

The history of pollen competition as an evolutionarily interesting phenomenon goes back to the beginning of the twentieth century. At first, the term 'certation' was used to describe the phenomenon where competition between different genotypes during pollen germination changed the sex ratio in the offspring (Correns 1928, reviewed by Walsh & Charlesworth 1992 and Delph & Havens 1998). Some years later, Haldane (1932) observed that competition among pollen grains is likely to occur due to 'serious overcrowding' of pollen grains on stigmas (reviewed by Walsh & Charlesworth 1992 and Delph & Havens 1998). Most researchers studying pollen competition nowadays, refer to Mulcahy (1979) who formulated the 'modern' theory of pollen competition and argued that pollen competition might have had an important role in the evolution of higher plants. The first requirement for pollen competition to occur is that there are more pollen grains on the stigmas than there are ovules in the ovary. It is believed that under conditions of pollen competition, only pollen grains with the fastest-growing pollen tubes can achieve fertilization. From this perspective, pollen-tube growth rate should be an important component of male reproductive success.

The large population size and the haploid status of pollen provide a fundamental basis for considerations on the evolutionary significance of pollen competition because selection during the gametophytic stage of life cycle can be very effective (Mulcahy 1979). For pollen competition to drive the evolution of higher plants (Mulcahy 1979) several conditions must be met. Most importantly, there must exist additive genetic variation in pollen-tube growth rate (Mulcahy 1979). Secondly, pollen donors with the fastest-growing pollen tubes must sire a disproportionate large number of seeds (Mulcahy 1979). And thirdly, differences among pollen donors in pollen performance, and generally, in mating success, should be consistent across different maternal

plants (Charlesworth et al. 1987). No evolutionary changes will occur if pollen donors exhibit unconsistent mating success across recipient plants.

1.2 Gametophytic-sporophytic genetic overlap

Already in the 1930s' Haldane argued that pollen grains have a physiology of their own and that pollen tube growth depends mostly on its own genes (reviewed by Delph & Havens 1998). This view is supported by the findings of Brink and MacGillivray (1924) and Demerec (1924) who found that maize plants that were heterozygous for a waxy endosperm produced waxy and starchy pollen grains in approximately equal numbers, indicating gametophytic expression of these genes (reviewed by Walsh & Charlesworth 1992). Today it is known that gene expression in pollen may be either gametophytic (expressing the genes carried by a pollen grain) or sporophytic (expressing the genes of a pollen parent), or both (e.g. Walsh & Charlesworth 1992). The most important and interesting finding in the field of pollen gene expression has been that most of the genes, up to 90%, expressed by a pollen grain are also expressed by the resulting seedling (e.g. Tanksley et al. 1981, Willing & Mascarenhas 1984). This kind of overlapping in the gene expression during the two stages of life cycle is called gametophytic-sporophytic genetic overlap.

Gametophytic-sporophytic genetic overlap has provided a fundamental background for many studies on pollen competition and pollen selection. The basic idea is that if pollen tube growth and plant growth both depend on the basic metabolic activities controlled by the same genes, the fitness of the fertilizing pollen grains influences the vigour of the sporophyte (Ottaviano & Mulcahy 1989). In most cases, the relationship between pollen-tube growth rate and progeny performance has been studied by varying the intensity of pollen competition (e.g. Schlichting et al. 1990, Richardson & Stephenson 1992, Quesada et al. 1993). It has been expected that seeds sired by pollen from conditions of intense pollen competition are more vigorous and give rise to better offspring than seeds sired by pollen from conditions of less intense competition. In plant breeding it is possible to utilize the knowledge of gametophytic-sporophytic genetic overlap by selecting certain sporophytic traits, for example cold (Zamir et al. 1982, Zamir & Gadish 1987) or herbicide (Frascaroli et al. 1995) tolerance, by using pollen selection (e.g. Hormaza & Herrero 1992, 1996a). Pollen selection is an approach in which pollen is first exposed to stressful conditions and subsequently used in pollination. Only those pollen grains that have survived the exposition are able to sire seeds and transfer their genes to the next generation. Thus, viable pollen grains should give rise to viable seedlings. In a similar manner, environmental conditions can vary over space and time in natural populations of plant species and selection subjected to pollen grains can translate into correlated responses in the sporophytic level.

1.3 Production of genetically-improved birch seed

In Finland, genetically-improved birch seed for forestry is produced at seed orchards established in plastic greenhouses called polytunnels. Each seed orchard consists of two to fourty grafted *Betula pendula* clones which have originally been selected for seed production on the basis of superior, heritable growth characters, like seedling height, revealed by progeny trials (Raulo & Koski 1977). Clones in a same seed orchard originate from similar climatic conditions. The purpose of the plastic greenhouses is to isolate the seed orchard clones from outside pollen sources, promote flowering and increase the seed crop. Temperature during pollination and seed development in the greenhouses is substantially higher than outdoors and, during vulnerable periods of flowering, frost damages can be prevented by heating. Realized breeding gains in growth and quality traits of the seed orchard material have been observed to be substantial when compared with the material of stand origin collected in the corresponding breeding zones (Hagqvist & Hahl 1997, 1998). At the moment, significant part of the birch seed material used at nurseries has been produced in plastic house seed orchards, and according to the recent national seed production programme, all birch seed material needed at nurseries and half of the seed material needed in forestry are planned to be produced in seed orchards (Hagqvist 1998).

1.4 Objectives of this thesis

The main objective of this thesis was to obtain new information about pollen competition and nonrandom fertilization, and evaluate the consequences of pollen competition for sexual selection in an anemophilous tree, *Betula pendula.* Clonal *B. pendula* seed orchards established in plastic greenhouses provided a controlled environment to study evolutionarily important phenomena in sexual reproduction of this commercially important tree species. From the practical point of view, I considered the possible consequences of pollen competition for commercial production of genetically-improved *B. pendula* seed. Paying more attention to the biological processes between pollination and seed development can enhance seed production and possibly provide new methods for selecting genotypes for seed orchads in future. By the studies included in this thesis I searched answers to the following questions:

1. Is pollen competition likely to occur among the seed orchard clones?
2. What are the consequences of pollen competition for the genetic composition of the seed crop?
3. Can the quality of seeds and seedlings be predicted on the basis of the performance of the pollen that sired the seeds?

4. How environmental conditions during pollination and pollen tube growth affect the outcome of pollen competition?
5. What are the possible consequences of pollen competition in natural birch stands?

2 EVOLUTIONARY PERSPECTIVE

2.1 Sexual selection in plants

The concept of sexual selection was originally developed on the basis of Darwin's recordings of animal behavior, especially observations of complicated courtship displays and unusual morphological traits (Darwin 1859, 1871). Sexual selection has usually been defined as selection that arises from differences among individuals in mating success (Arnold 1994). With respect to sexual selection, Darwin did not pay much attention to plants (Arnold 1994), which is one reason for the late awakening of the interest for studying plant sexual selection. However, the idea that sexual selection also occurred in plants took slowly root, and the number of studies related to plant sexual selection began to increase in the 1980s. Inspired by the thoughts of Mulcahy (1979), these studies have primarily attempted to demonstrate a positive relationship between pollen and progeny performance (e.g. Mulcahy & Mulcahy 1975, Davis et al. 1987, Winsor et al. 1987, Bertin 1990, Schlichting et al. 1990, Snow 1990, Quesada et al. 1993). Gradually it has also been paid more attention to the possible occurrence of female choice in plants, especially nonrandom seed abortion, (Stephenson & Bertin 1983, Marshall & Ellstrand 1986, Marshall & Ellstrand 1988, Rocha & Stephenson 1991, O'Donnel & Bawa 1993, Baker & Shore 1995), although detecting it has proven to be difficult.

In plants, sexual selection can occur at three temporally separated stages during sexual reproduction, including e.g. pollinator attraction before pollination, pollen competition between pollination and fertilization, and embryo and seed abortion, which occur after fertilization and are the most frequently studied forms of female choice (Stephenson & Bertin 1983). All these three stages of plant sexual selection can bias the mating success of an individual from random to nonrandom. At the moment, after twenty vital years of studying plant sexual selection, there is an increasing body of evidence to date that nonrandom mating is a frequent phenomenon in many

plant species. One of the first studies in which genetic markers (allozymes) were used to examine the paternity of the seeds was carried out by Marshall & Ellstrand (1986). They found out that two of three pollen donors always sired a disproportionately larger number of seeds than one of the pollen donors across different maternal plants after mixed-pollinations, which is an indication of nonrandom fertilization success of the pollen donors. Unequal paternal success among pollen donors has also been found in many conifers, when equal amounts of pollen from different pollen donors have been applied to female inflorescences in mixed pollen loads (Moran & Griffin 1985, Schoen & Cheliak 1987, Apsit et al. 1989).

Some years later, more attention was paid to the consistency of the ranking orders of the pollen donors across several maternal plants with respect to pollen- tube growth rate (Snow & Spira 1991) and seed siring success (Snow & Spira 1996, Marshall 1998, Mitchell & Marshall 1998). The rankings of the pollen donors have been found to be concordant across maternal parents with respect to pollen-tube growth rate in *Hibiscus moscheutos* (Snow & Spira 1991), and with respect to seed siring success in *H. moscheutos* (Snow & Spira 1996), *Raphanus sativus* (Marshall 1998) and *Lesquerella fendleri* (Mitchell & Marshall 1998). In papers I and IV, I demonstrated a positive relationship between pollen-tube growth rate and seed siring success among *Betula pendula* clones in a greenhouse. These results indicate that sexual selection is possible to occur among the studied plant species.

2.2 Pollen competition and evolution

An underlying assumption for pollen competition to occur, is that pollen deposition exceeds that necessary for full seed set (e.g. Marshall & Folsom 1991). To assess the potential for pollen competition, it should be determined, how many pollen grains are required to fertilize all of the ovules in the ovary and what is the rate of pollen accumulation on stigmas (Snow 1986). In some species seed set has been observed to be pollen limited (e.g. Bertin 1982, Snow 1982, Dudash & Fenster 1997) and competition among pollen grains for ovules is thus not possible to occur. Although the excess of pollen on the stigmas is a basic requirement for pollen competition, there are relatively few studies in which the number of pollen grains and the rate of pollen accumulation on stigmas have been recorded in nature. There is, however, evidence that excess pollen is frequently deposited on most flowers in natural populations of *Hibiscus moscheutos* (Spira et al. 1992) and *Cucurbita foetidissima* (Winsor et al. 2000). The sizes of pollen loads deposited on the stigmas of different flowers can be remarkably variable between years and populations (Dudash & Fenster 1997) and even within a population (Snow 1986). Consequently, possibilities for pollen competition should be separately determined for each study population.

When evolutionary consequences of pollen competition are considered, the magnitude of genetic basis of pollen-tube growth rate should be known. Although many studies have reported substantial phenotypic variation among pollen donors in pollen-tube growth rate (e.g. Ottaviano et al. 1980, Cruzan 1990, Snow & Spira 1991, Björkman et al. 1995), surprisingly little is known about the heritability of this trait. Evidence for genetic basis of pollen-tube growth rate has been searched by 1) comparing pollen-tube growth of progeny from parental lines differing in pollen tube-growth rates (Ottaviano et al. 1975, Sari-Gorla et al. 1975), 2) comparing pollen performance of parental plants produced under varying levels of pollen competition (Snow & Mazer 1988, Schlichting et al. 1990), and 3) analysing the clonal repeatability of pollen-tube growth rates to estimate the broad sense heritability (Havens 1994). There is evidence that pollen tube growth is under genetic control (Sari-Gorla et al. 1975, Sari-Gorla et al. 1992, Johannsson and Stephenson 1998) but in some species, heritability of pollen-tube growth rate appears to be fairly low (Snow & Mazer 1988, Havens 1994). If the variation detected in pollen performance was at least partly heritable, differences among pollen donors in pollen-tube growth rate would be transferred to the next generations and allele frequences in a population would gradually change, when pollen donors with the fastest-growing pollen tubes would always sire a disproportionate large number of seeds.

In sum, to interpret the evolutionary significance of pollen competition and nonrandom fertilization, more has to be known about the relative importance of genetic and environmental effects on pollen-tube growth rate (Stephenson and Bertin 1983, Walsh & Charlesworth 1992). Although many studies provide evidence for pollen-tube growth rate as an important component of male fitness, the heritability of pollen-tube growth rate remains frequently a puzzle that prevents researchers from making very far-going conclusions of the evolutionary significance of pollen competition. It is also important to assure that pollen loads deposited to the stigmas are large enough to make pollen competition possible before consequences of pollen competition in a given population will be evaluated. Every species has a pollination ecology of its own and there might be differences among populations in pollen-ovule ratios even within one species. Consequently, the possibilities for pollen competition can vary among species and populations. Despite of the strong evidence for the occurrence of sexual selection among some plant species, the consequences of that selection over several generations are difficult to verify.

2.3 Maintenance of variation in pollen performance

The question of heritability of pollen-tube growth rate is the biggest stumbling block in the considerations of evolutionary significance of pollen competition.

Firstly, drawing conclusions on the evolutionary consequences of pollen competition is prevented in many studies due to the lack of information about the heritability of pollen-tube growth rate. Secondly, detecting high or moderate heritabilities for pollen-tube growth rate gives rise to another problem; how does variation in pollen-tube growth rate persists if selection for faster pollen tube growth is effective? Theoretical considerations predict that genetic variation in traits closely related to fitness should have largely been eliminated by natural selection (Thomson 1989, Walsh & Charlesworth 1992). Pollen-tube growth rate has in many studies been considered as an important component of male fitness (e.g. Richardson & Stephenson 1992, Quesada et al. 1996a and b, Snow & Spira 1996, Johannsson & Stephenson 1997). If selection during gametophytic stage of life cycle is effective, alleles for the best competing phenotype would most likely have been fixed, which in turn, would have led to loss of genetic variation in pollen performance. Why, then, can differences among individuals in pollen-tube growth rate be observed?

Several mechanisms have been suggested to explain the maintenance of variation in pollen performance (see e.g. Snow & Mazer 1988). Two of the most important are, naturally, mutation and recombination. Due to the large population sizes of pollen grains, many new mutations are produced in each generation (Schlichting et al. 1990). Also recombination "among well-adapted and segregating" genomes can produce gametes which range from highly functional to nonviable generating a continual inflow of new variation (Mulcahy et al. 1996). The inconstancy of selective values provides also part of the answer. Environmental conditions are not constant across space and time, and alleles that are favored by selection in one environment can be disfavored in another environment (Mulcahy et al. 1996). In other words, interactions between genotype and environment can change the ranking orders of the pollen donors in pollen-tube growth rate in a way that different donors have the best performance in different environmental conditions. Further causes for maintenance of variation in pollen performance are possibly provided by gene flow and negative genetic correlations between gametophytic and sporophytic stages of life cycle. Gene flow from one population to another can be quite frequent in nature, especially when wind-pollinated plant species are concerned (e.g. Starfinger & Stöcklin 1996). The selection pressure of the original population of incoming pollen grains should, however, be relaxed, so that differences in pollen-tube growth rates would have persisted (Snow and Mazer 1988). It is possible that alleles for faster pollen tube growth may have unfavorable effects at other stages in the life cycle (Falconer 1981). This means that genotypes with fastest-growing pollen tubes would have lower fitness at some other stage of life, which is opposite of Mulcahy's (1979) view (Walsh and Charlesworth 1992). At the moment, there is no clear evidence for such effects.

Interactions between pollen donors and recipients in pollen competitive ability have been reported (e.g. Cruzan 1990, Johnston 1993, Hormaza & Herrero 1999) indicating that rankings of the pollen donors can change across

different maternal plants. Maternal plants can affect the competitive ability of pollen donors by regulating pollen tube growth on a stigma and in a style and ovary (Cruzan 1993, Herrero & Hormaza 1996) as well as aborting embryos and seeds nonrandomly (e.g. Marshall & Ellstrand 1988). Genetically determined interactions between pollen and style may play an important role in angiosperm evolution (Walsh & Charlesworth 1992) and partly explain the maintenance of variation in pollen performance. On the other hand, if interactions between donors and recipients in pollen competitive ability were constantly occurring, no evolutionary changes could be caused by pollen competition. Also direct pollen-pollen interactions have been reported (Sari-Gorla et al. 1975, Marshall et al. 1996). Consequently, the seed siring success of a pollen donor might not depend only on its own genetic potentiality but also on the identity of the competing pollen grains (Moran & Griffin 1985, Radha et al. 1993). In conclusion, there are several mechanisms that can generate and maintain additive genetic variance in pollen-tube growth rate, although the relative importance of the different mechanisms is difficult to estimate.

3 MATERIALS AND METHODS

3.1 Study species

In the field of pollen competition, the most frequently studied species have been cultivated zucchini (*Cucurbita pepo*) (e.g. Davis et al. 1987, Winsor et al. 1987, Schlichting et al. 1987, Schlichting et al. 1990, Wilson & Payne 1994, Johannsson & Stephenson 1998), its wild progenitor *Cucurbita texana* (Johannsson and Stephenson 1997) and their hybrids (Quesada et al. 1993, Quesada et al. 1996a and b), weedy annuals like *Raphanus sativus* (Marshall & Ellstrand 1986, Marshall & Whittaker 1989, Karron & Marshall 1990, Marshall 1998) and *Raphanus raphanistrum* (Snow 1990) as well as perennial *Hibiscus mosceutos* (Snow & Spira 1991, Spira et al. 1992, Snow & Spira 1996). Studies on pollen competition have focussed strongly on animal-pollinated, herbaceous plant species. Pollen competition has been assumed to be more frequent among animal-pollinated species because excess pollen can be delivered on the stigmas by one pollinator visit and mixed-donor pollen loads have appeared to be common in nature (Mulcahy 1979, Marshall & Ellstrand 1985). The opportunities for pollen competition among wind-pollinated species, in contrast, have been considered to be limited mainly due to gradual accumulation of pollen grains on the stigmas and low pollination intensities (Faegri & van der Pijl 1971). The number of studies on pollen competition carried out in wind-pollinated species is extremely low compared to studies performed in animal-pollinated species. Variation in male reproductive success has quite often been studied in some wind-pollinated conifers (Moran & Griffin 1985, Apsit et al. 1989, El-Kassaby & Ritland 1992, Nakamura & Wheeler 1992a and b) but much less frequently in hardwoods. Pollen tube growth has been studied in *Betula* (Holm 1994a, Dahl & Fredrikson 1996) and *Quercus* (Cecich 1997, Boavida et al. 1999) without attemptions to demostrate the variation in male reproductive success. Instead, pollen competition has been more frequently studied in insect-pollinated, cultivated tree species, like

Prunus (Hormaza & Herrero 1996b, 1999), *Malus* and *Pyrus* (Janse & Verhaegh 1993).

Although pollination intensities in natural stands of some tree species are known to be occasionally low (e.g. Holm 1994a, b), there are reasons to assume that pollen competition might occur also among anemophilous species. In spring 1998, I collected female inflorescences from natural birch stands and from birches growing on open habitats and found 1-5 germinating pollen tubes in most receptive stigmas (unpublished data). This indicates that there are possibilities for pollen competition to occur in natural birch stands although the intensity of competition is likely to vary substantially between years. In wind-pollinated species, pollen can be carried over long distances (e.g. Hjelmroos 1991) and the probability of mixed pollen loads is increased by the synchronism of pollen release (Rabinowitz et al. 1981). Female inflorescences of wind-pollinated species have also different kinds of structures for catching pollen, such as the closely fitting catkin scales of *Betula* and *Corylus* species, which make pollination more effective (Faegri & van der Pijl 1971). Furthermore, in several deciduous tree species, pollen tube growth is arrested at the base of the style until ovary has matured (Arbeloa & Herrero 1987, Dahl & Fredrikson 1996, Boavida et al. 1999). This kind of 'resting state' has been suggested to provide a 'fair start' for pollen tubes to ovules regardless of their different arrival times and the original positions at the stigma and enhance to possibilities for pollen competition even when pollination intensities are low (Dahl & Fredrikson 1996).

Betula pendula Roth (silver birch) was used as a study species in this thesis. *B. pendula* is an anemophilous, deciduous and self-incompatible tree belonging to the circumpolar *B. alba* complex. It has male and female flowers in separate inflorescences. The flowers of *Betula pendula* are initiated during spring and early summer in the year previous to flowering. In Finland, pollen is usually shed in May and receptivity of the female flowers depends on the temperature accumulation (Dahl & Fredrikson 1996). One female flower consists of a single two-locular ovary with two linear, dry stigmas and two ovules of which only one develops into mature seed (Sulkinoja & Valanne 1980, Dahl & Fredrikson 1996) (Figure 1). *B. pendula* seed orchards established in greenhouses made it possible to study evolutionarily important phenomena involved in sexual reproduction in controlled conditions. In greenhouse conditions, *B. pendula* produces usually large amounts of female inflorescences, which makes it possible to pollinate the same maternal plants by pollen from several different pollen donors. In a greenhouse, *B. pendula* pollen can also easily be collected and stored without a substantial decrease in the viability of pollen. Furthermore, effects of competition among pollen grains and embryos can not be a confounded in this species because only one of the two ovules will develop into mature seed.

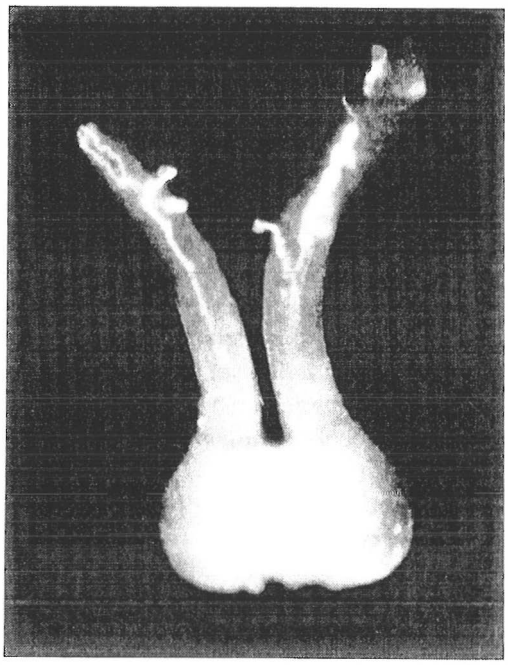

FIGURE 1 A female flower of *Betula pendula* and germinating pollen tubes (photo by H-L.P.)

3.2 Empirical approaches

3.2.1 Occurrence of pollen competition

To evaluate the possibilities for pollen competition to occur in nature, it is important to estimate the pollination intensities and the rate of pollen accumulation on stigmas in natural populations of plant species. This can be done by monitoring pollinator visits to individual flowers, collecting the flowers and quantifying stigmatic pollen loads after fixed time intervals or after variable times of pollinator visits (e.g. Spira et al. 1992). Many studies have been criticized due to the use of cultivated plant species as study species and relying on hand-pollination experiments to create differences in the sizes of pollen loads without relevant data on natural pollination intensities (see e.g. Winsor et al. 2000). The experiments included in this thesis were also mainly hand-pollination experiments carried out in the greenhouse. Natural pollination conditions were represented in this thesis by two outdoor clone collections in which weather and growing conditions were similar to natural. Although the pollination conditions were not natural, greenhouses provided a controlled environment for experimental procedures without confounding random factors. Pollination intensities in the greenhouse were guaranteed to be sufficient for pollen competition by technical assistance of pollen dispersal.

3.2.2 Nonrandom reproductive success

Demonstrating nonrandomness in the reproductive success of different pollen donors has been an objective of many studies (e.g. Moran & Griffin 1985, Marshall & Ellstrand 1986, Apsit et al. 1989) and it is one of the basic requirements for pollen competition to have evolutionary consequences. Components of male reproductive success have been studied by measuring the amount of pollen produced, quantifying rates of pollen removal, observing pollinator movements, tracking the fate of labelled pollen grains, or measuring paternal success following mixed-donor hand-pollinations (reviewed by Snow & Lewis 1993). In addition, different aspects of pollen performance, like pollen grain size (Cruzan 1990) and pollen-tube growth rate (Snow & Spira 1991) have been considered as components of male reproductive success. These all are, however, only indirect measures of total male reproductive success and should be interpreted with caution as estimates of male fitness (Snow & Lewis 1993). The best approach for estimating male reproductive success in a population is to analyze paternity from open-pollinated seeds collected from different maternal plants using highly polymorphic, codominant molecular markers, like microsatellites (e.g. Dow & Ashley 1996, Cruzan 1998, Karhu et al. 2000). However, the use of microsatellite markers, for example, has been limited in many cases by the enormous work needed for finding suitable microsatellite sequences.

Although molecular markers are likely to provide substantial advantages for analysing paternity in open-pollinated natural populations and investigating pollen dispersal, the most frequently used genetic markers, at the moment, are still allozymes. Because in many species only a few allozyme loci are polymorphic and it is uncommon to find more than two or three alleles per locus (Hamrick & Godt 1989), the use of allozymes as genetic markers is limited. A useful and commonly used approach for comparing the seed siring success of different pollen donors has been to employ two- or three donor pollen mixtures and analyze the percentage of seeds sired by each pollen donor in the mixture (e.g. Marshall & Ellstrand 1986, Snow & Spira 1996). In two-donor pollen mixtures one pollen donor can be used as a standard donor which provides a similar comparison material for all the other pollen donors (tester donors) so that they can be compared with each other's (e.g. Apsit et al. 1989). This approach was used also in this thesis. Pollen from five to seven different pollen donors was mixed in equal amounts with pollen from a standard donor in a way that five to seven different two-donor pollen mixtures were available. These pollen mixtures were used to pollinate several maternal plants. Pollen from the standard clone exhibited a rare isozyme genotype and could thus be unambiguously distinguished from the common genotypes that all the other donors and the maternal plants exhibited. This method provided no estimates for total reproductive success of the studied clones but allowed us to compare the seed siring success of different pollen donors and correlate seed siring success with pollen-tube growth rate.

3.2.3 Relationship between pollen-tube growth rate and progeny performance

The most frequently studied aspect of pollen competition is the relationship between pollen and progeny performance. Since 1970s, numerous studies have been carried out to test the predictions of the idea of gametophytic-sporophytic genetic overlap. The effects of pollen-tube growth rate on sporophyte fitness have usually been studied by varying the intensity of pollen competition by applying different amounts of pollen to stigmas (e.g. Schlichting et al. 1990, Snow 1990, Palmer & Zimmerman 1994, Aizen & Searcy 1998) or by varying the distance pollen tubes must travel to the ovules (Mulcahy & Mulcahy 1975, Ter-Avanesian 1978, Lassere et al. 1996). It has been assumed that under conditions of intense pollen competition only the fastest-growing pollen tubes achieve fertilization (Mulcahy 1979). Studies employing small and large pollen loads have been criticized due to the fact that high pollen loads alone may stimulate maternal parents to allocate more resources to seeds and fruits (Charlesworth 1988). Thus, better quality offspring may be a result of the pollen load itself, rather than competition among pollen tubes. Differences in sporophyte performance could also be due to undetected, selective abortions of embryos or seeds (Walsh & Charlesworth 1992). Furthermore, it has been suggested that it is the order of fertilizations rather than pollen-tube growth rate that affects the vigour of the progeny because the ovules fertilized early are better provisioned by the maternal plant than later-fertilized ovules (Delph et al. 1998). It is also possible that low pollen loads might not have been low enough to exclude all possibilities for pollen competition (Mitchell 1997).

For these reasons, direct pollen-tube growth rate measurements and the analysis of the performance of progeny sired by equal amounts of pollen from each pollen donor were employed in this thesis. Maternal plants were pollinated by pollen from several single pollen donors, some of the female inflorescences were collected after a fixed time of germination and some were allowed to develop into mature seed, pollen tube lengths in the collected inflorescences were measured and the performance of the resulting progeny was analyzed.

4 RESULTS AND DISCUSSION

4.1 Can pollen-tube growth rate be a predictor of seed siring success (I, IV)?

The role of pollen-tube growth rate as a predictor of male reproductive success has puzzled researches over twenty years but the true evidence for direct connection between pollen performance and seed siring success is still missing. The relationship between pollen performance and seed siring success is also an important consideration for plant breeders. At open-pollinated seed orchards, each seed orchard clone is expected to be equally represented in a seed crop (Schoen & Stewart 1987). Fertilizations at seed orchards are not, however, random and the reproductive outcomes of the seed orchard clones not equal, if certain genotypes produce better quality pollen that sires a disproportionate large number of seeds.

In the experiments described in papers I and IV, several maternal plants were pollinated with pollen from single pollen donors and two-donor pollen mixtures in a *B. pendula* greenhouse seed orchard. By correlating pollen-tube growth rates from single-donor pollinations with the relative seed siring success of the same pollen donors from two-donor pollinations, information about the relationship between pollen performance and seed siring success could be obtained. The two greenhouse studies revealed a significant positive relationship between pollen-tube growth rate and the relative seed siring success of the pollen donors. Significant differences in pollen-tube growth rate among the studied clones were found. Furthermore, the rankings of the pollen donors with regard to pollen-tube growth rate were statistically consistent across the studied maternal plants, which is a necessary condition for pollen competition to lead to sexual selection. These results indicate that pollen competition operates in *B. pendula* seed orchards, because fertilizations are likely to be nonrandom and associated with pollen competitive ability. In previous studies, no relationship between pollen-tube growth rate and seed siring success has been found (Melser et al. 1997).

To obtain a more reliable picture of the relationship between pollen-tube growth rate and seed siring success in an environment resembling natural pollination conditions, similar hand-pollination experiment was conducted at an outdoor clone collection. Clone collections are collections of seed orchard clones without commercial seed production purposes. Clone collections have usually been founded on abandoned fields where weather and pollination conditions resemble the conditions in natural birch stands. On the contrary to the expectations, the relationship between pollen-tube growth rate and seed siring success was significantly negative in heterogeneous outdoor conditions. It is difficult to find any probable explanation for this result. In natural birch stands, the relationship between pollen-tube growth rate and seed siring success might not be as straightforward as in controlled greenhouse conditions. Microclimatic variability and maternal effects can have substantial and unexpected influence on pollen-tube growth rate. For example, maternal plants are likely to provide pollen tubes with nutrients during pollen germination (Labarca & Loewus 1973, Sanders & Lord 1989, Wu et al. 1995, Herrero & Hormaza 1996), and their ability to do this may vary from one microsite to another depending, for example, on the nutrient content of the soil. Thus, pollen-tube growth rate can be a predictor of seed siring success in controlled greenhouse conditions where differences among maternal plants are mainly of genetic origin but the situation in heterogeneous outdoor conditions is more complicated.

4.2 Can pollen-tube growth rate be a predictor of progeny performance (II)?

The idea of the positive relationship between pollen-tube growth rate and progeny performance has attracted attention especially since Mulcahy (1979) formulated the "modern" hypothesis of pollen competition. Many studies have been carried out but the evidence for pollen-tube growth rate as an indicator of progeny performance remains equivocal. In paper II, I employed single-donor crosses and direct pollen-tube growth rate measurements to study whether crosses with fast pollen tube growth resulted in better quality seeds and faster growing seedlings. Maternal and paternal effects on seed and seedling performance were also studied.

The results of paper II revealed a significant positive relationship between pollen-tube growth rate and seed weight. No relationship between pollen-tube growth rate and percentage of germinable and embryonic seeds and between pollen-tube growth rate and seedling growth rate was found. Due to the effects of seed weight on seed and seedling performance, the most reliable insight into the relationship between pollen and progeny performance is provided by partial correlations in which seed weight or the number of seeds per inflorescence have been used as controlling factors. In previous

studies, in which the effect of pollen-tube growth rate on progeny performance has been studied indirectly by varying the intensity of pollen competition, no evidence for positive effects on seed weight has been found (Bertin 1990, Snow 1990, Björkman 1995, Quesada et al. 1996a and b, Johansson & Stephenson 1997). Instead, there are some studies that document a positive relationship between the intensity of pollen competition and percentage of germinable seeds (Davis et al. 1987, Bertin 1990, Palmer & Zimmerman 1994). The effects of the intensity of pollen competition on seedling growth rate have rarely been studied and the results from the existing studies reveal no significant trend (Ter-Avanesian 1978, Bertin 1990, Janse & Verhaegh 1993, Palmer & Zimmerman 1994).

Differences in seed size are known to affect many seedling fitness characters (Schaal 1980, Weis 1982, Zimmerman & Weis 1982) which can cause problems in interpreting the results from the studies on the relationship between pollen and progeny performance. Variation in seed size is often associated with the variation in seed number in a way that average seed weight decreases as the number of seeds within fruits increases (e.g. Stanton 1984). In this thesis, the average number of seeds within an inflorescence varied between the crosses and there was a negative relationship between seed number per inflorescence and seed weight, as expected. Furthermore, a substantial positive correlation between seed weight and percentage of germinable and embryonic seeds and a significant negative correlation between seed weight and seedling growth were detected. Longer period of recording seedling growth would have been relevant because the effects of seed weight on seedling performance can be transient (e.g. Richardson & Stephenson 1992). *B. pendula* seeds contain very little endosperm for early seedling growth. That is why the relationship between seed weight and seedling growth in *B. pendula* might not be as clear as in many other species, which can partly explain the negative correlation between seed weight and seedling growth.

In paper II, significant maternal effects were found on the number of seeds per inflorescence, percent germinable and embryonic seeds and seedling height. Paternal effects were detected only on seedling height after 85 days of growth. Paternal effects may not be easily recognized in the presence of maternal effects because they are usually confounded with them (Schmid & Dolt 1994). However, genetic maternal effects generally decline through the life cycle (Schmid & Dolt 1994) and recording seedling growth for a longer period would have possibly revealed decreased maternal and more pronounced paternal effects. The results of paper II are in concordance with many of the previous studies in the fact that paternal effects are rarely found in plants (Roach & Wulff 1987) and that verifying the positive association between pollen-tube growth rate and progeny performance is difficult. Although many confounding effects could be excluded from the study descriped in paper II by using equal amounts of pollen in each cross, the puzzle about the relationship between pollen and progeny performance

remains unsolved. Because the positive effects of pollen-tube growth rate on seed quality did not translate into faster growing seedlings, no strong evidence for the theory of pollen-tube growth rate as an indicator of progeny fitness is provided by this thesis.

4.3 Genotype-environment interactions in pollen competitive ability (III, IV)

Pollen competitive ability is usually expressed as pollen-tube growth rate (e.g. Mulcahy 1979, Snow & Spira 1991) or proportion of seeds sired by a given pollen donor (Snow & Spira 1996, Mitchell & Marshall 1998, Marshall 1998) which is a more direct measure of male reproductive success (Snow & Lewis 1993). Genotype-environment interactions have been suggested to be one of the possible mechanisms that maintains genetic variation in pollen-tube growth rates. There are, however, very few studies that report genotype-environment interactions in pollen-tube growth rate (but see Travers 1999) and none that report genotype-environment interactions in seed siring success. The greenhouse seed orchard and the outdoor clone collections of *B. pendula* provided a possibility to use the same genotypes in hand-pollination experiments in two different environments. By comparing pollen-tube growth rate and seed siring success of the same pollen donors on the same maternal clones across different environmental conditions, information about genotype-environment interactions in pollen competitive ability could be obtained.

The results of paper IV revealed significant interactions between genotype and environment in both pollen-tube growth rate and seed siring success indicating that the ranking orders of the pollen donors with regard to pollen-tube growth rate and seed siring success change across the two pollinations sites. If there is a positive relationship between pollen-tube growth rate and seed siring success, the changes in the rankings of the pollen donors in pollen performance indicate that different donors will be selected during pollen tube growth in different environmental conditions. Selection among the seed orchard clones is possible to occur in a greenhouse where a positive relationship between pollen-tube growth rate and seed siring success was observed. Because no positive relationship between pollen-tube growth rate and seed siring success was detected outdoors, changes in pollen-tube growth rates do not lead to parallel changes in seed siring success. It is also obvious that the changes in the rankings of the pollen donors in pollen-tube growth rate do not translate into parallel changes in the rankings in seed siring success across the two studied environments.

In natural forest stands, microenvironmental variation can be substantial even within a small range which is likely to cause differences in the physiological condition of the plants. Nongenetic differences among maternal plants and environmental heterogeneity is likely to result in random variation

in the outcome of pollen competition. That is why pollen competition might not be of crucial importance in sexual selection in natural birch stands. From this perspective, environmental variability and genotype-environment interactions are likely to explain a large part of the variation observed in pollen-tube growth rates.

4.4 Pollen-pollen interactions (V)

Interactions between pollen tube and stylar environment are well known in many species (Bowman 1987, Cruzan 1989, Aizen et al. 1990). Much less attention has been paid to pollen-pollen interactions, i.e. interactions between pollen from two or more genetically different pollen donors. Direct pollen-pollen interactions can, however, have significant and largely unknown effects on pollen performance (Mulcahy et al. 1996) and they can partly explain the maintenance of genetic variation in pollen-tube growth rates. At seed orchards, pollen-pollen interactions can cause deviations from random fertilizations if pollen from different donors have influence on each other's germination ability and, subsequently, fertilization success. In the study described in paper V, I demonstrated direct interactions in pollen germination ability between pollen from two genetically different donors. Equal amounts of pollen from two donors was mixed and germinated on agar medium. Germination percentages of the pollen mixtures were compared to the average germination percentages of the two pollen donors germinated separately. In addition, I studied whether pollen population effect existed in *B. pendula* and whether there were possibilities to overcome it in the pollen germination tests.

The germination percentages of three out of eight pollen mixtures differed significantly from average germination percentages of the two pollen germinated separately. If there was no interaction, the germination percentage of pollen from two clones tested in mixture was expected to be equal to the average germination percentage of the same two clones germinated separately. Thus, the results of paper V revealed that positive pollen-pollen interactions existed between pollen from certain clones. Positive interactions will lead to more intense competition among pollen grains which is advantageous to female component but presumably not to male component of reproduction. However, evidence for negative pollen-pollen interactions was not found in this thesis. Negative pollen-pollen interactions have usually been observed between different species (Galen & Gregory 1989, Murphy & Aarssen 1995). On the whole, in natural pollination conditions direct pollen-pollen interactions can easily be confounded by the interactions mediated by the style which makes them extremely difficult to detect.

Density-dependent germination of pollen grains *in vitro* can be considered to be an example of direct pollen-pollen interactions due to the fact that stimulation of germination is caused by pollen grains themselves.

Germination of *B. pendula* pollen was found to be density-dependent on agar medium. The addition of a pollen extract to the germination medium increased the germination percentages of the small pollen populations. Brewbaker and Kwack (1963) have postulated that the factor stimulating pollen germination is the calcium ion, interacting with K, Na and Mg ions. Given that calcium is essential for pollen tube growth in many species and that pollen grains are low in calcium content (Brewbaker & Kwack 1963), pollen population effect might be only a passive response to increasing calcium content when many pollen grains are present. Anyway, addition of pollen extract to the germination medium might provide a way to overcome the pollen population effect and increase the comparability of the germination percentages of different sized pollen samples.

4.5 Implications for practical tree breeding

In greenhouse seed orchards pollination takes place as mass pollination and fertilizations are assumed to be random. Traditionally, no attention has been paid to the functioning of pollen and phenomena between pollination and fertilization in commercial seed production. The results of this thesis reveal, however, that pollen quality expressed as pollen-tube growth rate can be an important factor controlling the paternity of the seeds in controlled greenhouse conditions. The significant positive relationship between pollen-tube growth rate and seed siring success described in papers I and IV indicates that pollen donors with the fastest-growing pollen tubes sire more seeds than pollen donors with slower-growing pollen tubes. Furthermore, significant differences among paternal clones in pollen-tube growth rates were detected. Given that the number of pollen grains far exceeds the number of ovules at stigmas during mass pollination and the differences among pollen donors in pollen-tube growth rate translate into differences in seed siring success, pollen competition in *B. pendula* seed orchards is a real phenomenon that is likely to affect the genetic composition of the seed crop.

An interesting application to tree breeding was provided by the possibility of predicting the performance of the seedlings on the basis of pollen-tube growth rate. A significant positive relationship between pollen-tube growth rate and seed weight and a substantial positive relationship between pollen-tube growth rate and percentage of germinable seeds were found. These results indicate that pollen donors with fast growing pollen tubes give rise to better quality seeds which, in turn, should give rise to more vigorous seedlings. From this perspective, the results are promising because seed quality expressed as seed germination percentages is an important consideration in the commercial production of forest tree seeds. Because no relationship between pollen-tube growth rate and seedling growth rate was

detected, very far-going conclusions about the possibilities to improve the progeny quality by pollen selection in birch seed production can not be drawn.

Genotype-environment interactions were found both in pollen-tube growth rate and seed siring success. Because there was a positive relationship between pollen-tube growth rate and seed siring success in the greenhouse, it is possible that different donors will be selected during pollen germination under different environmental conditions. For example, the reproductive outcome of seed orchard clones can vary from year to year depending on the weather conditions during pollination and pollen tube growth which, in turn, can cause unexpected deviations from the expected genetic composition of the seed crop. Selection among pollen donors during pollen tube growth in warm greenhouse conditions can also affect the adaptive properties of the seedlings. These kind of after-effects have only been documented among coniferous tree species (see e.g. Johnsen et al. 1995, 1996) but observations on decreased frost hardiness among *B. pendula* seedlings originating from seed orchards have been made. One possibility to explain after-effects in *B. pendula* is selection among pollen donors during pollen tube growth. In paper III, differential effects of temperature on pollen-tube growth rates of different pollen donors were reported. Do to gametophytic-sporophytic genetic overlapping, it is possible that pollen donors that outcompete the other donors in warm greenhouse conditions sire offspring that have different adaptive characters than offspring sired in harsher conditions. Further studies are going on to gain some insight into the relationships between pollen-tube growth rate and frost hardiness of the progeny.

5 CONCLUDING REMARKS

The aim of the studies included in this thesis was to evaluate the possibilities for pollen competition to operate in a *B. pendula* greenhouse seed orchard and to obtain new information about the mechanisms involved in sexual selection among hardwoods. The results of this thesis reveal that pollen competition is a real phenomenon in *B. pendula* seed orchards. Pollen-tube growth rate is likely to be a determinative factor controlling the paternity of the seeds indicating that fertilizations are not random as has been expected. The possibility of screening birch clones for new seed orchards on the basis of pollen performance to produce better quality seeds and faster growing seedlings was not manifested by this thesis because the differences in seed weight did not translate in parallel differences in seedling growth rate. In sum, differences among pollen donors in pollen-tube growth rate are likely to influence the genetic composition of the seed crop via pollen competition in *B. pendula* greenhouse seed orchards but the effects of pollen-tube growth rate on progeny performance are obscure.

To obtain information about the possible role of pollen competition in sexual selection in natural stands of *B. pendula*, two outdoor clone collections, in which environmental conditions were nearly natural, were used in the study. The results of this thesis showed that the relationship between pollen-tube growth rate and seed siring success might not be very straightforward and easy to interpret in heterogeneous outdoor conditions. In natural pollination conditions, microclimatic variability and maternal environmental effects are likely to have random effects on pollen-tube growth rates and the outcome of pollen competition. Thus, it is likely that pollen competition might not have as an important role in sexual selection in nature as could be considered on the basis of the studies carried out in greenhouse conditions.

Many authors have called for more evidence for the genetic basis of the variation observed in pollen-tube growth rates (e.g. Walsh & Charlesworth 1992, Havens 1994). Phenotypic variation in pollen-tube growth rates has been demonstrated in numerous studies (e.g. Mulcahy et al. 1983, Marshall & Ellstrand 1986, Snow & Mazer 1988, Björkman 1995) but the question of

heritability of pollen performance has remained largely unanswered. Among long-lived forest trees the most practical way to obtain information about the heritability of pollen performance is to test the clonal repeatibility of in vitro pollen-tube growth rates which gives a measure of broad sense heritability (see Havens 1994). Experiments for testing the clonal repeatibility of pollen-tube growth rate in *B. pendula* are going on. At the moment, it is, however, still obscure how much of the variation observed in pollen-tube growth rates in *B. pendula* is actually genetic. To draw conclusions on the evolutionary consequences of pollen competition, more has to be known about the genetic basis of pollen-tube growth rate and the frequency and influences of pollen competition in natural populations of *B. pendula*.

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YHTEENVETO

Siitepölykilpailu koivulla

Suomessa jalostetun koivunsiemenen tuotanto on keskitetty muovihuoneisiin perustetuille siemenviljelyksille. Siemenviljelykset koostuvat yleensä useista kymmenistä erilaisista kasvuominaisuuksiltaan hyvälaatuisista klooneista. Pölytys muovihuoneessa tapahtuu massapölytyksenä, jonka aikana kaikilla siemenviljelyksen klooneilla on perinteisesti oletettu olevan yhtäläiset mahdollisuudet siementen hedelmöittämiseen siten, että kaikki kloonit olisivat tasavertaisesti edustettuina syntyvässä siemensadossa. Tällä työllä halusin tutkia, onko siitepölyn laadulla siiteputken kasvunopeutena mitattuna vaikutusta kloonien siementensiitoskykyyn, mikä puolestaan voi johtaa kloonien epäsattumanvaraiseen hedelmöitysmenestykseen ja siihen, että vain tietyt laadultaan parasta siitepölyä tuottavat kloonit ovat edustettuina siemensadossa. Teoreettisen pohjan tutkimukselle on luonut teoria siitepölykilpailusta, jonka mukaan vain nopeimmin itävät siitepölyhiukkaset pääsevät toimimaan hedelmöittäjinä. Siitepölykilpailua voi tapahtua olosuhteissa, joissa emin luotille kerääntyvien useilta eri siitepölyn luovuttajilta peräisin olevien siitepölyhiukkasten lukumäärä ylittää hedelmöitettävien siemenaiheiden lukumäärän. Tällaiset olosuhteet vallitsevat muovihuonesiemenviljelyksillä massapölytyksen aikana. Muovihuoneet ja kloonien käyttö risteytyskokeissa mahdollistivat useiden evolutiivisesti mielenkiintoisten biologisten prosessien tutkimisen suhteellisen kontrolloidussa ympäristössä. Tutkimuksessa kiinnitin erityisesti huomiota siitepölyn laadun ja siementensiitoskyvyn sekä siitepölyn laadun ja tuotettujen siementen ja taimien laadun väliseen yhteyteen; voidaanko siiteputken kasvunopeutta pitää koiraan lisääntymismenestyksen mittarina? Lisäksi tutkin, kuinka ympäristöolosuhteet vaikuttavat siitepölykilpailuun ja pohdin, mikä on siitepölykilpailun mahdollinen merkitys luonnon koivupopulaatioissa.

Muovihuoneessa tehtyjen risteytyskokeiden perusteella merkittävin tutkimustulos oli siiteputken kasvunopeuden ja siementen siitoskyvyn välinen tilastollisesti merkitsevä positiivinen korrelaatio. Tutkittujen kloonien välillä oli myös merkitseviä eroja siiteputken kasvunopeudessa. Näin ollen eri klooneista peräisin olevien siitepölyhiukkasten välillä esiintyy massapölytystilanteessa kilpailua, joka mitä todennäköisimmin johtaa epäsattumanvaraisiin hedelmöityksiin siten, että nopeimmin siiteputken kasvattavat hiukkaset myös siittävät suurimman osan siemenistä. Jalostetun koivunsiemenen tuotannon kannalta mielenkiintoinen tutkimuskohde oli siitepölyn ja jälkeläisten laadun välinen yhteys. Koska suurin osa siitepölyvaiheessa ilmentyneenä olevista geeneistä on ilmentyneenä myös siemen- ja taimivaiheessa, on oletettavaa, että siitepölyhiukkasen elinvoimaisuudesta kertovat ominaisuudet, kuten siiteputken nopea kasvu, heijastuvat myös taimivaiheeseen. Tässä tutkimuksessa löydettiin tilastollisesti merkitsevä positiivinen korrelaatio siiteputken kasvunopeuden ja siementen painon välille mutta ei muiden tutkittujen siementen ja

taimien ominaisuuksien välille. Siemenen painon on useissa tutkimuksissa todettu vaikuttavan positiivisesti siemenen muihin ominaisuuksiin sekä taimien kasvuun, mutta tässä tutkimuksessa ei vastaavaa yhteyttä löydetty. Tukea oletukselle siitepuken kasvunopeudesta siementen ja taimien laadun indikaattorina ei siis tällä tutkimuksella saatu.

Genotyypin ja ympäristön välisiä vuorovaikutuksia tutkittiin risteyttämällä samoja klooneja keskenään muovihuoneessa ja ulkona olevilla kloonikokoelmilla. Koska olosuhteet kloonikokelmilla vastaavat hyvin paljon olosuhteita luonnon koivumetsikössä, tulokset antoivat mahdollisuuden pohtia siitepölykilpailun mahdollisia vaikutuksia luonnonmetsiköissä. Genotyypin ja ympäristön välisiä vuorovaikutuksia havaittiin sekä siiteputken kasvunopeudessa että siementensiitoskyvyssä. Näin ollen eri genotyypien välinen paremmuusjärjestys muuttuu ympäristöolosuhteiden muuttuessa. Siiteputken kasvunopeuden ei kuitenkaan havaittu korreloivan positiivisesti siementen siitoskyvyn kanssa ulkona vaan päinvastoin; siiteputken kasvunopeuden ja siementen siitoskyvyn välillä todettiin tilastollisesti merkitsevä negatiivinen korrelaatio. Tällainen tulos saattaa selittyä satunnaisilla ympäristön heterogeenisyyden aiheuttamilla vaikutuksilla, jotka heikentävät siitepölykilpailun mahdollisuuksia toimia luonnonpopulaatioissa. Vaikka olosuhteet siitepölykilpailulle runsaina siitepölyvuosina olisivatkin olemassa, mahdollisuudet tiettyjen genotyyppien jatkuvaan valikoitumiseen ja evolutiivisiin seurauksiin luonnossa ovat luultavasti huomattavasti vähäisemmät kuin mitä kontrolloiduissa kasvihuoneolosuhteissa tehtyjen kokeiden perusteella voisi päätellä.

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Original papers

I

Pollen-tube growth rate and seed-siring success among *Betula pendula* clones

by

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Pollen-tube growth rate and seed-siring success among *Betula pendula* clones

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SUMMARY

The aim of this study was to investigate whether genetically different pollen donors (*Betula pendula* clones) differed in pollen-tube growth rate across 11 maternal plants and *in vitro*, and whether the differences between the donors were consistent across the recipients. To compare the seed-siring success of competing pollen donors, a two-donor hand-pollination experiment with six donors and six recipients was conducted. The experiments were performed at a plastic-house seed orchard. The donors showed significant variation in pollen-tube growth rate on all the 11 recipients. The rankings of the pollen donors were statistically consistent across different maternal plants. A significant positive correlation between pollen tube growth *in vivo* and *in vitro* was found. The seed-siring success of two competing pollen donors was unequal in 20 of 29 cases and there was a significant positive correlation between seed-siring success and pollen-tube growth rate *in vivo* and *in vitro*. The results show that fertilizations are not random and pollen competition operates in a *B. pendula* seed orchard population.

Key words: non-random fertilization, pollen competition, pollen tube growth, *Betula pendula* (silver birch).

INTRODUCTION

In plants, post-pollination sexual selection can occur by competition between pollen grains and by female choice (Stephenson & Bertin, 1983). Pollen grains are believed to compete for ovules when there are more pollen grains on the stigmas than there are ovules in the ovary. It is assumed that under conditions of pollen competition only the fastest-growing pollen tubes achieve fertilization (Mulcahy, 1979). Selection due to differences between pollen donors can occur either before fertilization, as competition between pollen grains on the stigma and in the style, or after fertilization between the embryos if there is more than one developing embryo in a single flower. Furthermore, male reproductive outcome is regulated by the maternal plant. Evidence is accumulating that maternal plants have several strategies for controlling pollen-tube growth rate, including support and constraint (Herrero & Hormaza, 1996).

Owing to the large population size and the haploid status of pollen, gamctophytic selection has been presumed to explain both the evolutionary success of the angiosperms (Mulcahy, 1979) and the adaptive significance of numerous plant reproductive characters (Queller, 1983). However, pollen competition can lead to gametophytic selection only if there is genetic variation in pollen-tube growth rate between pollen donors and if the differences in pollen-tube growth rate result in a differential fertilization success of pollen donors. Snow & Spira (1991) demonstrated consistent differences between pollen donors in pollen-tube growth rate across maternal genotypes in *Hibiscus moscheutos*. They concluded that sexual selection is possible because males with the fastest-growing pollen tubes can potentially sire a disproportionately large number of seeds across maternal plants. In some other studies, pollen competitive ability seemed to be influenced by interactions between donors and recipients (Marshall & Ellstrand, 1986; Johnston, 1993; Björkman *et al.*, 1995; J. I. Hormaza & M. Herrero, unpublished).

Studies on pollen competition have focused strongly on animal-pollinated species. Mixed-donor pollen loads seem to be common in animal-pollinated plants (Marshall & Ellstrand, 1985), which makes pollen competition possible. There are fewer studies on pollen competition in wind-pollinated species, although many wind-pollinated forest tree species

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are commercially important, for example in forestry. Male reproductive success has been studied in some wind-pollinated conifers (Moran & Griffin, 1985; Apsit *et al.*, 1989; El-Kassaby & Ritland, 1992; Nakamura & Wheeler, 1992a). However, there are major differences in post-pollination phenomena between hardwood and coniferous tree species (Owens & Blake, 1985). Among hardwood tree species, pollen tube growth has been studied in, for example, *Betula* (Dahl & Fredrikson, 1996) and in *Quercus* (L. Boavida & J. A. Feijó, unpublished), and pollen competition in, for example, apple and pear (Janse & Verhaegh, 1993) and in sweet cherry (*Prunus avium*) (J. I. Hormaza & M. Herrero, unpublished). Further knowledge of the post-pollination mechanisms of anemophilous hardwood tree species could give new insight into the evolution of tree species as well as offering more tools for practical tree breeding.

There are reasons to assume that pollen competition might occur also in natural stands of anemophilous tree species, although pollination intensities in natural stands of *Betula pendula* and *B. pubescens*, for example, are known to be occasionally low (Holm, 1994a,b). Among wind-pollinated species, pollen can be carried over long distances (Hjelmroos, 1991) and pollen release tends to occur quite synchronously (Rabinowitz *et al.*, 1981) in association with dry, warm conditions (Biancchi *et al.*, 1959; Käpylä, 1984), which increases the probability of mixed pollen loads. Wind-pollinated species have several kinds of mechanism for catching pollen, such as the closely fitting catkin scales of *Betula* and *Corylus* species, which make the pollination more effective (Faegri & van der Pijl, 1971). Furthermore, pollen tube growth in *Betula pendula* has a resting state at the base of the elongate stigma. This allows the slower pollen tubes, as well as pollen grains that have landed at different times and at a greater distance from the ovules, to catch up with other pollen tubes (Dahl & Fredrikson, 1996). A similar pattern has also been observed in sweet cherry (J. L. Hormaza, pers. comm.) and in cork oak (J. Boavida & J. A. Feijó, unpublished).

Genetically improved *B. pendula* seed for forestry has been produced at seed orchards established in plastic houses in Finland and to a small degree in Sweden. Pollination in plastic houses takes place as mass pollination; pollination and fertilization have generally been assumed to be random. The aim of this study was to investigate whether genetically different pollen donors differed in pollen-tube growth rate and whether there was any relationship between pollen-tube growth rate and the seed-siring success among competing pollen donors at a *B. pendula* plastic-house seed orchard. We also studied whether the differences between pollen donors are consistent across several maternal plants and *in vitro*. Our study provides new information not only about the function of a plastic-house seed orchard but also about the mechanisms that might be involved in the sexual selection of forest tree populations.

MATERIALS AND METHODS

Study species and the seed orchard

Betula pendula Roth is a common, deciduous tree up to 30 m high that ranges throughout most of Europe from Norway to Sicily (Tutin *et al.*, 1964). This species is anemophilous, self-incompatible and monoecious, with male and female flowers occurring in separate inflorescences. There are 200–300 male flowers in each catkin (Dahl & Fredrikson, 1996) and approx. 500–600 female flowers in each pistillate inflorescence (H. L. Pasonen, unpublished). One female flower consists of a single two-locular ovary with two linear, dry stigmas and two ovules of which only one develops into a mature seed (Sulkinoja & Valanne, 1980; Dahl & Fredrikson, 1996). Fertilization does not occur in more than one of the two ovules because the other ceases to develop once pollen tubes have penetrated the funiculus of the first ovule (Dahl & Fredrikson, 1996).

The study was performed in a plastic-house seed orchard. The *B. pendula* seed orchards had been established in plastic houses to promote flowering and to increase the seed crop. The clones were originally selected for the seed orchards on the basis of the results from field tests (Raulo & Koski, 1977). The most important selection criterion was genotypically superior growth; the amount of flowering was not a selection criterion. The clones in the seed orchard that we used originated from rather small area, between latitudes 60° 30′ N and 62° 30′ N.

Hand pollinations

Eleven maternal and six paternal clones bearing a specific isoenzyme marker were selected from a group of *B. pendula* seed orchard clones. Pollen used in this study was collected in April 1992, with paper bags into which mature pollen spontaneously fell after dehiscence of the anthers. Pollen was stored at −20°C in a freezer until used in May 1997. We were unable to use fresh pollen because the flowering of male and female flowers occurs almost at the same time and there would have been no time to determine the pollen germination *in vitro* before hand-pollination experiments. Pollen germination percentages were determined *in vitro* for pollen collected from several clones in 1992 and 1995. Pollen collected in 1992 had the highest germination percentages, perhaps owing to the favourable weather conditions during pollen collection. Only clones with germination percentages > 50 %, and as uniform as possible, were chosen for the experiment.

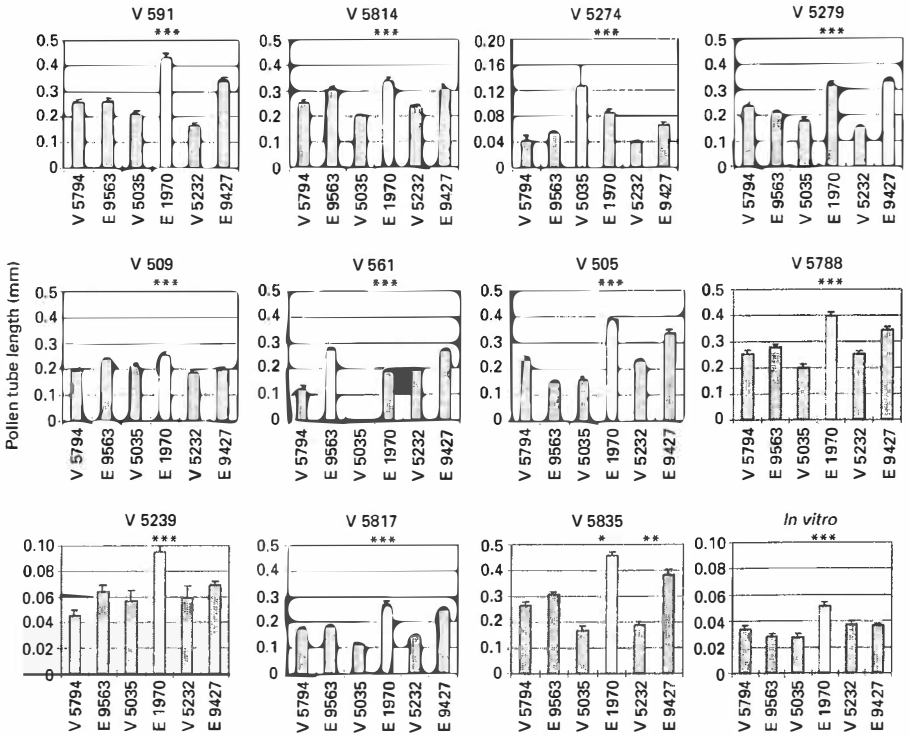

Fig. 1. The average lengths of pollen tubes of six pollen donors on 11 recipient *Betula pendula* plants and on agar medium 12 h after germination. The open bar represents the pollen donor with the longest pollen tubes. Error bars show the SE (n=80–120), one-way ANOVA; ***, $P<0.001$.

Pollen from five selected donors was mixed with standard pollen from clone E 1970 (=pollen donor 1) for mixed-donor hand-pollination experiment. The pollen mixtures were prepared by measuring out an equal volume of pollen from two clones into a small glass bottle, after which each bottle was shaken gently. The pollen from clone E 1970 was chosen as a standard pollen because clone E 1970 exhibited a rare isoenzyme genotype and could therefore be distinguished unambiguously from the common genotypes that all the other donors and the maternal plants exhibited.

Before the onset of flowering, branches with at least three female inflorescences for single-donor pollinations and 10 female inflorescences for mixed-donor pollinations were isolated with white paper bags to prevent uncontrolled pollinations. Once receptive, 11 maternal plants were hand-pollinated with pollen from six donors. Six maternal plants were additionally pollinated with five pollen mixtures. All the hand pollinations (66 single-donor pollinations and 30 mixed-donor pollinations) were performed at a plastic-house seed orchard at the beginning of May 1997 at Haapastensyrjä Breeding Centre in Läyliäinen, Finland (60° N, 24° E). The amount of pollen in each hand-pollination was kept as uniform as possible, because the density of pollen grains can influence pollen tube growth (Cruzan, 1990; Holm, 1994b).

Pollen-tube growth rate measurements

Twelve h after pollination, samples of female inflorescences pollinated with pollen from a single donor were collected. In 12 h the fastest pollen tubes had almost reached the base of the elongate stigma, but the resting state had not yet begun (Dahl & Fredrikson, 1996). At least three inflorescences per pollination were detached and immediately stored in glacial acetic acid and 60% (v/v) ethanol (1:9). The inflorescences were stored in a refrigerator at 4°C until they were examined. The flowers were scraped off with a scalpel and stained with a solution of 0.1% aniline blue in aqueous $K_3P\bullet_4$ (0.3 mol); 80–120 randomly chosen pollen tubes per maternal/paternal combination were measured by UV fluorescence microscopy. Pollen tube length after 12 h of germination was also determined for every pollen donor *in vitro* on agar medium (containing 1% agar, 0.01% boric acid and 0.5 M sucrose) (Käpylä, 1991).

Table 1. *Random-effects ANOVA of the effects of paternal and maternal* Betula pendula *clones on pollen tube length for single donor pollinations*

Source of variation	df	SS	*F*	*P*
Paternal clone	5	11.33	15.22	<0.001
Maternal clone	10	36.86	24.74	<0.001
Two-way interactions	49	7.28	14.47	<0.001
Error	4868	49.99		

SS, sum of squares.

Isoenzyme analysis

The seeds for isoenzyme analysis from mixed-donor pollinations were germinated under a plant lamp with a photoperiod of 15 h day and 9 h night on Petri dishes covered with sand and moist filter paper. The seeds were sown at the end of November 1997 (20 maternal/paternal combinations) and in February 1998 (10 maternal/paternal combinations). The samples were collected when the cotyledons had fully opened, and the whole plantlet was immediately ground in 50 µl of 0.12 M Tris-HCl extraction buffer, pH 7.5 (slightly modified from Bousquet *et al.* (1987), containing 100 ml 0.12 M Tris-HCl, 6.8 g sucrose, 20 mg $NADP^+$, 25 mg NAD^+, 15 mg EDTA, 80 mg dithiothreitol, 0.19 g cysteine-HCl, 0.44 g ascorbic acid, 0.1 g bovine serum albumin, 2 ml Tween 80 and 5 g poly(vinyl pyrrolidone)) and fine granular quartz, imbibed into wicks and stored for 1–2 wk at −20°C before electrophoresis. The samples were assayed for phosphoglucoisomerase (Pgi-2) by standard starch-gel electrophoresis (10% (w/v) Sigma hydrolysed starch) with a Tris-citrate buffer system (modified from Shaw & Prasad (1970)). The Tris-citrate buffer contained 0.0135 M Trizma base and 0.043 M citric acid, pH 7.8. The enzyme activity was stained by using the agar-overlay protocol of Cheliak & Pitel (1984), and the locus Pgi-2 was scored. Approximately 100 seedlings per sample were analysed.

Data analysis

Differences between pollen donors in the average lengths of pollen tubes in the pistils of the maternal plants and *in vitro* were analysed by one-way ANOVA. A weighted mean correlation coefficient was calculated by using a Schmidt-Hunter meta-analysis method with Fisher's *z*-transformation (Hedges & Olkin, 1985) to test the relationship between pollen-tube growth rate *in vitro* and on 11 maternal plants (*in vivo*). A random-effects ANOVA was performed to test the effect of the paternal and maternal clone on pollen tube growth and on the relative seed-siring success of pollen donor 2, i.e. the number of seeds sired by donor 2 divided by the total number of seeds sired by pollen mixtures. Pollen tube lengths were square-root-transformed and the proportions of seeds sired by pollen donor 2 were arcsine square-root-transformed to normalize the data. A concordance coefficient (W) (Sokal & Rohlf,

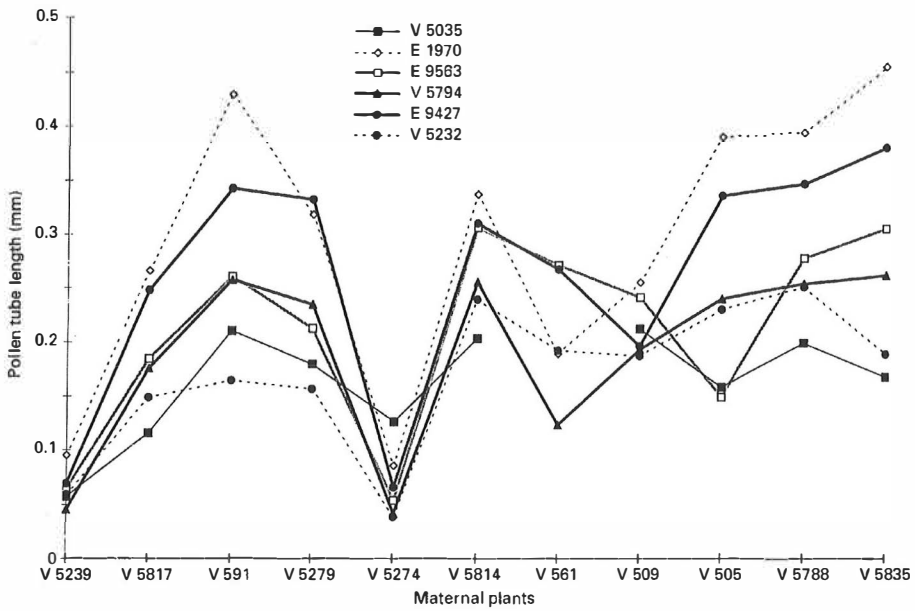

Fig. 2. The average lengths of pollen tubes of six pollen donors on 11 maternal *Betula pendula* plants.

1981, p. 609) was assessed to test whether the rankings of the pollen donors changed across different maternal plants. A weighted mean correlation coefficient (Hedges & Olkin, 1985) was calculated to test the relationship between pollen-tube growth rate and seed-siring success of the pollen donors across six maternal plants. A χ^2 test was performed to compare the seed-siring ability of the standard donor (E 1970) with the seed-siring ability of five other pollen donors on six different maternal plants. The deviations from 50:50 were interpreted as products of variation in pollen competitive ability. Most of the statistics was performed with the SPSS package for Windows 6.1.

RESULTS

Pollen tube growth

Differences between six pollen donors in average lengths of pollen tubes after 12 h of germination were statistically significant on every maternal plant and *in vitro* (Fig. 1). A weighted mean correlation coefficient for pollen tube lengths *in vivo* and *in vitro* was 0.60 ($P<0.001$). A concordance coefficient for the pollen tube lengths *in vivo* ($W=0.62$, $P<0.001$) indicates that there was significant consistency in the rankings of the pollen donors across maternal plants. However, a significant two-way interaction between paternal and maternal effects on pollen tube lengths was also found ($F=14.47$; $P<0.001$) (Fig. 2, Table 1). Two pollen donors (clones E 1970 and E 9427) were competitively superior across 8 of 11 maternal plants. Clone E 1970 was also competitively superior *in vitro*.

Seed-siring success

The seed-siring success of two competing pollen donors differed significantly in 20 of 29 cases (Table 2). Both paternal ($F=41.75$, $P<0.001$) and maternal ($F=5.75$, $P<0.01$) clones had an effect on the relative seed-siring success of pollen donor 2 in mixed pollinations (Fig. 3, Table 3.) The rankings of five pollen donors were statistically consistent across maternal plants ($W=0.61$, $P<0.01$), clone E 9427 being the most successful and clone V 5794 being the second successful across all the six maternal plants when clone E 1970 was used as a standard donor (Fig. 3). A significant relationship between pollen

Table 2. *Proportion of seeds sired by each pollen donor 2 compared with donor 1 (E* 1970 *in every mixture) in mixed pollinations, and the significance levels of the* χ^2 *test*

Recipient	Donor 2	*N*	Seeds (%)	Significance
V 505	V 5232	117	42	ns
V 505	V 5794	117	55	ns
V 505	E 9563	120	42	ns
V 505	V 5035	122	23	***
V 505	E 9427	117	66	***
V 5788	V 5232	142	30	***
V 5788	V 5794	160	57	ns
V 5788	E 9563	136	38	**
V 5788	V 5035	144	32	***
V 5788	E 9427	129	60	*
V 5835	V 5232	112	41	ns
V 5835	V 5794	103	72	***
V 5835	E 9563	1	–	–
V 5835	V 5035	119	34	***
V 5835	E 9427	128	72	***
V 5814	V 5232	116	27	***
V 5814	V 5794	115	61	*
V 5814	E 9563	137	48	ns
V 5814	V 5035	113	32	***
V 5814	E 9427	122	70	***
V 509	V 5232	110	34	***
V 509	V 5794	110	35	**
V 509	E 9563	109	30	***
V 509	V 5035	110	14	***
V 509	E 9427	108	57	ns
V 591	V 5232	119	37	**
V 591	V 5794	110	55	ns
V 591	E 9563	110	45	ns
V 591	V 5035	110	14	***
V 591	E 9427	108	64	**

N, total number of seeds analysed. *, $0.01 < P \leqslant 0.05$; **, $0.001 < P \leqslant 0.001$; ***, $P \leqslant 0.001$.

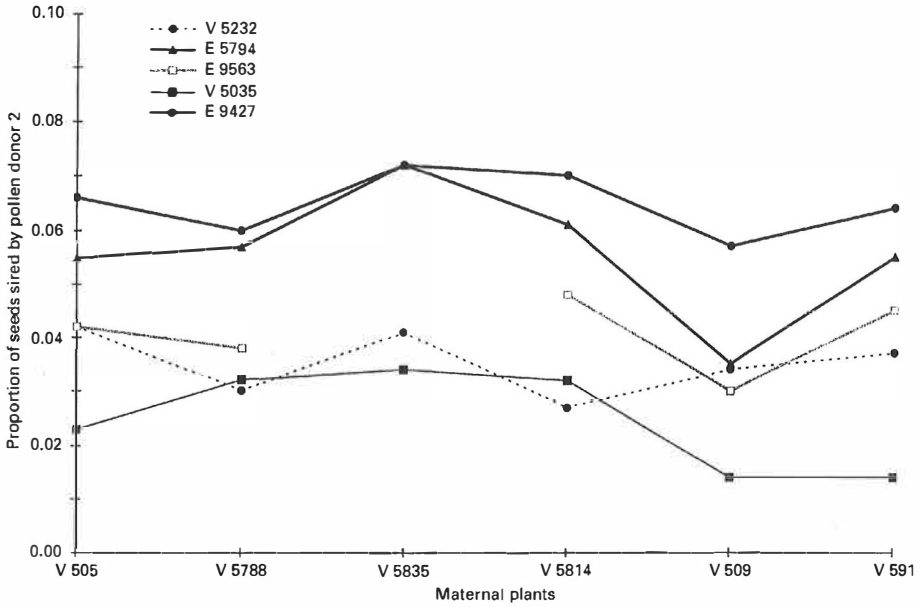

Fig. 3. The relative seed-siring success of five pollen donors on six maternal *Betula pendula* plants (one maternal/paternal combination produced no seeds).

Table 3. *Random-effects ANOVA of paternal and maternal effects on the relative seed-siring success of pollen donor 2 in mixed pollinations*

Source of variation	df	SS	*F*	*P*
Paternal clone	4	0.67	41.75	<0.001
Maternal clone	5	0.11	5.75	<0.01
Two-way interactions	19	0.083		
Error	0	0		

SS, sum of squares.

tube growth and the relative seed-siring success of pollen donor 2 was found (Fig. 4). A weighted mean correlation coefficient for pollen tube length *in vivo* and seed-siring success was 0.63 ($P<0.001$) and for pollen tube length *in vitro* and seed-siring success was 0.53 ($P<0.01$).

DISCUSSION

The *B. pendula* pollen donors showed significant variation in pollen-tube growth rates. Most importantly, there was a significant consistency in the rankings of the pollen donors across 11 maternal plants. Variation in pollen performance within a population has been reported by several authors (Sari-Gorla *et al.*, 1975; Mulcahy, 1979; Ottaviano *et al.*, 1980; Cruzan, 1990; Snow & Spira, 1991; Björkman *et al.*, 1995), but few studies have shown that differences in pollen-tube growth rate are

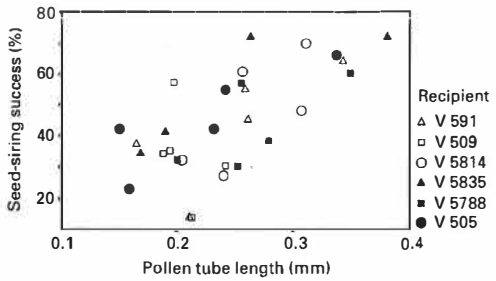

Fig. 4. The relationship between pollen tube length and seed-siring success in *Betula pendula*. Mean $r=0.63$; $P<0.001$.

consistent across several recipients. Snow & Spira (1991) were the first to show that differences in pollen-tube growth rates between pairs of individuals were consistent across maternal plants. Their later study with *Hibiscus moscheutos* (Snow & Spira, 1996) has provided convincing evidence that pollen tube competitive ability varies within a population and can have a substantial influence on male reproductive success when large, mixed-donor pollen loads are deposited on stigmas. Marshall (1998) has shown in her study that the rank order of pollen donor performance, in terms of number of seeds sired after mixed pollination, is highly consistent across maternal plants. She did not, however, study the pollen-tube growth rates.

Although many studies (e.g. Björkman *et al.*, 1995; Snow & Spira, 1996) provide evidence for non-random fertilization, the association between pollen tube growth and seed-siring success has not been straightforward (Melser *et al.*, 1997; J. I. Hormaza & M. Herrero, unpublished; see also Thomson *et al.*, 1994). Melser *et al.* (1997) found that the parental combinations that sired relatively many seeds did not have high pollen-tube growth rates in *Echium vulgare*. In *P. avium* the speed of pollen germination on the stigma was not a determinant factor affecting fertilization success (J. I. Hormaza & M. Herrero, unpublished). In our study, we found a significant positive correlation between pollen-tube growth rate and seed-siring success in *B. pendula*. Furthermore, the differences in relative seed-siring success of five pollen donors, when compared with that of the standard donor E 1970, were statistically consistent across all the six maternal plants studied. These results indicate that pollen-tube growth rate can be an important component of male fitness and a determinative factor controlling the paternity of the seeds in a *B. pendula* seed orchard.

In many studies, the competitive ability of pollen has been affected by both paternal and maternal genotypes. Marshall & Ellstrand (1986) showed in their study with *Raphanus sativus* that the fertilization of seeds in a single fruit by two kinds of pollen is not random. Fruits sired by one pollen donor were more likely to mature; this pattern was consistent across four of the five maternal plants. They suggested that the pollen parent, the maternal parent and the competing pollen types can all affect the proportion of seeds fertilized by a particular pollen parent. In a study by Johnston (1993), the competitive status of pollen changed with a pollen recipient. The pollen-tube growth rate, as well as the seed-siring success of a pollen donor, was affected by both the paternal parent and the maternal parent in our study. However, the significant positive correlation between pollen tube growth *in vivo* and *in vitro* indicates that the rankings of the pollen donors are largely the same without any maternal influence.

It is difficult to distinguish between pollen competition and female choice in plant sexual selection (Snow & Lewis, 1993). If a certain male consistently outcompetes other males across a wide range of maternal plants, it could be due to the superiority of that male or to the fact that all the females are choosing in the same way (Marshall & Ellstrand, 1986; Stanton, 1994). Females have several strategies to regulate pollen tube growth on a stigma and in a style and ovary. These stategies involve nutritive support, directional guidance of pollen tubes towards the ovules and constraints on pollen tube growth (Herrero & Hormaza, 1996). An interesting mechanism of regulating pollen-tube growth rate by the pistil is transmitting tissue-specific (TTS) glycoprotein (Wang *et al.*, 1993) that attracts pollen tubes (Cheung *et al.*, 1995) and possibly gives some nutritive support (Wu *et al.*, 1995). A low level of TTS protein in the style causes decreased pollen tube growth (Cheung *et al.*, 1995). The different growth rates of *B. pendula* pollen tubes on different recipients might be due to the different levels of glycoprotein like TTS provided by the maternal clone (A. Y. Cheung, pers. comm.). It would be interesting to know whether TTS protein is able to attract pollen tubes differently depending on the genotype of the pollen donor.

The best-known examples of active constraint of pollen tube growth by the pistil are different incompatibility reactions. Intraspecific incompatibility between *Betula* trees has been observed (Hagman, 1971) and it might partly explain why some pollen donors germinate better than others on certain recipients. Intraspecific incompatibility in hardwood forest tree species has been studied infrequently (Owens & Blake, 1985) and is difficult to detect because there are numerous other factors that might be involved in pollen tube growth on the stigmas (Hagman, 1971). However, it is known that intraspecific incompatibility between *Betula* species occurs at very early stages of germination, usually as an inhibition of pollen tube penetration into the stylar tissue. Even when this occurs occasionally, the growth of the incompatible pollen tube is soon arrested (Hagman, 1971). None of the pollen donors in our study showed retarded pollen tube growth on any of the maternal plants, and no abnormalities in pollen tube growth were observed, indicating compatibility between pollen donors and recipients.

It is possible that pollen germination ability (that is, competitive ability) is dependent on prevailing environmental conditions during pollen development (Young & Stanton, 1990; Delph *et al.*, 1997) or during pollen germination (Zamir *et al.*, 1981; Luza *et al.*, 1987; Polito *et al.*, 1988; Kristjansdottir, 1990). If this is so, male-male competition might be occurring, but no net evolutionary change will result if the effect is due entirely to environmental variation (Snow & Mazer, 1988). In our study all the pollen

donors and maternal clones were grown under uniform conditions in a plastic house, so all variation in the reproductive success of the clones resulting from environmental variation during pollen development or pollination should have been excluded.

The resting state in pollen tube growth in *B. pendula* (and in some other species) provides a fair start for pollen grains to the ovules, regardless of their different arrival times and their original positions on the stigmatic surface, thus increasing the possibility for pollen competition. We did not measure the pollen-tube growth rate after the resting state, which is a shortcoming in our study. Despite this there was a significant positive correlation between pollen tube growth and seed-siring success, indicating that the resting state has no effect on the competitive outcome of the pollen. Another problem in our study was that stored pollen was used in hand-pollination experiments; there is therefore a slight possibility that differences between the pollen donors were affected by the storage. However, our aim was to describe a situation of pollen competition and its effects on the fertilization success of the pollen donors in a seed orchard population. Further studies will be needed to determine whether the differences between pollen donors are genetically based, and whether pollen competition leads to sexual selection in a population.

If, after all, pollen-tube growth rate is an important component of male fitness, how can variation in this growth rate persist in a population? Maintainance of variation in pollen-tube growth rate might include several mechanisms, such as recombination, inconstancy of selective values, direct pollen-pollen interactions and interactions between pollen grains and the stigmatic tissue (Mulcahy *et al.*, 1996). Direct pollen-pollen interactions might have significant and largely unknown effects on pollen performance (Mulcahy *et al.*, 1996). It has been shown in many studies that the seed-siring success of a pollen donor in mixed-donor pollinations is dependent not only on its intrinsic genetic potentiality but also on the identity of the competing pollen grains on the stigma (Moran & Griffin, 1985; Bertin, 1986; Cruzan, 1990; Nakamura & Wheeler, 1992b; Radha *et al.*, 1993). Pollen-pollen interactions have also been found between certain *B. pendula* clones *in vitro* (Pasonen & Käpylä, 1998). The effect of these interactions on male reproductive success is, however, unknown.

Given that there are significant differences in pollen-tube growth rate between different pollen donors and that the differences between donors are largely consistent across recipients, we suggest that pollen competition operates in a *B. pendula* seed orchard population. A significant positive correlation between pollen-tube growth rate and seed-siring success indicates that pollen-tube growth rate can be a determinative factor controlling the paternity of the seeds. A similar mechanism can be involved in the sexual selection of natural hardwood forest tree populations if pollination intensities are high enough. However, there are many other factors besides pollen-tube growth rate that can influence the reproductive success of a pollen donor. Further investigations will be required to determine whether pollen competition leads to sexual selection and long-term change in allele frequencies in a population.

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II

Do pollen donors with fastest-growing pollen tubes sire the best offspring in an anemophilous tree, *Betula pendula* (Betulaceae)?

by

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Do pollen donors with fastest-growing pollen tubes sire the best offspring in an anemophilous tree, *Betula pendula* (Betulaceae)?

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Abstract

The relationship between pollen and progeny performance has been a subject of many studies but the evidence for pollen-tube growth rate as an indicator of progeny fitness is equivokal. We used an anemophilous tree, *Betula pendula,* to examine the relationship between pollen-tube growth rate and seed and seedling performance. We crossed nine maternal plants with pollen from six pollen donors in a clonal *B. pendula* seed orchard, measured the pollen-tube growth rates for every cross, and analyzed the performance of the resulting seeds and seedlings. The only significant positive correlation was found between pollen-tube growth rate and seed mass when seed number per inflorescence was controlled for. Using seed mass as a covariate, we found that only maternal parent had a significant effect on the number of seeds per inflorescence, the percentage of germinable and embryonic seeds, and early seedling growth. Both maternal and paternal parents had significant effects on seedling height after 85 d of growth. These results are in concordance with the general view that maternal effects are usually most apparent in seed characters and during early plant growth. This study does not provide strong evidence for the theory of pollen tube growth rate as an indicator of progeny quality.

Key words: Betulaceae, Betula pendula, maternal effects, paternal effects, pollen-tube growth rate, progeny performance, seed abortion.

Introduction

During the last three decades many studies have been carried out on the relationship between pollen and progeny performance, but the evidence for pollen-tube growth rate as an indicator of progeny performance remains equivocal. The idea of a positive relationship between pollen and progeny performance is based on an overlap in gene expression between gametophytic and sporophytic stages of life cycle (Mulcahy 1979). The effects of pollen-tube growth rate on progeny quality have usually been studied by varying the intensity of pollen competition by applying different amounts of pollen to the stigmas (Winsor, Davis & Stephenson 1987, Bertin 1990, Snow 1990, Richardson & Stephenson 1992, Janse & Verhaegh 1993, Quesada, Winsor & Stephenson 1993, Palmer & Zimmerman 1994, Quesada, Winsor & Stephenson 1996a, Johannsson & Stephenson, 1997, Mitchell 1997) or by varying the distance pollen tubes must travel to the ovules (Mulcahy & Mulcahy 1975, Ter-Avanesian 1978). It has been assumed that under conditions of intense pollen competition only the fastest-growing pollen tubes achieve fertilization (Mulcahy 1979).

Studies employing small and large pollen loads have been criticized due to the fact that results from these studies are not always easy to interpret (Schlichting, Stephenson & Small 1990, Mitchell 1997). For example, high pollen loads alone may stimulate maternal parent to allocate more resources to seeds and fruits and thus better quality offspring may be a result of the pollen load itself, rather than competition among pollen tubes (Charlesworth 1988). Differences in sporophyte quality could also be due to undetected selective abortion of the embryos or the seeds (Walsh & Charlesworth 1992). Furthermore, it has been suggested that low pollen loads might not have been low enough to exclude all possibilities for pollen competition (Mitchell 1997). For these reasons, it would be informative to employ direct pollen-tube growth rate measurements and the analysis of progeny quality from different maternal plants pollinated by the same pollen donors.

In previous studies we have found evidence for the positive relationship between pollen-tube growth rate and seed siring success of the pollen donors in a self-incompatible, deciduous tree, *Betula pendula* Roth (Pasonen et al. 1999). The aim of the present study was to investigate the maternal and paternal effects on seed and seedling performance, with particular attention to the relationship between pollen-tube growth rate and progeny performance. We also attempted to find out whether selective abortions of the seeds were likely to occur in *Betula pendula*. The clonal seed orchards of *B. pendula* established in plastic greenhouses provided an excellent controlled environment for studying evolutionarily important processes that might have been confounded by environmental variation in natural pollination conditions. No previous studies on the relationship between pollen and progeny performance have been performed in this commercially important tree species. To our knowledge, this study is also the first one in which direct pollen-tube growth rate measurements have been employed to examine the relationship between pollen and progeny performance.

Materials and methods

Study species and study site

Betula pendula Roth is a common, anemophilous, monoecious, and self-incompatible tree ranging throughout most of Europe from Norway to Sicilia (Tutin et al., 1964). It has 200-300 male flowers in each catkin (Dahl & Fredrikson 1996) and ~ 600 female flowers in each pistillate inflorescence (personal observation of H-L.P.). Each female flower consists of a single two-locular ovary with two linear, dry stigmas and two ovules of which only one develops into a mature seed (Sulkinoja & Valanne 1980, Dahl & Fredrikson 1996).

The study was carried out in a plastic house seed orchard at Haapastensyrjä Forest Tree Breeding Centre in Läyliäinen (60°N, 24°E), Finland. The seed orchard consists of 38 *B. pendula* clones originating from 11 closely situated populations. The clones were originally selected for the seed orchard on the basis of the results from the field trials (Raulo & Koski 1977). These field trials were done to select genotypes with superior heritable growth characters (e.g., straight stem). A plastic house provides favorable conditions for flowering and seed development and isolates seed orchard clones from outside pollen sources.

Hand-pollinations and pollen-tube growth rate measurements

To study the pollen-tube growth rates of different pollen donors on several maternal plants, a single-donor hand-pollination experiment was conducted. Nine maternal plants (different *B. pendula* clones) were selected from a group of seed orchard clones and were later pollinated with pollen from six paternal clones. Maternal clones were selected on the basis of the number of female inflorescences (only the clones with large amounts of female inflorescences were used) and paternal clones were selected on the basis of the germination ability of pollen. Before any pollen was shed and before female inflorescences were receptive, twelve adjacent branches (two for each pollen donor) from each maternal plant were isolated with paper bags to prevent uncontrolled pollinations. Each branch was isolated with one paper bag and each bag contained three (for pollen-tube growth rate measurements) or ten (for analysing the progeny performance) female inflorescences. All of the male inflorescences in the bagged branches were removed to prevent self-pollinations. Adjacent branches were isolated to minimize the differences among the branches. When female inflorescences became receptive, an equal volume of pollen was applied to each pollination bag by using a pollination syringe. The amount of pollen applied into the pollination bags exceeded the number of ovules in the bags. Each maternal plant had a total of 12 hand-pollinations (six for pollen-tube growth rate measurements and six for the analysis of progeny performance). The single-donor hand-pollinations for pollen-tube growth rate measurements and for the analysis of progeny performance were performed in the plastic house seed orchard at the same

time during a period of 3 d (maternal plants became receptive at different times) in the beginning of May 1997.

Pollen stored at –20°C was used in hand-pollinations. There was not enough time to collect fresh pollen before hand-pollination experiments because the flowering of male and female flowers occurs almost synchronously in *B. pendula*. Germination percentages of stored pollen from several donors were determined in vitro on agar medium (containing 1% agar, 0.01% boric acid and 0.5 mol/L sucrose) (Käpylä 1991). In previous experiments we found that in vitro pollen tube growth of stored pollen correlates positively with pollen tube growth in vivo (Pasonen et al. 1999). We have also found that if *B. pendula* pollen is properly collected (in dry conditions) and dried in room temperature (24°C) for 24 h before storage at –20°C, germination ability of the pollen samples remains fairly unchanged for several years. Finally, six paternal clones with as equal germination percentages as possible were chosen for the experiment. Germination percentages of the selected pollen donors varied between 60 – 70%. Paternal clones with equal germination percentages were selected because germination tests in vitro have revealed that high germination percentages can also enhance the pollen tube growth rate (Pasonen, Käpylä & Pulkkinen 1997).

Twelve hours after pollination, three pollinated female inflorescences per cross were collected. After 12 h the fastest pollen tubes had almost reached the base of the elongate stigma, but not entered the funiculus. In *B. pendula* there is a resting state in pollen tube growth after which all the pollen tubes have a "fair start" to the ovules regardless of their original positions and arrival times on the stigma (Dahl & Fredrikson 1996). We measured the pollen tubes before the resting state had begun. In our previous studies we have found that there is a significant positive correlation between pollen tube length after 12 h of germination and seed siring success (Pasonen et al. 1999). The female inflorescences were detached and immediately stored in glacial acetic acid and 60% ethanol (1:9). The inflorescences were stored in a refrigerator at 4°C until they were examined. The single flowers were detached from the inflorescence with a scalpel and stained with a solution of 0.1% aniline blue in aqueous K_3PO_4 (0.3 mol/L). After staining, pollen tube callose became fluorescent and distinguishable in the darker stylar tissue when examined with UV fluorescence microscopy. Between 80 and 120 pollen tubes (from three inflorescences and several flowers) were measured per cross so that a total of 162 (54 crosses x 3 inflorescences) female inflorescences was examined. Pollen tubes were measured by focussing the scale of the ocular on the pollen tube and counting the intervals of the scale from the base to the tip of the pollen tube. If the pollen tube was not straight, the scale was moved along the pollen tube while counting the intervals so that a reliable measure of pollen tube length was obtained. Pollen tubes were measured only in styles that had no more than 1-2 pollen tubes because it is known that the density of pollen tubes in the stylar tissue may influence pollen tube growth (Cruzan 1990, Holm 1994)

Analyzing the progeny performance

Seeds from the pollinated inflorescences were collected in July 1997. Total seed mass per cross, mass of random sample of 100 seeds, and percentage of germinable and embryonic seeds (= seeds with fully developed embryos) were determined in the following autumn. A microfilm reading device was used to determine whether a seed contained a fully developed embryo. A fully developed embryo could be seen to fill a large part of a seed while seeds without embryos were fairly transparent. The number of seeds per cross was estimated by dividing the total seed mass per cross by the average mass of a single seed of the same cross. The number of seeds per inflorescence in each cross was estimated by dividing the total number of seeds per cross by the number of pollinated inflorescences. Seeds were stored in 4 °C until all of the analyses were carried out. To determine the seed germination percentages, moist pieces of paper were placed on petri dishes and a random sample of 100 seeds per cross were counted on each piece of paper. The petri dishes were placed in a germination chamber on a 12 h light/12 h dark cycle in room temperature (=23°C). The number of germinated seeds was counted every day during a period of 12 d. A seed was considered germinated if it had a clearly visible primordial cotyledon whose length was at least 1 mm. The germination percentage on the 12th d of germination was used in the statistical analyses.

On the 1 June 1998, seeds from every cross were sown on peat substrata in a greenhouse at Haapastensyrjä Forest Tree Breeding Centre to study the seedling growth rate. Twenty to 25 seedlings per cross were raised as two replicates of 10 – 15 seedlings in natural light conditions (2 x 10-15 seedlings per cross). The replicates were scattered randomly throughout the greenhouse to minimize the effects of possible microclimatic differences in the greenhouse. Temperature in the greenhouse was not controlled, although it was higher than outdoors on sunny days. One month later the seedlings were transferred outdoors. The height of the seedlings was measured twice, in the beginning of July (growing time 30 d) and in the end of August (growing time 85 d) 1998.

Estimation of seed abortion frequency

Several (10-20) female inflorescences were collected from different parts of four randomly chosen maternal plants that were also used in the hand-pollination experiment. The number of female flowers in each inflorescence was counted to estimate: (1) whether the number of flowers per inflorescence varied within a tree and (2) the average number of flowers per inflorescence per tree. The number of female flowers within an inflorescence did not differ in different parts of the tree among three out of four maternal plants. Among these three maternal plants, it could be estimated whether the maternal plants aborted differentially seeds sired by different pollen donors (see Table 7). The frequency for seed abortion was estimated by comparing the average number of female flowers per inflorescence with the average number of seeds per inflorescence in each cross. Because excess pollen was applied to the stigmas it was assumed that all the female flowers in each inflorescence were pollinated

and fertilized. However, this could not be verified and thus all the values for the frequency of seed abortion should be considered as rough estimates of the number of aborted or undeveloped seeds.

Data analysis

Spearman correlation coefficients were calculated separately for each maternal plant to test the relationship between pollen tube length (PTL) and percentage of germinable and embryonic seeds, PTL and mean seed mass and PTL and seedling height. Spearman correlations were also calculated for each maternal plant between seed mass and the other sporophytic traits. Furthermore, Spearman correlations between PTL and seed and seedling performance and between seed mass and the other sporophytic traits were calculated on the basis of the means of nine recipient plants for each pollen donor.

Partial correlations between PTL and seed and seedling performance were calculated using seed mass as a controlling factor due to the fact that seed mass was found to correlate with progeny performance. To assess the relationship between PTL and seed mass, the number of seeds per inflorescence (total number of seeds per cross divided by the number of pollinated inflorescences) was used as a controlling factor. To determine whether the rankings of the pollen donors in the form of percentage of germinable and embryonic seeds, seed mass and seedling height changed depending on the maternal plant, a Kendall's coefficient of concordance (W) was calculated (Sokal & Rohlf 1981, p. 609).

Two-way (random-effects) ANOVAs using seed mass or seed number per inflorescence as covariates were performed to test the effect of maternal and paternal parent on seed number per inflorescence, percentage of germinable and embryonic seeds and mean seed mass. Number of seeds per inflorescence was used as a covariate when the effect of parental clones on seed mass was tested. Percentages of germinable and embryonic seeds were arcsine square-root transformed to normalize the data. Two-way (random-effects) ANOVA of the effects of parental clones on seedling height was performed using the original heights of each measured seedling (not the means of the seedlings heights for each cross). Seed mass could not be used as a covariate in this analysis due to the fact that only one value for seed mass was available in each cross.

One-way ANOVAs were performed to study whether the number of female flowers per inflorescence varied within and between the maternal plants. A Kendall's coefficient of concordance was calculated to study whether the ranking orders of the pollen donors differed in the number of aborted or undeveloped seeds across the maternal plants. In addition, a Spearman correlation coefficient between PTL and the number of aborted or undeveloped seeds was separately calculated for the three maternal plants.

Results

Seed quality

A positive relationship between pollen tube length and seed quality expressed as seed mass and percentage of germinable and embryonic seeds was detected on most maternal plants. However, the significance of the correlation coefficients varied across the maternal plants and was mostly non significant (Table 1). The only significant positive Spearman correlation calculated on the basis of the means of the nine maternal plants for each pollen donor (see Table 2a) was found between pollen tube length and percentage of germinable seeds (Table 1). Means of the six pollen donors on nine maternal plants have been summarized in Table 2b.

A positive correlation between seed mass and percentage of embryonic seeds was found (Table 3). There was also a positive, but not significant, correlation between seed mass and percentage of germinable seeds, indicating that at least part of the variation observed in seed performance can result from the variation observed in seed mass. A negative correlation (although not statistically significant) between seed mass and the number of seeds per inflorescence (Spearman r = -0.62, P = 0.191) indicated that seed mass, in turn, could be affected by the number of seeds per inflorescence. Partial correlations between pollen tube length and seed performance using seed mass and the number of seeds per inflorescence as controlling factors revealed a significant positive correlation between pollen tube length and seed mass and a substantial but not statistically significant positive correlation between pollen tube length and percentage of germinable seeds (Table 4).

Kendall's coefficients of concordance for six pollen donors on nine maternal plants were 0.11 ($P > 0.05$) for percentage germinable seeds, -0.02 ($P > 0.05$) for seed mass and 0.05 ($P > 0.05$) for percentage embryonic seeds. Coefficients of concordance indicate that there is no concordance in the rankings of the pollen donors in terms of the quality of the seeds sired.

Two-way ANOVA using seed mass as a covariate revealed significant maternal effects on seed number per inflorescence and percentage of germinable and embryonic seeds. Paternal clone had no effect on any of these traits. Neither maternal nor paternal clone had significant effect on seed mass (Table 5).

TABLE 1 Spearman correlations between pollen tube length and the sporophytic traits on nine maternal plants (seedling height was measured after 30 (I) and 85 (II) d of growth). Mean = Spearman correlation between pollen tube length and the sporophytic traits calculated on the basis of the means of the nine recipient plants for each pollen donor.

Recipient	Germinable seeds %	Embryonic seeds %	Mass of 100 seeds	Seedling height I	Seedling height II
V 5814	-0.35	-0.46	0.03	-0.15	-0.84*
V 509	0.84*	0.70	0.63	-0.81*	-0.41
V 5239	0.53	0.41	0.55	0.37	0.26
V 5835	0.54	0.09	0.12	-0.89*	-0.37
V 505	0.66	0.94**	0.54	0.26	-0.49
V 5279	0.77	0.84*	0.83*	-0.52	-0.43
V 5817	0.54	0.89*	0.58	0.09	-0.49
V 5788	0.89*	0.71	0.67	-0.20	-0.20
V 591	0.64	0.13	0.46	-0.03	-0.49
Mean	1.00***	0.77	0.74	-0.09	-0.60

* $0.01 < P \leq 0.05$;
** $0.001 < P \leq 0.01$;
*** $P \leq 0.001$

TABLE 2a Means (and standard deviations) of pollen tube length after 12 h of germination, mass of 100 seeds, percentage of germinable and embryonic seeds and seedling height after 30 (I) and 85 (I) d of growth of nine maternal plants for six pollen donors ($N = 9$).

Paternal clone	Pollen tube length (mm)	Mass of 100 seeds (g)	Germinable seeds (%)	Embryonic seeds (%)	Seedling height I (cm)	Seedling height II (cm)
V 5035	0.17	0.019	17.2	15.1	3.28	41.74
	(0.05)	(0.004)	(14.91)	(11.49)	(0.41)	(2.35)
E 1970	0.33	0.026	43.2	35.1	3.34	35.87
	(0.11)	(0.007)	(26.92)	(26.06)	(0.41)	(3.66)
E 9563	0.22	0.019	28.1	18.8	3.33	42.18
	(0.08)	(0.005)	(24.29)	(16.20)	(0.48)	(4.95)
V 5794	0.21	0.021	24.8	19.4	3.11	40.06
	(0.07)	(0.006)	(16.93)	(20.15)	(0.64)	(1.90)
E 9427	0.29	0.023	34.8	28.7	3.00	36.41
	(0.10)	(0.004)	(19.52)	(7.47)	(0.33)	(5.44)
V 5232	0.18	0.021	24.4	22.4	3.41	38.72
	(0.06)	(0.006)	(14.55)	(14.46)	(0.33)	(4.88)

TABLE 2b Means (and standard deviations) of pollen tube length after 12 h of germination, mass of 100 seeds, percentage of germinable and embryonic seeds and seedling height after 30 (I) and 85 (I) d of growth of six pollen donors on nine maternal plants ($N = 6$).

Maternal plants	Pollen tube length (mm)	Mass of 100 seeds (g)	Germinable seeds (%)	Embryonic seeds (%)	Seedling height I (cm)	Seedling height II (cm)
V 5814	0.28	0.023	32.83	33.00	2.97	38.59
	(0.05)	(0.005)	(7.41)	(16.21)	(0.37)	(3.37)
V 509	0.22	0.019	31.33	20.83	3.04	35.97
	(0.03)	(0.002)	(24.99)	(14.37)	(0.39)	(4.22)
V 5239	0.07	0.023	10.33	12.83	2.84	45.49
	(0.02)	(0.006)	(15.57)	(19.18)	(0.72)	(3.19)
V 5835	0.30	0.027	31.67	30.5	3.56	42.64
	(0.11)	(0.005)	(21.01)	(15.83)	(0.21)	(2.35)
V 505	0.25	0.020	44.33	26.67	3.11	35.73
	(0.10)	(0.003)	(18.49)	(12.96)	(0.37)	(4.15)
V 5279	0.24	0.019	17.17	14.00	3.32	37.88
	(0.07)	(0.007)	(16.08)	(15.01)	(0.36)	(3.33)
V 5817	0.19	0.020	8.00	10.33	3.61	39.38
	(0.06)	(0.007)	(4.73)	(8.80)	(0.25)	(4.09)
V 5788	0.29	0.025	52.17	43.5	3.50	37.53
	(0.07)	(0.006)	(16.44)	(21.78)	(0.35)	(3.76)
V 591	0.28	0.019	31.00	17.67	3.26	39.28
	(0.09)	(0.005)	(16.59)	(9.27)	(0.31)	(5.03)

TABLE 3 Spearman correlations between seed mass and the other sporophytic traits on nine maternal plants (seedling height was measured after 30 (I) and 85 (II) d of growth). Mean = Spearman correlation between seed mass and the other sporophytic traits calculated on the basis of the means of the nine recipient plants for each pollen donor.

Recipient	Germinable seeds %	Embryonic seeds %	Seedling height I	Seedling height II
V 5814	0.66	0.83*	0.83*	-0.09
V 509	0.46	0.99***	-0.67	-0.35
V 5239	0.84*	0.96**	0.75	0.35
V 5835	0.75	0.99***	-0.06	-0.75
V 505	0.09	0.49	0.26	-0.26
V 5279	0.60	0.90*	-0.29	-0.49
V 5817	0.99***	0.75	-0.49	-0.75
V 5788	0.55	0.75	0.17	-0.46
V 591	0.60	0.49	0.49	-0.03
Mean	0.74	0.97***	0.00	-0.97***

* $0.01 < P \leq 0.05$;
** $0.001 < P \leq 0.01$;
*** $P \leq 0.001$

TABLE 4 Partial correlations (controlling for seed mass) between pollen tube length and the sporophytic traits on nine maternal plants (seedling height was measured after 30 (I) and 85 (II) d of growth). Mean = partial correlation (controlling for seed mass) between pollen tube length and the sporophytic traits calculated on the basis of the means of the nine recipient plants for each pollen donor.

Recipient	Germinable seeds %	Embryonic seeds %	Seed mass [a]	Seedling height I	Seedling height II
V 5814	-0.53	-0.74	0.10	0.07	-0.93*
V 509	0.86	0.22	0.98**	-0.56	-0.58
V 5239	-0.77	-0.81	0.29	0.57	-0.42
V 5835	0.72	0.29	0.09	-0.81	-0.30
V 505	0.89*	0.67	0.81	-0.33	-0.80
V 5279	0.92*	0.79	0.76	-0.79	-0.26
V 5817	0.31	0.74	0.95*	0.50	-0.04
V 5788	0.39	0.26	0.82	-0.66	-0.29
V 591	0.57	0.21	0.32	-0.41	-0.44
Mean	0.86	0.58	0.88*	-0.37	0.19

* $0.01 < P \leq 0.05$;
** $0.001 < P \leq 0.01$;
*** $P \leq 0.001$
[a] number of seeds per inflorescence was used as a controlling factor

TABLE 5 Two-way ANOVA (random-effects) of the effects of maternal and paternal clones on seed performance using seed mass as a covariate. The analysis is based on the mean values of the traits for each cross. Two-way interactions could not be calculated due to the lack of replication.

Source of variation	df	MS	*F*	*P*
Number of seeds per inflorescence [a]				
Mother	6	55977.0	4.00	0.005
Father	5	9923.9	0.71	0.621
Germinable seeds %				
Mother	8	0.21	8.28	<0.001
Father	5	0.03	1.22	0.316
Embryonic seeds %				
Mother	8	0.095	8.09	<0.001
Father	5	0.006	0.55	0.740
Seed mass [b]				
Mother	6	0.0003	1.22	0.326
Father	5	0.0005	2.03	0.104

[a] No covariate was used.
[b] Number of seeds per inflorescence (known only for seven maternal plants) was used as a covariate.

Seedling performance

Only one trait, seedling height, was used to express seedling performance. Spearman correlations revealed a negative relationship between pollen tube length and seedling height on most maternal plants in both measurements (after 30 and 85 d of growth), but no significant mean correlations were detected (Table 1). There was no relationship between seed mass and seedling height after 30 d of growth, but a significant negative mean correlation between seed mass and seedling height after 85 d was found (Table 3). Partial correlations using seed mass as a controlling factor revealed a negative relationship between pollen tube length and seedling height after 30 and 85 d of growth on most maternal plants (Table 4). However, no significant mean correlations between pollen tube growth rate and seedling height were detected (Table 4).

Kendall's coefficients of concordance for six pollen donors on nine maternal plants were 0.12 ($P > 0.05$) for seedling height after 30 d of growth and 0.43 ($0.001 < P < 0.01$) for seedling height after 85 d, indicating concordance in the rankings of the pollen donors in later seedling growth. Two-way ANOVA of the effects of parental clones on seedling height revealed that only the maternal parent had significant effects on early seedling growth (growing time = 30 d) but both maternal and paternal parents had significant effects on later seedling growth (growing time = 85 d) (Table 6).

TABLE 6 Two-way ANOVA (random effects) of the effects of parental clones on seedling height after 30 (I) and 85 (II) d of growth.

Source of variation	df	MS	*F*	*P*
Height I				
Mother	8	8.82	3.06	0.009
Father	5	4.33	1.50	0.210
Mother x father	40	2.89	7.04	<0.001
Error	1013	0.41		
Height II				
Mother	8	1110.78	6.90	<0.001
Father	5	1239.38	7.70	<0.001
Mother x father	40	161.43	3.41	<0.001
Error	998	47.40		

Seed abortion frequency

The average number of female flowers per inflorescence varied depending on the maternal plant between 475 and 668 ($F = 16.39$, $P < 0.001$) (see Table 7 for three maternal plants). There was no difference in the number of female flowers per inflorescence within a maternal plant in three out of four maternal plants ($F = 2.81$, $P = 0.110$; $F = 0.41$, $P = 0.798$; $F = 1.98$, $P = 0.136$; $F = 12.40$, $P = 0.001$). These three maternal plants differed significantly in the estimated number of aborted or undeveloped seeds ($F = 10.43$, $P = 0.002$). There was a significant concordance in the rankings of the pollen donors in terms of

aborted or undeveloped seeds on three maternal plants ($W = 0.66$, $P < 0.01$), which can indicate that all maternal plants favor seeds sired by the same pollen donors. The Spearman correlations between pollen tube length and the estimated number of aborted or undeveloped seeds were –0.26 ($P = 0.620$) on maternal plant V 5239, -0.26 ($P = 0.620$) on maternal plant V 5835, and 0.43 ($P = 0.400$) on maternal plant V 5817.

Discussion

Relationship between pollen tube growth rate and progeny performance

In the present study, direct pollen-tube growth rate measurements were employed to study the relationship between pollen-tube growth rate and seed and seedling performance. A positive correlation was found between pollen-tube growth rate and seed mass when the number of seeds per inflorescence was controlled for. No significant correlations between pollen-tube growth rate and percentage of germinable and embryonic seeds and between pollen-tube growth rate and seedling height were detected. Previously, the effect of pollen-tube growth rate on progeny performance had been studied indirectly by varying the intensity of pollen competition. Many previous studies revealed no effect of the intensity of pollen competition on seed mass (Bertin 1990, Snow 1990, Björkman 1995, Johansson & Stephanson 1997, Quesada, Winsor & Stephenson, 1996a, b) or on percentage of germinable seeds (Mulcahy & Mulcahy 1975, Snow 1990, Johansson & Stephenson 1997). There are some studies that document a positive effect of the intensity of pollen competition on percentage of germinable seeds (Davis, Stephenson & Winsor 1987, Bertin 1990, Palmer & Zimmerman 1994), but evidence for positive effects on seed mass was not found. The effects of the intensity of pollen competition on seedling growth rate have rarely been studied and the results from the existing studies reveal no significant trend (Ter-Avanesian 1978, Bertin 1990, Janse & Verhaegh 1993, Palmer & Zimmerman 1994).

Delph, Weinig, and Sullivan (1998) have hypothesized that it is the order of fertilization rather than speed of pollen tube growth that affects the vigor of the resulting progeny because the ovules fertilized early are better provisioned by the maternal plant than later fertilized ovules. Many studies show that faster growing pollen tubes fertilize ovules in different regions in the ovary than slower growing pollen tubes (Marshall & Ellstrand 1988, Stephenson, Winsor & Schlichting 1988, Rocha & Stephenson 1991) and it has been suggested that different regions in the ovary might have nutritional advantages, which, in turn, could lead to increased vigor in progeny (Stephenson, Winsor & Schlichting 1988). The eliminating of maternal effects by comparing the progeny resulting from small and large pollen loads in different regions of the ovary, as was done by Quesada, Winsor, and Stephenson (1993), does not rule out the possibility that high pollen loads alone may stimulate the maternal parent to allocate more resources to seeds and fruits.

TABLE 7 Estimated numbers of aborted or undeveloped seeds among three maternal plants. Among these maternal plants, no difference in the number of female flowers within an inflorescence in different parts of the tree was observed.

Maternal parent	Paternal parent	No. of seeds per inflorescence per cross	Average no. of female flowers per inflorescence within the maternal plant (sd)	Estimated no. of aborted or undeveloped seeds per cross	Average number of aborted or undeveloped seeds per maternal plant (sd)
V 5239	V 5053	380	668	288	289
	E 1970	304	(42.51)	364	(80.46)
	E 9563	450		218	
	V 5794	260		408	
	E 9427	433		235	
	V 5232	447		221	
V 5835	V 5053	461	579	118	152
	E 1970	381	(41.22)	198	(54.92)
	E 9563	470		109	
	V 5794	346		233	
	E 9427	421		158	
	V 5232	485		94	
V 5817	V 5053	470	620	150	118
	E 1970	563	(50.33)	57	(56.45)
	E 9563	777		-	
	V 5794	479		141	
	E 9427	437		183	
	V 5232	559		61	

In our study, the differences in progeny performance cannot be due to greater maternal allocation to higher pollen loads because the amount of pollen did not vary between pollinations. Furthermore, the differences in progeny performance cannot be due to differential maternal allocation to different regions of the ovary because there is only one ovule in the ovary that develops into mature seed in *Betula pendula*. It is, however, not known whether flowers in different parts of the inflorescence are differentially provisioned by the maternal plant. In this study, a random sample of pollen tubes and seeds from several flowers in different parts of the inflorescences were analyzed so that the results should not be biased by the location of the flowers in inflorescences.

In addition to differential maternal allocation, another widely discussed problem (Charlesworth 1988, Stephenson et al. 1988, Walsh & Charlesworth 1992) linked with studies of pollen competition is the relationship between seed number and seed size. In many plants, average seed weight decreases as the number of seeds within a fruit increases (Stanton, 1984) and differences in seed size, in turn, are known to affect many seedling fitness characters (Schaal 1980, Weis 1982, Zimmerman & Weis 1982). Seed size has sometimes been matched with the pollination treatments by comparing only the groups of same sized-seeds from two treatments (Winsor, Davis & Stephenson 1987) or comparing the fruits containing the same number of seeds (Mulcahy & Mulcahy 1975). We found that the average number of seeds within an inflorescence varied between the crosses and that there was a negative relationship between seed number per inflorescence and seed mass as expected. Furthermore, a substantial positive correlation between seed mass and percentage of germinable and embryonic seeds was detected. Instead, a significant negative correlation between seed mass and later seedling growth was found. Effects of seed mass on seedling performance can, however, be transient, and a longer period for recording seedling growth in *Betula pendula* is needed. In *Campanula americana*, for example, seed size correlated positively with early seedling performance but not with any of the later vegetative measures or reproductive output (Richardson & Stephenson 1992). Furthermore, due to the fact that *B. pendula* seeds contain very little endosperm for early seedling growth, there might not be as clear a relationship between seed mass and seedling performance as observed in many other species.

An interesting finding in this study was the negative relationship between pollen-tube growth rate and early seedling growth when seed mass was used as a controlling factor. Many authors have addressed the question of the mechanisms that might maintain the variation in pollen performance if pollen tube growth rate is an important component of male fitness (Snow & Mazer 1988, Walsh & Charlesworth 1992). In our previous study, we concluded that pollen-tube growth rate is an important factor controlling the paternity of the seeds in *B. pendula* seed orchards (Pasonen et al. 1999). If this is the case also in natural populations, there have to be mechanisms that maintain the variation in pollen competitive ability. It has been suggested that mechanisms like gene flow, mutations, genotype-environment interactions,

pollen-pollen interactions, and negative genetic correlations between gametophytic and sporophytic stages of life cycle could explain the maintenance of genetic variation in pollen-tube growth rate (see Schlichting, Stephenson & Small 1990, Walsh & Charlesworth 1992, Mulcahy, Sari-Gorla & Mulcahy 1996). Pollen-pollen interactions (Pasonen & Käpylä 1998) and genotype-environment interactions in pollen-tube growth rate (Pasonen, Käpylä & Pulkkinen 2000) have already been documented in *Betula pendula*. The negative relationship observed in this study between pollen-tube growth rate and early seedling height can be due to negative epistatic effects and have an evolutionary significance in maintaining the variation in pollen performance. Although the relationship was not statistically significant, even minor effects on the competitive ability of the seedlings can be biologically important during a vulnerable period of very early growth.

Parental effects on progeny performance

The results of the present study reveal substantial maternal effects on the number of seeds per inflorescence, percent germinable and embryonic seeds and seedling height. Paternal effects were detected only for seedling height after 85 d of growth. These results are in concordance with the general view that paternal effects are rarely found in plants (Roach & Wulff 1987), and they may not easily be recognized in the presence of maternal effects because they are usually confounded with them (Schmid & Dolt 1994). Maternal effects are usually most apparent in seed characters and during early plant growth, due to the intimate dependence of the developing seed on the maternal plant (Roach & Wulff 1987). Seed traits are also more influenced by genes expressed in the maternal parent than by genes expressed in the embryo (Thiede 1998). Also paternal effects on seed development are known, though they are of lesser magnitude than maternal influences (Nakamura & Stanton 1989, Richardson & Stephenson 1991). The lack of paternal effects on seed performance and early seedling growth in this study can partly be explained by the fact that the experiment was not primarily designed to test for small paternal and maternal effects.

Maternal effects on seed development and on later progeny performance can be either environmental or genetic (or both). Because maternal plants were raised in uniform conditions in the plastic house, maternal effects observed in this study are more likely to be due to genetic than to environmental effects. Genetic maternal effects generally decline through the life cycle (Schmid & Dolt 1994) and studying later vegetative characters of the seedlings can reveal decreased maternal and more pronounced paternal effects. Recall that in the present study only the maternal parent had significant effect on early seedling growth but both maternal and paternal parents had significant effects on later seedling growth.

Seed abortion and maternal provisioning

It is possible that maternal plants can influence the quality of their offspring by aborting seeds nonrandomly with respect to paternal genotypes (Lee 1984, Marshall & Ellstrand 1988). Such maternal effects have rarely been demonstrated because pollen competition is always a potentially confounding effect (Marshall & Ellstrand 1988). We found that maternal plants differed significantly in the number of aborted or undeveloped seeds in *Betula pendula*. Because the number of flowers per inflorescence can only be counted by destroying the inflorescence, the initial number of flowers of pollinated inflorescences is not known, and thus, it is not known whether the variation in the number of seeds per inflorescence is due to the original differences in the number of flowers within an inflorescence or due to the abortion of the seeds sired by different pollen donors. Among three maternal plants, no differences in the number of female flowers within an inflorescence was detected in different parts of the tree but the number of seeds sired by different pollen donors varied remarkably. This may indicate that selective abortions occur. However, because it could not be verified whether all the flowers in inflorescences were fertilized or not, the number of aborted seeds should be considered as a rough estimate of the number of aborted or undeveloped seeds. No correlation between pollen-tube growth rate and the number of aborted or undeveloped seeds was found. The significant concordance in the rankings of the pollen donors in terms of aborted or undeveloped seeds on different maternal plants may indicate that all three maternal plants favor seeds sired by the same pollen donors. It is also possible that maternal plants allocate resources in different ways to seeds sired by different pollen donors during seed development. We found no evidence for the idea that maternal plants should allocate more resources to certain pollen donors because no concordance among pollen donors was detected in terms of seed mass and percentage of germinable and embryonic seeds on different maternal plants.

Conclusions

Due to the effects of seed mass on seed and seedling performance, the most reliable insight into the effects of pollen-tube growth rate on progeny performance is provided by the partial correlations in which seed mass has been used as a controlling factor. Although a positive relationship between pollen-tube growth rate and seed quality was found, the only significant positive correlation was detected between pollen-tube growth rate and seed mass. No correlation between pollen-tube growth rate and seedling height was detected. Because the positive effects of pollen-tube growth rate on seed quality did not translate into faster growing seedlings, no strong evidence for the theory of pollen-tube growth rate as an indicator of progeny quality is provided by this study. The slight negative relationship between pollen-tube growth rate and early seedling growth can be due to negative epistatic effects and have an evolutionary significance in maintaining the variation in pollen-tube growth rates. The result of substantial maternal and largely undetectable

paternal effects on seed and seedling performance is in concordance with the general view that paternal effects are rarely found in plants (Roach & Wulff 1987), and they may not easily be recognized in the presence of maternal effects (Schmid & Dolt 1994). In conclusion, the answer to the question raised in the title of this paper is that pollen-tube growth rate is not a good predictor of progeny performance in *Betula pendula.*

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III

Effects of temperature and pollination site on pollen performance in *Betula pendula* Roth – Evidence for genotype-environment interactions

by

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Effects of temperature and pollination site on pollen performance in *Betula pendula* Roth – evidence for genotype-environment interactions

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Abstract We studied whether the differences between genetically different pollen donors (*Betula pendula* Roth clones) with respect to pollen-tube growth rate were consistent under different thermal conditions during pollen germination in vivo and in vitro. We conducted a single-donor hand-pollination experiment with same pollen donors and recipients in a plastic house seed orchard and at an outdoor clone collection. The prevailing daily mean temperature during pollen germination was 13°C higher in the plastic house than outdoors. The pollen-tube growth rate of each pollen donor was additionally determined in vitro on agar medium at five temperatures (10°, 15°, 22°, 30° and 35°C). A significant interaction between paternal clone and pollination site as well as between paternal clone and temperature was found, which provides evidence for genotype-environment interactions. Genotype-environment interactions can have evolutionary significance in maintaining the variation in pollen-tube growth rates. At seed orchards, genotype-environment interactions can cause deviations from the expected genetic composition of the seed crop depending on the prevailing environmental conditions during pollen-tube growth.

Key words Pollen-tube growth rate · Pollen competition · Temperature · Seed orchard · *Betula pendula* Roth (silver birch)

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Introduction

According to the pollen competition hypothesis, pollen grains have to compete for access to the ovules when there are more pollen grains on the stigmas than there are ovules in the ovary. Under conditions of pollen competition only the fastest growing pollen grains are assumed to achieve fertilization (Mulcahy 1979). In addition to the genotypes of the pollen donors and recipients, pollen competitive ability is affected by several external factors (Stephenson et al. 1992). The competitive ability of pollen donors has been an object of numerous studies (e.g. Marshall and Ellstrand 1986; Johnston 1993; Björkman et al. 1995; Snow and Spira 1996; Hormaza and Herrero 1998), but the consistency of the competitive status under different environmental conditions has received less attention.

Changes in pollen competitive ability caused by environmental or weather conditions might have unexpected consequences at seed orchards that have usually been founded to improve the seed production of commercially important tree species. Seed orchards of coniferous tree species have been founded at sites of favourable conditions for seed production, quite often far away from the original growing places of the seed orchard trees. In open-pollinated seed orchards, each seed orchard genotype is expected to be approximately equally represented in a seed crop, i.e. it is assumed that male fertilities are equal and pairwise mating probabilities are identical (Schoen and Stewart 1987). Seed orchards of *Betula pendula* have been established in plastic houses to isolate them from outside pollen sources and to increase seed production. Similarly, fertilizations are assumed to be random, and the reproductive outcome of each clone is expected to be fairly equal.

In previous studies we have obtained evidence for significant differences between seed orchard clones with respect to pollen-tube growth rates and for the fact that the pollen-tube growth rate can be a determinative factor controlling the paternity of the seeds (Pasonen et al. 1999). If pollen donors with the fastest growing pollen

tubes sire most of the seeds and environmental conditions significantly affect pollen-tube growth rate, the reproductive outcome of seed orchard clones is not equal, and it can vary depending on the prevailing environmental conditions during pollen-tube growth. In the present study we examined whether the competitive ability (pollen-tube growth rate) of six pollen donors varied depending on the pollination environment and prevailing temperature during pollen-tube growth in *Betula pendula* Roth. We discuss the possible effects of genotype-environment interactions on pollen competition and on the functioning of plastic house seed orchards.

Materials and methods

Species and study site

Betula pendula Roth is an anemophilous, self-incompatible and monoecious tree ranging throughout most of Europe from Norway to Sicilia (Tutin et al. 1964). Its male and female flowers occur in separate inflorescences. There are 200–300 male flowers in each catkin (Dahl and Fredrikson 1996) and, on average, 600 female flowers (personal observation) in each pistillate inflorescence. One female flower consists of a single two-locular ovary with two linear, dry stigmas and two ovules of which only one develops into a mature seed (Sulkinoja and Valanne 1980; Dahl and Fredrikson 1996).

The study was carried out in a plastic house seed orchard at Haapastensyrjä Breeding Centre in Läyliäinen (lat. 60° N, long. 24° E) and at an outdoor clone collection in Kangasniemi (lat. 61° N, long. 26° E), southern Finland. The clones were originally selected for the seed orchards on the basis of superior vegetative growth. Those used in the present study originated from rather a small area, between latitudes 60°30′ N and 62°30′ N.

Hand-pollinations

Four maternal and six paternal clones were selected from a group of *Betula pendula* seed orchard clones for a single-donor, hand-pollination experiment. The same clones were used in a plastic house seed orchard and at an outdoor clone collection. Prior to the onset of flowering, branches with at least three female inflorescences were isolated from each maternal plant by bagging with paper bags to prevent uncontrolled pollinations. Once receptive, maternal plants were hand-pollinated with pollen from the six paternal clones. Frozen pollen was used because male and female flowering occurs almost synchronously in *Betula pendula*, and there would have been no time to both collect fresh pollen and test the germination percentages of the pollen before the hand-pollinations. The germination percentages of frozen pollen from several donors were determined in vitro on agar medium (1% agar, 0.01% boric acid, 0.5 *M* sucrose) (Käpylä 1991). Six paternal clones with germination percentages over 50% and as similar as possible were chosen for the experiment. All the hand-pollinations (4×6 crosses in both places) were carried out in the plastic house seed orchard on 5 May, 1997, and at the outdoor clone collection on 19 May, 1997. The temperature during pollen-tube growth varied substantially between these two sites. In the plastic house, the maximum daily temperature was 28°C, the minimum temperature 11°C and the daily mean temperature 17°C; at the clone collection the temperatures were 8°C, 2°C and 4°C, respectively. The volume of pollen used in the hand-pollinations was kept as equal as possible in each cross, because it is known that the density of pollen grains on the stigma may influence pollen-tube growth rate (e.g. Cruzan 1990; Holm 1994).

Pollen-tube growth-rate measurements

Three female inflorescences were analysed and, on average, 65 randomly chosen pollen tubes (from several flowers) per maternal/paternal combination were measured by UV fluorescence microscopy. For details, see Pasonen et al. (1999). Pollen from the same donors that were used in the hand-pollination experiment (the same pollen samples) was germinated on agar medium (Käpylä 1991) at five different temperatures (10°C, 15°C, 22°C=RT, 30°C, 35°C) for 12 h to study the effect of temperature on pollen-tube growth rate. On average, 70 randomly chosen pollen tubes per sample were measured.

Data analysis

A mixed-effects ANOVA was performed to test the effect of the maternal and paternal parent (random effects) and pollination site (fixed effect) on pollen-tube growth rate. In addition, a *t*-test was performed separately for each paternal clone to test the differences in pollen-tube lengths between two pollination sites on two maternal plants. Pollen-tube lengths were measured only on two maternal plants because the other two recipients had suffered from frost during the night prior to the hand-pollinations (frost damage could only be observed when the female inflorescences were analysed under a microscope). The pollen-tube lengths were square root-transformed to normalize the data. To study whether the ranking orders of the pollen donors changed between the pollination sites, we calculated a Spearman rank correlation coefficient (r) separately for two maternal plants. A random-effects ANOVA was performed to test the effect of pollen-donor genotype and temperature on pollen-tube length in vitro. In addition, one-way ANOVA was performed to test whether the differences between the pollen donors with respect to pollen-tube growth rate were significant at different temperatures. To test whether the rankings of the pollen donors changed across four different germination temperatures in vitro, we calculated a concordance coefficient (*W*) (Sokal and Rohlf 1981, p 609).

Results

Mixed-effects ANOVA revealed significant interactions between maternal and paternal clones and the pollination site (F=3.43, P<0.01) (Table 1). Due to these interactions, maternal and paternal effects on pollen-tube growth rate were tested separately in both places (Table 2a, b), and the differences in pollen-tube growth rates between pollination sites were tested separately for each pollen donor on two maternal plants (Table 3). Significant interaction between maternal and paternal parents with respect to pollen-tube growth rate was detected at

Table 1 Mixed-effects ANOVA of the effects of maternal and paternal clones and pollination site (plastic house vs. outdoors) on pollen-tube length in *Betula pendula* Roth

Source of variation	*df*	MS	*F*	*P*
Maternal clone	1	1.62	123.44	<0.001
Paternal clone	5	0.56	42.67	<0.001
Pollination site	1	0.92	20.35	<0.01
Interactions				
Mother×father	5	0.05	4.10	<0.01
Mother×site	1	0.21	16.11	<0.001
Father×site	5	0.24	18.20	<0.001
Mother×father×site	5	0.05	3.43	<0.01
Error	1532	0.01		

Table 2 Random-effects ANOVA of maternal and paternal effects on pollen-tube length

a) At the outdoor clone collection:

Source of variation	*df*	MS	*F*	*P*
Mother	1	0.26	3.34	0.13
Father	5	0.20	2.41	0.18
Mother x father	5	0.08	4.74	<0.001
Error	610	0.02		

b) In the plastic house:

Source of variation	*df*	MS	*F*	*P*
Mother	1	2.05	329.91	<0.001
Father	5	0.66	107.41	<0.001
Mother×father	5	0.01	0.60	0.701
Error	922	0.01		

the outdoor clone collection (F=4.74, P<0.001) (Table 2a) but not in the plastic house (F=0.60, P>0.05) (Table 2b). Pollen-tube growth rate of four out of six pollen donors on the first maternal plant (clone V 5788) and three out of six pollen donors on the other maternal plant (clone V 5718) differed significantly depending on the pollination site (Table 3). A Spearman rank correlation coefficient (r) between two pollination sites on the maternal clone V 5788 was 0.60 (P>0.05) and on the maternal clone V 5817, –0.06 (P>0.05), indicating that there was no significant correlation in the rankings of the pollen donors between two pollination sites.

After 12 h of germination, the optimum germination temperature in vitro appeared to be 30°C for most of the clones (Fig. 1). Pollen from none of the pollen donors germinated at 10°C. The random-effects ANOVA revealed a significant interaction between pollen-donor genotype and temperature (F=29.85, P<0.001) (Table 4). There were also significant differences between the pol-

Table 3 The average lengths of the pollen tubes of six pollen donors on two maternal plants in the plastic house and outdoors (number of pollen tubes measured), and Student's t-test

Maternal plant	Pollen donor	Pollen-tube length (mm)		*T*	*P*
		Plastic house	Outdoors		
V 5788	E 9427	0.35 (87)	0.21 (65)	–6.66	<0.001
	E 9563	0.29 (79)	0.22 (67)	–3.89	<0.001
	E 1970	0.40 (86)	0.29 (64)	–5.06	<0.001
	V 5035	0.20 (76)	0.17 (74)	–1.96	0.053
	V 5232	0.25 (69)	0.22 (65)	–1.48	0.142
	V 5794	0.26 (82)	0.20 (60)	–2.97	<0.01
V 5817	E 9427	0.25 (99)	0.16 (55)	–7.09	<0.001
	E 9563	0.19 (88)	0.19 (16)	–0.25	0.802
	E 1970	0.27 (38)	0.16 (38)	–5.03	<0.001
	V 5035	0.12 (87)	0.12 (58)	–0.88	0.383
	V 5232	0.15 (71)	0.25 (34)	6.60	<0.001
	V 5794	0.18 (72)	0.16 (26)	–0.86	0.395

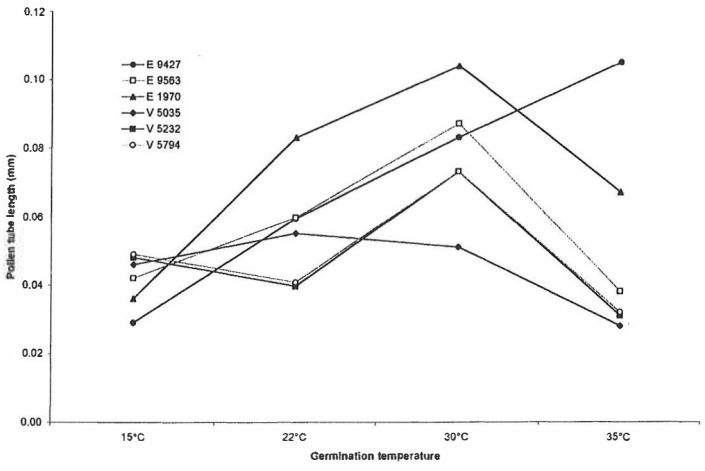

Figure 1 Average lengths of the pollen tubes of six pollen donors after 12 h of germination at four different temperatures in vitro

Table 4 Random-effects ANOVA of the effects of pollen donor and temperature on pollen-tube length

Source of variation	*df*	MS	*F*	*P*
Pollen donor	5	4.85	5.11	0.012
Temperature	3	1.14	1.20	0.354
Temperature ×donor	15	1.01	29.85	<0.001
Error	1217	0.03		

Table 5 One-way ANOVA of the effect of pollen-donor genotype on pollen-tube length at four different temperatures in vitro

Temperature (°C)	*df*	MS	*F*	*P*
15	5	0.40	13.05	<0.001
22 (RT)	5	0.55	16.15	<0.001
30	5	0.61	16.33	<0.001
35	5	2.77	85.19	<0.001

len donors with respect to pollen-tube length at four different temperatures (Table 5). The concordance coefficient (W) for the six pollen donors at four temperatures was 0.27 (P>0.05), indicating that there was no significant concordance in the rankings of the pollen donors across different germination temperatures.

Discussion

Among deciduous tree species, pollen germination in different temperature regimes has been studied in several commercially important species including almond (Weinbaum et al. 1984), pear (e.g. Mellenthin et al. 1972; Vasilakakis and Porlingis 1985), walnut (Luza et al. 1987) and pistachio (Polito et al. 1988). Temperature ranges and optima for pollen germination are known to vary among species (Luza et al. 1987; McKee and Richards 1998), but they can also vary among clones (Luza et al. 1987) and morphs (McKee and Richards 1998) within the same species. Although gametophytic selection in general has been the subject of many studies by evolutionary biologists (e.g. Stephenson and Bertin 1983; Mulcahy and Mulcahy 1987; Snow 1994; Stanton 1994; Willson 1994) and plant breeders (see Hormaza and Herrero 1992, 1994, 1996), differential temperature preferences among individuals within a same population with respect to pollen-tube growth rate have not explicitly attracted much attention. Many external factors are known to affect pollen-tube growth rate (see e.g. Stephenson et al. 1992). However, it is the order of the pollen donors and the consistency (or inconsistency) of the competitive status of the donors under different external conditions that matters (Charlesworth et al. 1987).

In the present study, significant pollen donor-environment interaction was detected, and the ranking orders of the pollen donors changed depending on the pollination environment and prevailing temperature during pollen germination, indicating that different donors can be selected under different conditions during pollen-tube growth. Given that pollen-tube growth rate can be a determinative factor controlling the paternity of the seeds (Pasonen et al. 1999), donors that sire most of the seeds might differ depending on whether they are in a plastic house or outdoors, and the genetic composition of seeds from a plastic house might differ from that of seeds from outdoors. Furthermore, the reproductive outcome of seed orchard clones can vary from year to year depending on the weather conditions during pollination and pollen-tube growth.

Many studies document a decreased frost hardiness of coniferous seedlings originating from seed orchard seeds when the seedlings are transferred to harsher growing places (e.g. Johnsen et al. 1995). These kind of influences of weather conditions during sexual reproduction on the adaptive properties of the seedlings are called after-effects (see e.g. Johnsen et al. 1995, 1996). The after-effects have only been documented among coniferous tree species but observations on decreased frost hardiness among *B. pendula* seedlings originating from seed orchard seeds has been made. Johnsen et al. (1996) suggested that some reproductive stages during female flowering may be sensitive to conditions of the female flowering environment, leading to a decreased frost hardiness of the progeny. It is also possible that the effects of greenhouse conditions on gametophytic selection can partly explain the after-effects. Although the potential effect of gametophytic selection on after-effects in *Picea abies* is rather limited due to the low number of pollen grains in each pollen chamber (Johnsen et al. 1996), the situation in *Betula pendula* is quite different. In *Betula pendula*, the number of pollen grains on the stigmas usually exceeds the number of ovules in the ovary during mass pollination in a plastic house (personal observation), and pollen competition is thus likely to occur. If pollen donors that outcompete the other donors in warm greenhouse conditions sire offspring with a decreased frost hardiness, there should be a negative correlation between pollen-tube growth rate and frost hardiness among seedlings originating from seeds produced in plastic houses. Further studies are needed to gain some insight into relationships between pollen-tube growth rate and frost hardiness of the progeny.

Intense pollen competition and gametophytic selection should, in theory, lead to a decrease in the genetic variation in pollen-tube growth rate (e.g. Walsh and Charlesworth 1992). Many studies have reported a substantial variation in pollen tube growth rates within a population (Sari-Gorla et al. 1975; Mulcahy 1979; Ottaviano et al. 1980; Cruzan 1990; Snow and Spira 1991; Björkman et al. 1995) which has led to speculations over the mechanism that might retain the variability in pollen performance. However, the question on the degree of genetic versus phenotypic variation in pollen-tube growth rates has remained largely unanswered in many studies (Havens 1994). In some species the micro-environment in which a plant grows may be more important in determining its mating success than its genotype (e.g. Havens 1994). However, it is usually assumed that at least part of the observed variation in pollen-tube

growth rate is genetic, and one possibility to maintain this variability is genotype-environment interactions (Gillespie and Turelli 1989; Delph et al. 1997). Alleles which are favoured by selection in one environment can be at a disadvantage in another environment (see Mulcahy et al. 1996). Although we do not know as yet just how much of the variation in pollen-tube growth rate in *Betula pendula* is actually genetic, the results of the present study provide evidence for genotype-environment interactions in pollen-tube growth rate. In addition to have evolutionary significance in maintaining the variation in pollen-tube growth rate, genotype-environment interactions can cause unexpected deviations from the expected genetic composition of the seed crop at seed orchards depending on the prevailing environmental conditions during pollen-tube growth.

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IV

Genotype-environment interactions in pollen competitive ability

by

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Genotype-environment interactions in pollen competitive ability

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Abstract

This study describes genotype-environment interactions in pollen competitive ability expressed as pollen-tube growth rate and seed siring success in *Betula pendula* Roth. Pollen collected from several clones in the greenhouse was used to pollinate the same maternal clones in different environmental conditions. Pollen tube lengths were measured for each cross and paternity of the seeds sired by two-donor pollen mixtures was analyzed. Genotype-environment interactions were found in pollen-tube growth rate and seed siring success. A highly significant positive correlation between pollen-tube growth rate and seed siring success was found in a greenhouse but not in more heterogeneous outdoor conditions. Pollen-tube growth rate can be a predictor of the seed siring success in the greenhouse but the situation at natural *B. pendula* stands is likely to be more complicated. Environmental variability and genotype-environment interactions can partly explain the maintenance of variation in pollen-tube growth rates.

Key words: Betula pendula, genotype-environment interactions, pollen competition, pollen-tube growth rate, seed siring success, environmental effects.

Introduction

In plants, sexual selection may occur by prepollination mechanisms involved in pollinator attraction or by postpollination selection acting in the form of pollen competition and female choice (Stephenson & Bertin 1983). Competition among pollen grains on the stigmas and in the styles has often been referred to sperm competition in animals due to the fact that both pollen grains and sperm cells are haploid male gametes that have to compete for access to eggs (Delph & Havens 1998). Pollen competitive ability, usually expressed as pollen-tube growth rate or seed siring success, has often been cited as a trait that can influence male reproductive success and lead to nonrandom fertilization (Stephenson & Bertin 1983, Marshall & Ellstrand 1986, Snow & Spira 1991 and 1996, Marshall 1998, Mitchell & Marshall 1998, Pasonen et al. 1999).

According to the theoretical predictions, genetic variation in traits closely related to fitness, such as pollen-tube growth rate, should have largely been eliminated by natural selection (Thomson 1989, Walsh & Charlesworth 1992). There are, however, several mechanisms that have been suggested to explain the maintenance of variation in pollen performance. These mechanisms include mutation, recombination, gene flow, antagonistic pleiotropic effects between gametophytic and sporophytic stages of the life cycle, pollen-pistil interactions, direct pollen-pollen interactions and genotype-environment interactions (Snow & Mazer 1988, Walsh & Charlesworth 1992, Mulcahy et al. 1996). Pollen-pistil interactions have been widely studied and observed in many species (Cruzan 1993, Johnston 1993, Herrero & Hormaza 1996, Hormaza & Herrero 1999) and gene flow can be substantial especially among wind-pollinated species (e.g. Starfinger & Stöcklin 1996). On the other hand, the effects of direct pollen-pollen interactions are largely unknown and there is no evidence for antagonistic pleiotropic effects. Because many species cover a range of different environmental conditions and significant environmental variation is often observed even within a range of one population, genotype-environment interactions can have an important role in maintaining the variation in pollen-tube growth rates.

The existence of genotype-environment interaction may mean that the best genotype in one environment is not the best in another environment. Due to the fact that different alleles can be favored by selection in different environmental conditions, genotype-environment interactions can have significance in adaptation to local conditions (Falconer & Mackay 1996). To interpret the significance of pollen competition in adaptation and modifying the genetic structure of populations, more has to be known about the effects of environmental variation on the outcome of pollen competition. It is known that environmental conditions during pollen development (see e.g. Delph et al. 1997) and pollen tube growth (Stephenson et al. 1992, Pasonen et al. 2000) have influence on pollen performance but there are no studies on the immediate changes in gametophytic selection caused by environmental variation.

In this study we examined the effects of environmental conditions on pollen competitive ability expressed as pollen-tube growth rate and seed siring success with particular attention to genotype-environment interactions. The

clonal seed orchards and clone collections of an anemophilous tree, *Betula pendula* Roth, situating in three environmentally variable sites in southern Finland provided an opportunity to study pollen competition among the same genotypes in different environmental conditions. In this paper we present the relationship between pollen-tube growth rate and seed siring success among the same maternal and paternal genotypes in different environments and discuss the possible consequences of genotype-environment interactions in pollen competitive ability for gametophytic selection. We focus especially on the following questions: 1) Do the ranking orders of the pollen donors with regard to pollen-tube growth rate and seed siring success change across different environments, and 2) What kind is the relationship between pollen-tube growth rate and seed siring success in different environmental conditions.

Materials and methods

Study species and study sites

Betula pendula is a common, anemophilous, monoecious and self-incompatible tree ranging throughout most of Europe from Norway to Sicilia (Tutin et al. 1964). It has 200-300 male flowers in each catkin (Dahl & Fredrikson 1996) and approximately 600 female flowers in each pistillate inflorescence (personal observation by H-L. Pasonen). Each female flower consists of a single two-locular ovary with two linear, dry stigmas. A mature ovary generally contains two ovules of which only one develops into a mature seed (Sulkinoja & Valanne 1980, Dahl & Fredrikson 1996).

The study was carried out in a plastic house seed orchard (= in a greenhouse) at Haapastensyrjä Forest Tree Breeding Centre of Finnish Forest Research Institute in Läyliäinen (lat. 60°30′ N, long. 24° E), and at two outdoor clone collections, in Röykkä (lat. 60°30′ N, long. 24°39′ E), and in Kangasniemi (lat. 61°59′ N, long. 26°38′ E). The outdoor clone collections in Röykkä and Kangasniemi will be named as clone collections I and II, respectively. The seed orchard consists of 36 *B. pendula* clones originating from southern Finland. The clones were originally selected for the seed orchard on the basis of the results from the field trials (Raulo & Koski 1977). These field trials aimed to select genotypes with superior heritable growth characters (e.g. straight stem). Plastic house provides favourable conditions for flowering and seed development and isolates the seed orchard clones from outside pollen sources. The paternal clones used in the present study originated from an area between latitudes 60°30′ and 62°10′ and maternal clones between latitudes 60°15′ and 63°15′.

Hand-pollination experiments

Pollen for the hand-pollination experiments was collected in 1995 and 1997 from several paternal clones growing in the greenhouse and subsequently stored at –20°C. Germination percentages of the pollen samples varied between 31 – 51 %. No difference in the germination percentages with respect to year of pollen collection was observed. We have found that if *B. pendula* pollen is

properly collected (in dry conditions) and dried in room temperature for 24 hours before storage at –20°C, germination ability of the pollen samples remains fairly unchanged for several years. Pollen from the same paternal clones (and the same pollen samples) collected in the greenhouse was used in all hand-pollinations in the greenhouse and at two outdoor clone collections.

Single-donor hand-pollinations were conducted at three different sites to study the effects of environmental conditions during pollen tube growth on pollen-tube growth rate. In the greenhouse, eleven maternal plants (different *B. pendula* clones) were pollinated with pollen from eight paternal clones. At the outdoor clone collection I (in Röykkä), seven maternal plants were pollinated with pollen from eight paternal clones, and at the outdoor clone collection II (in Kangasniemi), six maternal plants were pollinated with pollen from seven paternal clones. The maternal clones used at the outdoor clone collections were the same as in the greenhouse but only two of them were common for all the three pollination sites (Table 1a). It would have been ideal to have all the genotypes at three different sites but the two outdoor clone collections had only very few common clones of which only two had enough female inflorescences for the hand-pollination experiment. For this reason, pollen-tube growth rate of the same pollen donors can only be compared between two pollination sites at the time; greenhouse vs. clone collection I and greenhouse vs. clone collection II.

TABLE 1a Single-donor pollinations in the greenhouse (G) and at two outdoor clone collections (clone collection I = ●, clone collection II = ■).

Pollen donors	Maternal plants										
	V 5239	V 5279	V 509	V 505	V 5010	V 5818	V 5938	V 5817	V 532	V 5828	V 54
E 1970	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 9434	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 9435	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 9576	G●	G	G●	G●	G●	G●	G■	G	G●	G	G●
E 9615	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 9563	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 9670	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 9568	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■

Before any pollen was shed and before female inflorescences were receptive, branches with three or ten female inflorescences were isolated with paper bags from each maternal plant to prevent uncontrolled pollinations. The isolated female inflorescences were pollinated with pollen from each pollen donor. The three female inflorescences were collected 12 hours after pollination in order to measure the pollen tube lengths of each cross. The ten pollinated female inflorescences were let to develop into seeds in order to analyze the seed

germination percentages. Self-pollinations were prevented by removing all male inflorescences from the bagged branches. When female inflorescences became receptive, an equal volume of pollen was applied to each pollination bag by using a pollination syringe. The amount of pollen applied to the pollination bags exceeded the number of ovules in the bags.

Two-donor hand-pollinations were conducted in the greenhouse and at the outdoor clone collections to examine the G-E-interactions in seed siring success and the relationship between pollen-tube growth rate and seed siring success at three different pollination sites. The same maternal and paternal clones were used in the two-donor hand-pollinations as in the single-donor hand-pollinations (Tables 1a and b). All the two-donor hand-pollinations were performed at the same time than single-donor hand-pollinations.

TABLE 1b Two-donor pollinations in the greenhouse (G) and at two outdoor clone collections (clone collection I = ●, clone collection II = ■).

Pollen mixture (1:1)	Maternal plants V 5239	V 5279	V 509	V 505	V 5010	V 5818	V 5938	V 5817	V 532	V 5828	V 54
E 1970+ E 9434	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 1970+ E 9435	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 1970+ E 9576	G●	G	G●	G●	G●	G●	G■	G	G●	G	G●
E 1970+ E 9615	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 1970+ E 9563	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 1970+ E 9670	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■
E 1970+ E 9568	G●	G■	G●■	G●	G●	G●	G■	G■	G●	G■	G●■

Pollen from seven paternal clones was mixed with pollen from a standard donor (clone E 1970) by measuring out an equal volume of two pollen into a small glass bottle. Clone E 1970 was chosen as a standard donor (= pollen donor 1) because it exhibited a rare isozyme genotype (11 in Pgi-2) and could thus be unambigiously distinguished from the common genotypes that all the other donors (pollen donor 2) and the maternal plants exhibited. Prior to the onset of flowering, seven branches (one for each pollen mixture) with ten female inflorescences were isolated with paper bags from each maternal plant to prevent uncontrolled pollinations. Once receptive, each maternal plant was hand-pollinated by seven different pollen mixtures. At the outdoor clone collection II, only six pollen mixtures were used because we did not have enough pollen from one of the paternal clones. All the hand pollinations were performed in the greenhouse on the 28th – 30th of April, at the outdoor clone collection I on the 7th - 8th of May, and at the outdoor clone collection II on the

13^{th}-14^{th} of May, 1998. Temperature during pollen tube growth varied substantially between these three sites (Table 2).

TABLE 2 Minimum, maximum and mean temperatures (°C) during 12 hours of pollen tube growth at three pollination sites.

Date	Pollination site								
	Greenhouse (60°30'N, 24°E)			Clone collection I (60°15'N, 24°30'E)			Clone collection II (61°59'N, 26°38'E)		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
28.4.	19.0	36.0	27.0						
29.4.	13.0	31.0	23.4						
30.4.	14.0	27.0	21.2						
7.5.				3.5	15.5	8.9			
8.5.				3.5	16.0	8.6			
13.5.							10.0	18.0	13.5
14.5.							5.1	11.0	9.1

Pollen-tube growth rate measurements

In this study, pollen-tube growth rate is expressed by pollen tube length after twelve hours of germination. Samples of female inflorescences pollinated by pollen from a single pollen donor were collected at each of the three pollination sites twelve hours after pollination. In the sampled inflorescences, the fastest pollen tubes had almost reached the base of the elongate stigma, but not entered the funiculus. Three inflorescences per cross were detached and immediately stored in glacial acetic acid and 60% ethanol (1:9). The inflorescences were stored in a refrigerator at 4 °C until they were examined. The flowers were scraped off with a scalpel and stained with a solution of 0.1 % aniline blue in aqueous K_3PO_4 (0.3 mol). As a result pollen tube callose became fluorescent and distinguishable in the darker stylar tissue when examined by UV fluorecence microscopy. Approximately 50 pollen tubes (from three inflorescences and from several flowers) per cross were measured.

Analysing the seed siring success

The seed siring success of the studied clones in two-donor pollinations was expressed as a proportion of seeds sired by pollen donor 2 when compared to the number of seeds sired by the standard pollen donor. Seeds from the pollinated inflorescences (from single- and two-donor crosses) were collected in July, 1998, in the greenhouse and at the outdoor clone collections and subsequently stored at 4 °C. The two-donor crosses at the outdoor clone collection II yielded no seeds and paternity could only be analyzed from the seeds collected in the greenhouse and at the clone collection I. The seeds from the two-donor pollinations were germinated for the isozyme analyses under a plant lamp with a photoperiod of 15 h day and 9 h night on petri-dishes covered with sand and moist filter paper. The samples were collected when the cotyledons had fully opened and the whole plantlet was immediately ground in 50 µl of 0.12 M Tris-HCl extraction buffer, pH 7.5 (slightly modified from

Bousquet et al. 1987), and fine granular quartz, imbibed into wicks and stored for 1-2 weeks in -20° C prior to electrophoresis. The samples were assayed for phosphoglucoisomerase (Pgi-2) by standard starch gel electrophoresis (10% Sigma Hydrolyzed Starch) using a Tris-citrate buffersystem (modified from Shaw & Prasad (1970)) (for details, see Pasonen et al. 1999).

Analysing the seed germinability

Germination percentages were determined from the seeds from the single-donor crosses to examine the relationship between seed germinability and seed siring success of the pollen donors. Due to the fact that paternity could only be analyzed from the cotyledons of the germinated seeds, variation in seed germination percentages between the two pollen donors could have resulted in respective variation in seed siring success of the pollen donors. To determine the seed germination percentages, moist pieces of paper were placed on petri-dishes and a random sample of 100 seeds per cross were counted on each piece of paper. The petri-dishes were placed in a germination chamber on a 12 hr light/12 hr dark cycle in room temperature (=23 °C). The number of germinated seeds was counted after 14 days of germination.

Data analysis

A mixed-effects ANOVA was performed to study the effect of parentage (random effects) and pollination site (fixed effect) on pollen-tube growth rate and the relative seed siring success of the pollen donors. In addition, one-way ANOVAs were performed to test the effect of pollination site on pollen-tube growth rate. Pollen tube lengths were square-root transformed and the proportion of seeds sired by pollen donor 2 was arcsine square-root transformed to normalize the data. Coefficients of concordance (W) were calculated for pollen tube lengths and seed siring success to study whether the rankings of the pollen donors changed across maternal plants (Sokal & Rohlf 1981, p. 609).

To study the relationship between pollen-tube growth rate and seed siring success, Spearman and Pearson correlation coefficients were separately calculated for each maternal parent in the greenhouse and at the outdoor clone collection I (two-donor crosses at the outdoor clone collection II yielded no seeds). To summarize the Pearson correlation coefficients, a Schmidt-Hunter meta-analysis method with Fishers z-transformation was used to obtain a weighted mean correlation coefficient between pollen tube length and seed siring success (Hunter et al. 1982, Hedges & Olkin 1985). The relationship between pollen germination percentage and seed siring success as well as between germination percentage of the seeds sired by pollen donor 2 and seed siring success of pollen donor 2 were studied by Pearson correlation coefficients calculated separately for each maternal plant. The Pearson correlations were summarized by using a Schmidt-Hunter meta-analysis method.

Results

Genotype-environment interactions in pollen-tube growth rate

Pollination site had significant effect on pollen-tube growth rate in the mixed-effects ANOVA (Tables 3a and b). Significant interaction between paternal parent and pollination site was found (Tables 3a and b) indicating that genotype-environment interactions exist in pollen-tube growth rate among the studied clones between two pollination sites (Figs. 1a and b). Paternal parent had no main effect on pollen-tube growth rate (Tables 3a and b) and maternal parent had significant main effect on pollen-tube growth rate only when pollen tube lengths in the greenhouse were compared to pollen tube lengths at the outdoor clone collection II (Table 3b). One-way ANOVAs revealed that pollen-tube growth rates at both outdoor clone collections differed significantly from pollen-tube growth rates in the greenhouse (greenhouse vs. clone collection I: $F = 1607.93$, $P < 0.001$; greenhouse vs. clone collection II: $F = 2031.20$, $P < 0.001$). A significant concordance in pollen-tube growth rates across different maternal plants was found in the greenhouse ($W = 0.55$, $P < 0.001$) but not at the outdoor clone collection II ($W = 0.12$, $P > 0.05$). Coefficient of concordance could not be calculated for clone collection I due to the fact that no germinated pollen tubes were found on some maternal plants. There was a significant negative correlation between pollen tube lengths in the greenhouse and at the outdoor clone collection I (weighted mean correlation = -0.39, $0.001 < P < 0.01$). No correlation between pollen tube lengths in the greenhouse and at the outdoor clone collection II was found (weighted mean correlation = -0.19, $P > 0.05$).

TABLE 3a Mixed-effects ANOVA of the effects of parentage (random effects) and pollination site (fixed effects; greenhouse and outdoor clone collection I) on pollen-tube growth rate.

Source	df	MS	F	P
Maternal parent	6	0.079	1.46	n.s.
Paternal parent	7	0.100	1.85	n.s
Pollination site	1	12.18	37.48	< 0.001
Mother x father	42	0.054	4.50	< 0.001
Mother x site	6	0.103	1.81	n.s.
Father x site	7	0.279	4.90	< 0.001
Mother x father x site	28	0.057	4.75	< 0.001
Error	3885	0.012		

Genotype-environment interactions in seed siring success

Mixed-effects ANOVA of the effect of parentage and pollination site on seed siring success of the pollen donors revealed significant main effects and two-way interactions between both parents and pollination site (Table 4). The significant interaction between paternal parent and pollination site is an evidence of genotype-environment interactions in seed siring success (Fig.2).

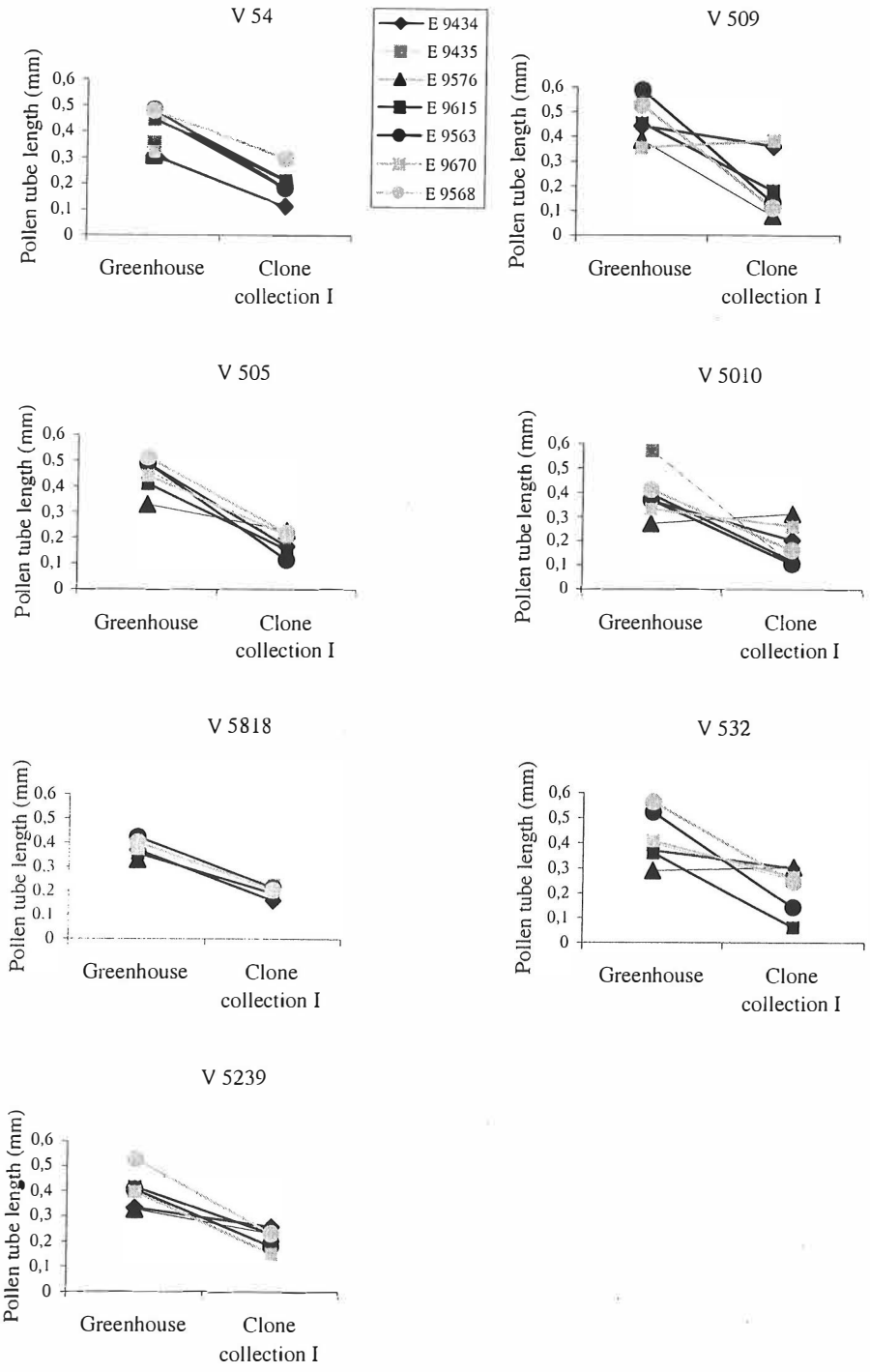

FIGURE 1a. Genotype-environment interactions in pollen-tube growth rate on different maternal plants (greenhouse vs. clone collection I).

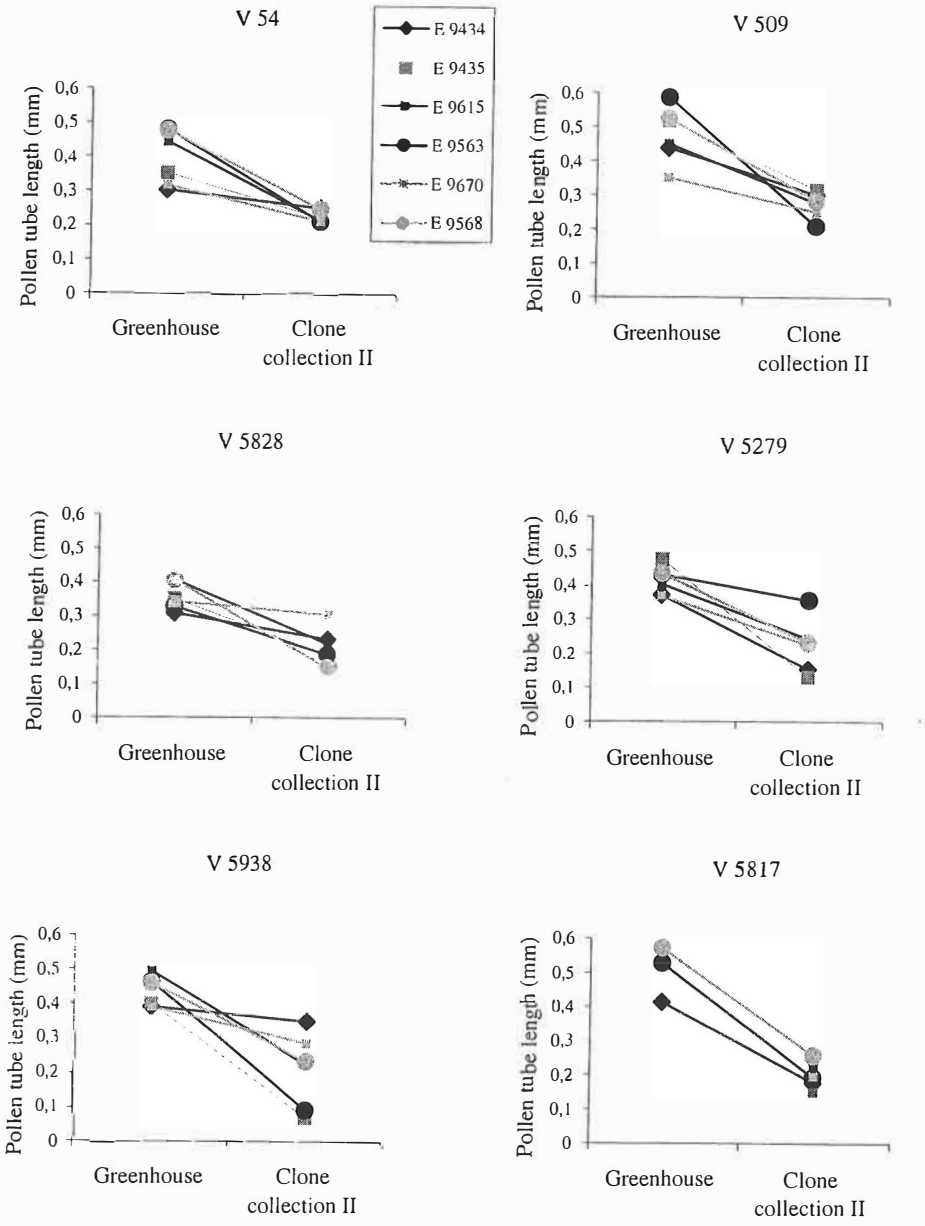

FIGURE 1b. Genotype-environment interactions in pollen-tube growth rate on different maternal plants (greenhouse vs. clone collection II).

TABLE 3b Mixed-effects ANOVA of the effects of parentage (random effects) and pollination site (fixed effects; greenhouse and outdoor clone collection II) on pollen-tube growth rate.

Source	df	MS	F	P
Maternal parent	5	0.393	6.66	< 0.001
Paternal parent	6	0.128	2.17	n.s.
Pollination site	1	15.650	74.52	< 0.001
Mother x father	30	0.059	4.92	< 0.001
Mother x site	5	0.112	1.90	n.s.
Father x site	6	0.157	2.66	< 0.05
Mother x father x site	26	0.059	4.92	< 0.001
Error	3813	0.012		

Although genotype-environment interactions in seed siring success were found, substantial parallelism in the rankings of the pollen donors between the greenhouse and outdoor clone collection I was observed (Fig.2). The parallelism in the rankings of the pollen donors across the two pollination sites is also supported by a significant positive correlation between the greenhouse and clone collection I in seed siring success (weighted mean correlation coefficient = 0.80, $P < 0.001$). This indicates that the same pollen donors, on the average, are superior in siring seeds at both sites. For example, clones E 9615 and E 9563 are performing well on most maternal plants in the greenhouse and outdoors while the proportion of of seeds sired by clones E 9576 and E 9670 is fairly low on most maternal plants at both sites (Fig. 2).

TABLE 4 Mixed-effects ANOVA of the effect of parentage (random) and pollination site (fixed effects; greenhouse and outdoor clone collection I) on seed siring success of the pollen donors. F-value of the three-way interaction (and mother x father interaction) could not be calculated due to the lack of replication.

Source	Df	MS	F	P
Maternal parent	6	0.025	3.68	< 0.01
Paternal parent	5	0.147	21.62	< 0.001
Pollination site	1	0.797	23.93	< 0.001
Mother x father	26	0.007		
Mother x site	6	0.024	6.49	< 0.001
Father x site	5	0.013	3.51	< 0.05

A coefficient of concordance (W) was calculated to study whether the same pollen donors sire most of the seeds across all the maternal plants. A concordance coefficient for the pollen donors in seed siring success was 0.68 ($0.001 < P < 0.01$) in the greenhouse and 0.56 ($0.01 < P < 0.05$) at the outdoor clone collection I indicating that the rank ordering of the pollen donors do not statistically change across maternal plants at either pollination sites. There was a slight positive relationship between germination percentage of the seeds sired by pollen donor 2 and the relative seed siring success of pollen donor 2 in the greenhouse (weighted mean correlation = 0.26, $0.01 < P < 0.05$) but not at the outdoor clone collection I (weighted mean correlation = -0.02, $P > 0.05$). Seed

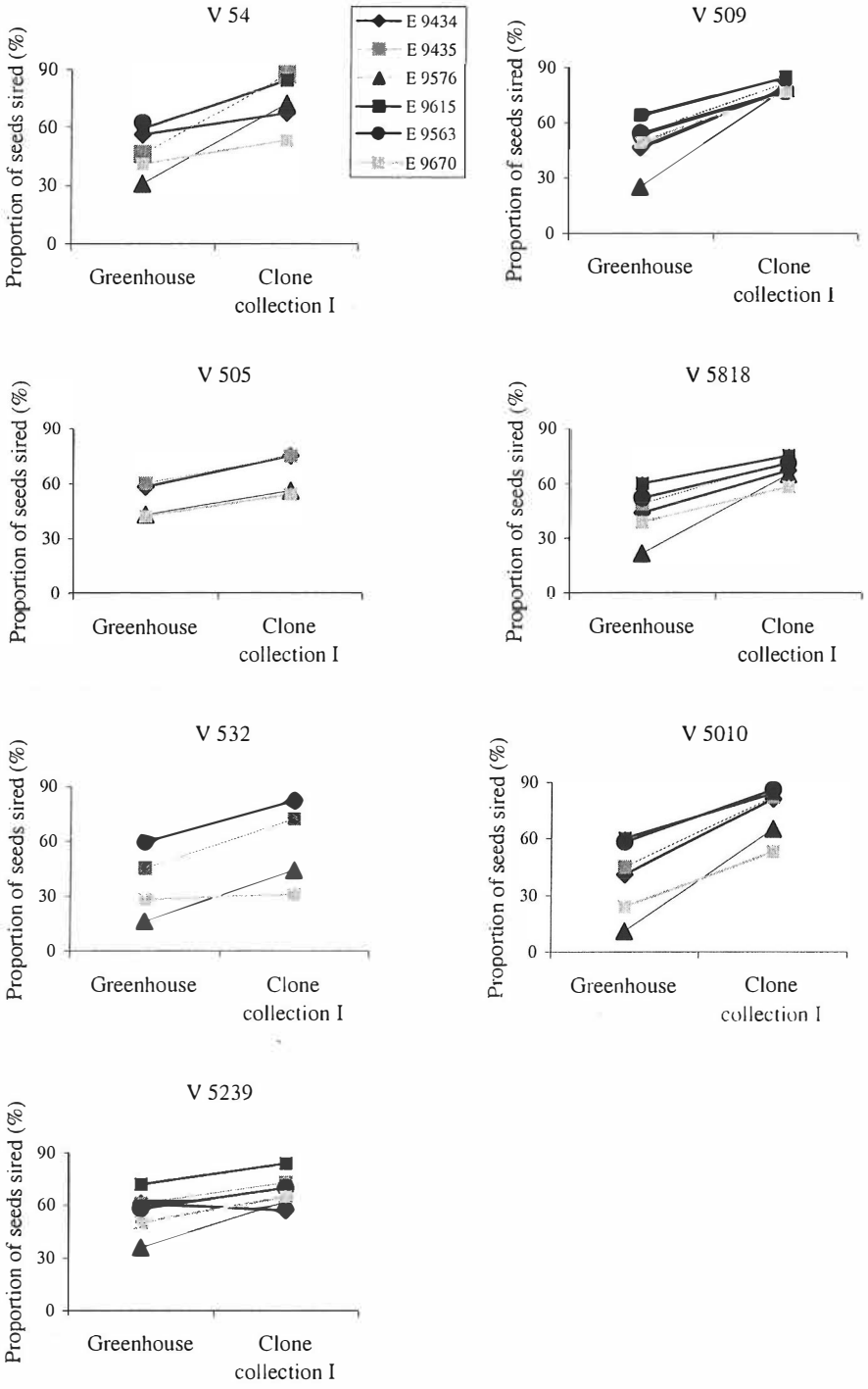

FIGURE 2. Genotype-environment interactions in seed siring success on different maternal plants (greenhouse vs. clone collection I).

germinability does not seem to have much influence on the relative seed siring success.

Relationship between pollen performance and seed siring success

Spearman correlation coefficients between pollen-tube growth rate and the proportion of seeds sired by pollen donor 2 varied between 0.26 and 0.83 in the greenhouse and between –0.87 and 0.40 at the outdoor clone collection I depending on the maternal plant (Table 5). A weighted mean correlation coefficient between pollen-tube growth rate and seed siring success was 0.64 ($P < 0.001$) in the greenhouse and –0.54 ($0.001 < P < 0.01$) at the clone collection I (Table 5). There seems to be a positive relationship between pollen-tube growth rate and seed siring success in the controlled greenhouse environment and a negative relationship in more heterogeneous outdoor conditions. A positive relationship between pollen germination percentage (in vitro) and seed siring success of pollen donor 2 was found in the greenhouse (weighted mean correlation = 0.36, $0.001 < P < 0.01$) but not at clone collection I (weighted mean correlation = 0.15, $P > 0.05$).

Discussion

In this paper, we report genotype-environment interactions in pollen-tube growth rate and seed siring success and discuss the possible consequences for pollen competition and gametophytic selection. Significant interactions between paternal parent and pollination site were found in both pollen-tube growth rate and seed siring success indicating that genotype-environment interactions

TABLE 5 Spearman correlation coefficients between pollen tube length and seed siring success in the greenhouse and at the outdoor clone collection I. Weighted mean correlations are based on the Pearson correlations between pollen tube length and seed siring success calculated separately for each maternal plant.

Recipient	Greenhouse			Outdoor clone collection I		
	Spearman r	p	n	Spearman r	p	n
V 5239	0.75	0.084	6	-0.60	0.285	5
V 509	0.64	0.173	6	-0.21	0.734	5
V 505	0.60	0.40	4	-0.74	0.262	4
V 5010	0.83	0.042	6	-0.87	0.019	6
V 5818	0.26	0.623	6	0.40	0.600	4
V 5938	0.56	0.322	5			
V 532	0.80	0.200	4	-0.80	0.200	4
V 54	0.66	0.156	6			
Weighted mean r	0.64	< 0.001	8	-0.54	< 0.01	6

occur in pollen competitive ability. To our knowledge, this is the first time when genotype-environment interactions in seed siring success were examined. There are only very few studies that report any interactions between genotype and environment in pollen-tube growth rate and none that report genotype-environment interactions in seed siring success. The interaction between pollen donor and environmental conditions during pollen tube growth has explicitly been studied only by Travers (1999) and Pasonen et al. (2000) but implicit information especially about genotype-temperature interactions in pollen germination ability and pollen-tube growth rate can also be obtained from other studies (e.g. Polito et al. 1988, Elgersma et al. 1989).

In the present study, environmental conditions during pollen tube growth had significant influence on pollen performance. It is known that environmental factors during pollen development affect pollen performance (see e.g. Delph et al. 1997). In natural habitats soil fertility varies from one microsite to another, which can lead to differential abilities of plants to provide developing pollen grains with resources (e.g. Lau & Stephenson 1993, Lau & Stephenson 1994). Storage products provided by the pollen producing parent are metabolized during pollen tube growth (e.g. Jackson & Linskens 1982) and are thought to play important roles during pollen germination (Mulcahy & Mulcahy 1982). Herbivory (Quesada et al. 1995, Mutikainen & Delph 1996) and temperature (Johannsson & Stephenson 1998) are also known to affect pollen-tube growth rates and the seed siring ability of pollen. In our study, pollen donor environment should not have caused variation in pollen performance because all the pollen donors were grown under similar greenhouse conditions. Thus, variation in pollen-tube growth rates of the pollen donors between different pollination sites is purely due environmental effects during pollen tube growth.

The result that there is a significant consistency in the rankings of the pollen donors in pollen tube length across different maternal plants in the greenhouse is in concordance with our previous results from the studies carried out in similar greenhouse conditions (Pasonen et al. 1999). No concordance in the rankings of the pollen donors in pollen tube length was found outdoors. This is most likely due to more heterogeneous outdoor conditions. In the greenhouse, all the maternal plants have been grown under similar temperature, water and nutrient conditions. At the outdoor clone collections, microclimatic differences and variation among microsites can be much more substantial than in the greenhouse. Because maternal plants are likely to provide pollen tubes with nutrients during pollen germination (Labarca & Loewus 1973, Sanders & Lord 1989, Herrero & Hormaza 1996, Wu et al. 1995), the ability of maternal plants to support pollen tube growth can vary from one microsite to another depending, for example, on the nutrient content of the land. There was also a substantial difference in the daily mean temperature between the two days when hand-pollination experiments were carried out at the outdoor clone collection II which can partly explain the lack of concordance in the rankings of the pollen donors.

An interesting finding was the significant concordance in the rankings of the pollen donors in seed siring success across different maternal plants in the greenhouse and at the clone collection I. This indicates that the same pollen

donors, on the average, are the most successful in siring seeds independent of the maternal parent. Consistent rank ordering of the pollen donors in seed siring success after mixed-pollinations across different maternal plants has previously been reported at least in *Hibiscus moscheutos* (Snow & Spira 1996), *Lesquerella fendleri* (Mitchell & Marshall 1998) and *Raphanus sativus* (Marshall 1998). In our study, a significant positive correlation in the seed siring success of the pollen donors was also detected between the greenhouse and outdoor clone collection I. Despite of the existence of genotype-environment interactions, there seem to be some consistency in the rankings of the pollen donors in their ability to sire seeds across two different pollination environments. The consistency of the rankings of the pollen donors in seed siring success across different maternal plants and pollination environments could be due to some form of female choice. Female choice can act during pollen tube growth allowing only some pollen tubes to achieve fertilization (e.g. Cruzan 1993, Herrero & Hormaza 1996), or during embryo and seed development when seeds sired by certain pollen donors are aborted more frequently than seeds sired by other donors (e.g. Marshall & Ellstrand 1988).

A significant positive correlation between pollen-tube growth rate and seed siring success in the greenhouse is in concordance with our previous results from the study carried out in similar greenhouse conditions (Pasonen et al. 1999). One of the main interests of the present study was to investigate whether the relationship between pollen-tube growth rate and seed siring success was also positive in more natural pollination conditions. Interestingly, the relationship between pollen-tube growth rate and seed siring success was not parallel at the two pollination sites used in this study. At clone collection, which represents more heterogeneous outdoor environment and conditions in natural *B. pendula* stands, the correlation between pollen-tube growth rate and seed siring success was significantly negative. It is difficult to find any probable biological explanation for this negative relationship. According to these results, pollen-tube growth rate can be a predictor of seed siring success in controlled greenhouse conditions where differences among maternal plants are mainly of genetic origin but not in more heterogeneous outdoor conditions. It seems also that environmentally induced changes in the rankings of the pollen donors in pollen-tube growth rate do not translate into parallel changes in seed siring success. That is why pollen competition might not be of crucial importance in sexual selection in natural populations of *B. pendula.* From this perspective, environmental variability and genotype-environment interactions are likely to explain a large part of the variation observed in pollen-tube growth rates. To obtain a more accurate insight into the consequences of pollen competition for sexual selection in natural stands of *B. pendula,* further studies are needed.

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Pollen-pollen interactions in *Betula pendula in vitro*

by

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Pollen–pollen interactions in *Betula pendula in vitro*

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SUMMARY

Direct pollen interactions, as well as interactions mediated by a recipient, can have a remarkable influence on pollen fertilization ability. Under conditions of pollen competition it could be advantageous if pollen grains interfered with the germination of other pollen. The aim of this study was to find out if there are direct negative or positive pollen–pollen interactions between pollen grains from genetically slightly different donors. The *in vitro* germinability of the pollen from several *Betula pendula* Roth clones was investigated. The pollen interactions between the clones were examined pairwise by using equal pollen mixtures. In three of the eight cases the germination percentage of the pollen mixture was significantly higher than the average germination percentage of the separate clones that formed the mixture, which indicates some type of interaction between the pollen populations. We found only positive interactions between the pollen of clones. This study also documented density-dependent germination of pollen grains *in vitro* (= pollen population effect). Adding an aqueous pollen extract to the incubation medium increased the germination percentages of poorly germinating pollen and small pollen populations. Germination-stimulating effects were found to exist both with fresh and dead pollen. Such direct pollen–pollen interactions could be explained by specific water-soluble substances diffusing from pollen grains.

Key words: Pollen interactions, pollen population effect, pollen competition, *Betula pendula* Roth (silver birch).

INTRODUCTION

Under natural pollination conditions there is usually an excess of pollen on the stigmas and mixed-donor pollen loads are common (Marshall & Ellstrand, 1985). Donors can differ in pollen performance, especially in pollen tube growth rate (Snow & Spira, 1991; Björkman, Samimy & Pearson, 1995), which leads to competition for available ovules (Mulcahy, 1979). The fertilization success of the pollen of a particular donor might vary depending on whether pollen from other plants is present on the stigma. In order to study pollen competition, it is important to know how pollen grains from different donors interact in germination and which factors are involved. Factors known to affect pollen tube growth rates include paternal and maternal genotype (Sari Gorla, Ottaviano & Faini, 1975), temperature (Jefferies & Brain, 1984), and the density of pollen grains on the stigma and in the style (Cruzan, 1986).

As well as interactions mediated by a recipient, direct pollen interactions can have a remarkable influence on pollen fertilization ability. Pollen interactions mediated by a recipient have been reported in literature by several authors (e.g. Visser, 1983; Bowman, 1987; Bertin & Sullivan, 1988). Evidence for direct pollen interactions is slight. Density-dependent germination of pollen grains *in vitro* could be considered to be an example of direct pollen interactions, because the germination stimulating effect comes purely from the pollen grains. The density-dependent germination of pollen grains *in vitro* was first discovered as early as 1924 by Brink. Since then, the phenomenon has been observed in many species (e.g. Brewbaker & Majumder (1961); Holm (1994)) and has been termed 'pollen population effect' by Brewbaker & Majumder (1961) who suggested that it results from the presence of a diffusible, water-soluble pollen growth factor in pollen grains and in other plant tissues. This factor was later proved to be the calcium ion (Brewbaker & Kwack, 1963). Density-dependent growth of pollen tubes has also been observed *in vivo* (Cruzan, 1986). Enhanced germination of large pollen populations *in vivo* might result from accelerated breakdown of intercellular substances by groups of pollen tubes, which makes the stylar environment more favourable for germinating pollen grains (Cruzan, 1986).

Inhibition of pollen-tube growth at high pollen densities has been observed by Thomson (1989)

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and by d'Eeckenbrugge (1990) who found that germination and pollen tube growth rates in *Cichorium intybus* were negatively affected by the number of pollen tubes growing in the style. He concluded that, when pollen densities are high, increasing inhibition of pollen tube growth might result from increasing competition between tubes. He suggested that some inhibitor substance diffuses from pollen tubes to surrounding cells of the stigma receptive epidermis and transmitting tissue. It has also been observed that the inhibition of pollen tube growth at high pollen densities might result from the physical blocking by other tubes (Cruzan, 1986).

If pollen grains compete for ovules, an ability to inhibit the germination of other pollen might be of selective advantage. After applying adjacent pollinations on the same stigma, Marshall *et al.* (1996) showed that mixed pollen loads germinated more slowly than single-donor pollen loads. Because interference occurred only when the two kinds of pollen were in direct contact, a pollen–pollen interaction was indicated. On the whole, negative interactions have rarely been found and they have usually been between different species (Galen & Gregory, 1989; Murphy & Aarssen, 1995). Furthermore, in many studies the role of direct pollen interactions *in vivo* has been difficult to separate from the interactions mediated by the style (e.g. Kearney, 1932; Marshall & Ellstrand, 1986; Radha *et al.* 1993). To search for direct pollen interactions, clearly it would be useful to employ *in vitro* experiments.

The present study attempted to test: (1) whether the *in vitro* germination of pollen mixtures consisting of pollen from two *Betula pendula* clones differs from the average germination of the two single clones; (2) whether it is possible to improve the germinability of non-germinating pollen by mixing it with well germinating pollen and (3) how strong is the pollen population effect among *Betula pendula* pollen and whether it is possible to overcome it in a germination test with pollen extracts made from fresh and dead pollen. The difference between germination percentage of a pollen mixture and the average germination percentage of pollen from two separate clones indicates some type of interaction between the pollen donors.

MATERIALS AND METHODS

Betula pendula Roth, a deciduous, anemophilous tree with male and female flowers occurring in separate inflorescences, produces abundant small binucleate pollen grains that might be transported by wind over long distances (e.g. Hjelmroos (1991)). The pollen used in this study was collected at the end of April 1995 from a plastic house seed orchard at the experimental station of the Foundation for Forest Tree Breeding in Pieksämäki (latitude 62° N, longitude 27° E), Finland. This collection has 35 *Betula pendula* clones chosen for their superior vegetative growth characteristics. In our study we used several genetically different clones all originating from Central Finland. Immediately before the onset of flowering, two or three branches with male flowers were isolated from every clone with white paper bags. After flowering, bags were detached and pollen from each bag was separately vacuumed into injection bottles. Pollen was refrigerated at +4 °C for 2–7 wk before the germination tests.

In the first pollen mixture experiment, pollen from nine clones was used to prepare eight pollen mixtures (1:1). In the second experiment, dead pollen was mixed (1:1) with fresh pollen from clone V 505, which, in a previous experiment, was found to have a high percentage germination. The germinabilities of pollen mixtures and of individual clones were separately determined. Using a Pasteur pipette a thin layer of melted incubation medium (containing 0·5 M sucrose, 0·01% boric acid and 1% agar) was placed on the middle of a glass microscope slide. The addition of $Ca(NO_3)_2$ to the incubation medium was tested, but lowered germinability of the pollen was found in such experiments. Pollen was placed on cellulose-ester membrane filters (Gelman Science Metrical GN-4) using a fine brush, after which the filters were placed on the incubation medium. Filters were used because, in preliminary experiments, pollen was found to germinate better on a filter than directly on agar. The microscope slides covered by the agar and the filters were placed in moist 12-cm Petri dishes and incubated for 24 h at room temperature (22 °C) (Käpylä, 1991). After incubation, pollen grains were stained with lactophenol cotton blue (Grimstone & Skaer, 1972, p. 47). Using a light microscope, at least 500 pollen grains were counted on each filter. Pollen grains with pollen tubes longer than the diameter of a pollen grain were considered to have germinated. The number of pollen grains on the filters could not be controlled precisely, but the amount of pollen used in every germination test was kept as equal as possible. Each test was carried out three times. The germination percentages in pollen mixtures were compared with those of the separate clones germinating in isolation. The germination tests were carried out during May and June, 1995.

To study the pollen population effect, pollen from two of the clones and genetically unspecified pollen collected from trees outdoors was used. Different amounts of pollen were incubated on agar media. In order to overcome the pollen population effect, an aqueous extract from both fresh and dead pollen was prepared and added to the incubation medium. 0·3 g of the pollen was mixed with 12 ml of distilled water. The pollen–water mixture was vigorously shaken with a Vortex-mixer and then filtered twice (Machery–Nagel filter no. 616). The filtered pollen

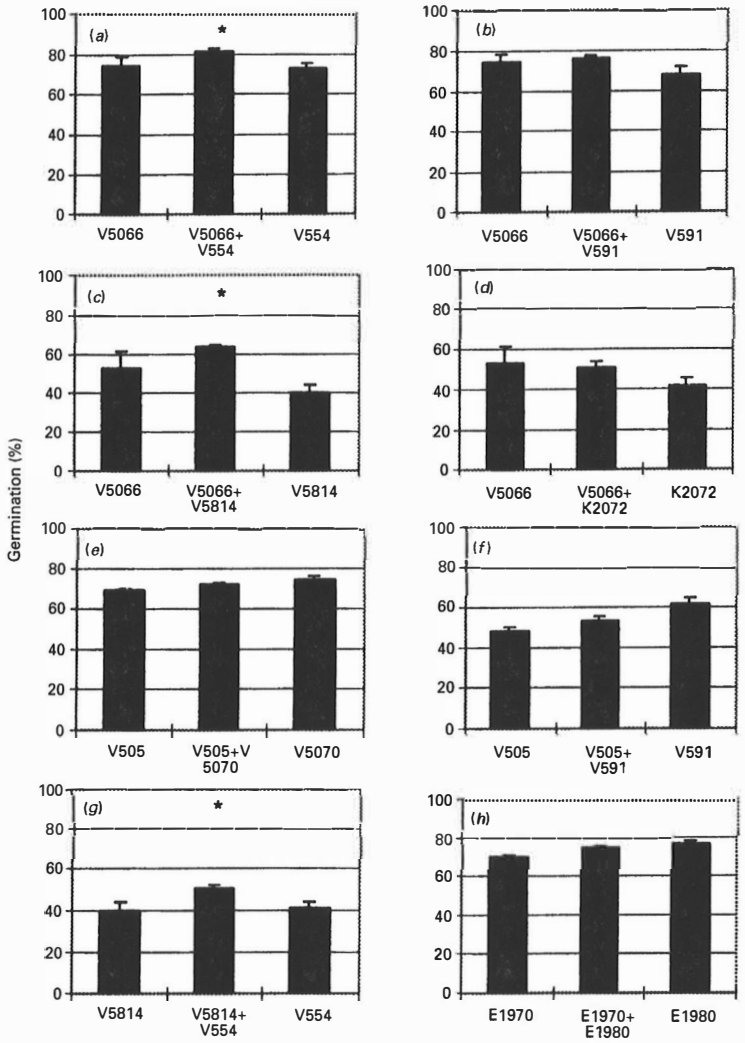

Figure 1. The germination percentages of eight pollen mixtures and separate clones used in the mixtures. Vertical bars: SE ($n = 3$). Significance levels as given in Table 1.

extract was immediately added to the incubation medium, which was subsequently autoclaved. Germination tests on the incubation medium containing pollen extract were carried out as described above.

Germination percentages of the pollen mixtures were compared to the expected germination percentages by one-way ANOVA using two separate clones and the pollen mixture as contrasts 1, 2 and 3 respectively (see Montgomery (1997), p. 97). H_0-hypothesis was: $\mu_3 = (\mu_1 + \mu_2)/2$. The effect of pollen density on the pollen germination ability was assessed by regression of the percent germinable pollen on the number of pollen grains per mm^2. Differences between regression coefficients in different treatments were examined statistically. All the statistical methods were carried out using the SPSS package for Windows® 6.1.

RESULTS

Interactions among different pollen donors

If there was no interaction, the germination percentage of pollen of two clones tested in mixture

Table 1. *The expected and observed germination percentages of eight pollen mixtures and one-way ANOVA with contrasts*

	Germination %			Statistics		
Pollen mixture	Expected	*n*	Observed	*n*	d.f.	*F*
V5066 + V554	73·94	8552	81·25	6847	1	5·73*
V5066 + V5814	46·42	1387	63·51	1910	1	10.37*
V5066 + V591	71·15	9097	75·78	5707	1	1·45 n.s.
K2072 + V5066	47·15	1582	51·03	1509	1	0·006 n.s.
V505 + V5070	71·58	4378	72·33	7144	1	0.002 n.s.
V505 + V591	54·94	2210	53·22	2157	1	0.005 n.s.
V5814 + V554	40·70	1613	50·74	2152	1	10·63*
E1970 + E1980	72·84	4395	74·05	5438	1	0.35 n.s.

* $P < 0.05$.
n = total number of pollen grains counted.

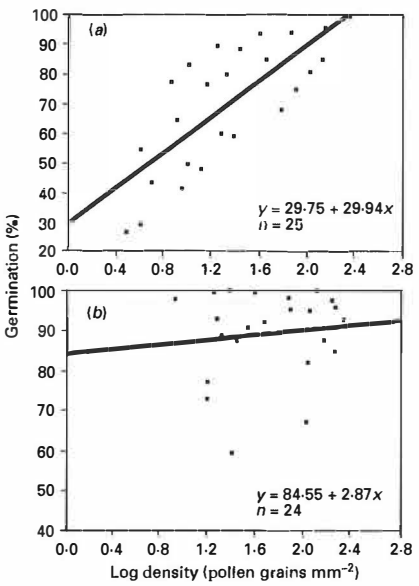

Figure 2. Relation between pollen densities and pollen germination percentages without (*a*) and with (*b*) the pollen extract for genetically unspecified pollen. The pollen extract was made from fresh pollen.

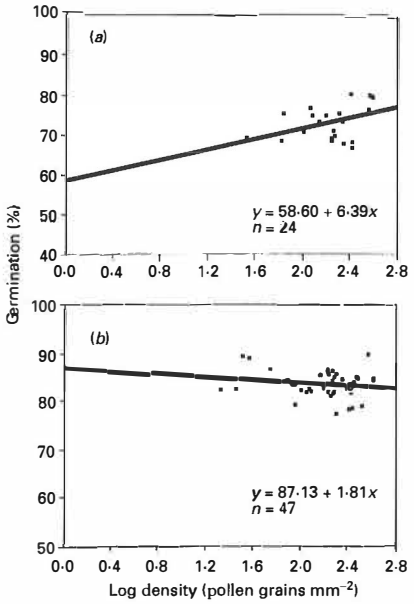

Figure 3. Relation between pollen densities and pollen germination percentages without (*a*) and with (*b*) the pollen extract for the pollen from clone E 1980. The pollen extract was made from fresh pollen.

would be expected to be equal to the mean germination percentage of the same two clones germinating in isolation. The germination percentage would be less if there was a negative interaction, and greater if there was a positive interaction. In three of the eight mixtures the germination percentages were significantly higher than the average germination percentage of the two separate clones. In the other five mixtures the germination percentages did not differ significantly from the mean germination of the two clones, and in no case was a negative interaction observed (Fig. 1, Table 1). There were some differences between the germinabilities of subsamples from the same clones in different experiments. This is most likely due to the fact that some of the experiments (panels Fig. 1 *a*, *b*, *e*, *h*) were carried out at the beginning of May 1995 (storage time 2 wk), while others (Fig. 1 *c*, *d*, *f*, *g*) were set up one month later (storage time 7 wk). The germinability of pollen decreased rapidly in a refrigerator. For instance, in another study, we observed, on average, 50% decrease in pollen germination percentages after 7 wk of storage in a

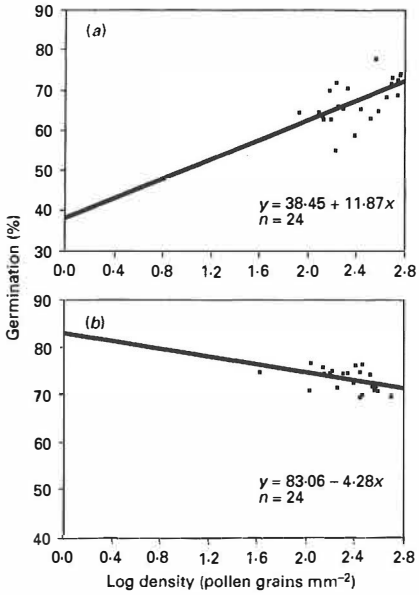

Figure 4. Relation between pollen densities and pollen germination percentages without (*a*) and with (*b*) the pollen extract for the pollen from clone V 505. The pollen extract was made from dead pollen.

refrigerator. Thus, in every experiment, the germination of pollen mixtures was tested simultaneously with corresponding controls (single clones germinating in isolation) using pollen all stored for the same length of time.

In the second pollen mixture experiment, non-germinating pollen (several years old with a germination percentage = 0%) was mixed (1:1) with the pollen from clone V 505. The expected germination percentage of the pollen mixture was the average of the germination percentages of two separate pollen samples. The observed germination percentage of the mixture (55·26%) was significantly higher than the expected germination percentage (34·44%) ($F = 411{\cdot}68$; d.f. = 1; $P < 0{\cdot}001$).

The pollen population effect

Pollen germination was found to be density dependent on agar medium, being strongest for the pollen collected outdoors (Fig. 2*a*) and slightly weaker for pollen from two seed orchard clones (Figs 3*a*, 4*a*). The latter two experiments did not include very low pollen densities, which diminishes the accuracy of the regression coefficient. For each of the three pollen samples studied, pollen germinability increased linearly with increasing pollen densities on the germination medium, but neither levelling nor negative effects were found at these densities.

Effect of pollen extracts

The addition of a pollen extract to the germination medium increased germination percentages in small pollen populations. Germination was not density-dependent when pollen extract was included in the germination medium (Figs 2*b*, 3*b*, 4*b*). In two of three cases, the pollen extract was made from fresh, viable pollen (Figs 2*b*, 3*b*) and in one of three cases from dead pollen (Fig. 4*b*).

The regression lines for germinations made with the pollen extract vs. germinations made without the pollen extract were significantly different in the three experiments. The difference between the slopes of the regression lines was statistically significant for the pollen collected outdoors ($t = -3{\cdot}45$, $P < 0{\cdot}01$) and for clones E 1980 ($t = -2{\cdot}86$, $P < 0{\cdot}01$) and V 505 ($t = -4{\cdot}24$, $P < 0{\cdot}001$). The intercepts of the regression lines were also significantly different for the pollen collected outdoors ($t = 4{\cdot}27$, $P < 0{\cdot}001$) and for clones E 1980 ($t = 4{\cdot}42$, $P < 0{\cdot}001$) and V 505 ($t = 4{\cdot}97$, $P < 0{\cdot}001$).

DISCUSSION

Pollen interactions

There are some reports in the literature of an alteration of the overall fertilization success of pollen when compatible pollen grains from two sources are mixed, but there is virtually no direct evidence for pollen interactions. Kearney (1932) found that the number of seeds fertilized by mixtures of pollen from two strains of cotton was apparently not simply a function of the pollen tube growth rate of each type of pollen, but involved more complicated interactions between the pollen populations. The results could be interpreted as being due to direct pollen interactions or interactions mediated by the style (see Cruzan (1990)). The germination patterns found in *Erythronium grandiflorum* were also not simply a function of different growth rates of pollen tubes, but involved some type of pollen interactions (Cruzan, 1990). Furthermore, it has been shown in mixed-donor pollinations that the success of pollen in siring seeds depends not only on the intrinsic genetic potentiality of the pollen, but also on the genetic composition of the competing pollen grains on the stigma (Bertin, 1986; Marshall & Ellstrand, 1986; Radha *et al.*, 1993; Marshall *et al.*, 1996), which might indicate interference between pollen from different donors.

Our results from *in vitro* experiments demonstrated that direct interactions between pollen of some *Betula pendula* clones exist. Evidence for direct pollen interactions was sought in experiments on *in vitro* pollen tube growth by Schemske & Fenster (1983), but was not found. In our study interactions

were found only between certain clones, which might indicate that not every combination is favourable. Negative interactions between the clones were not found. When dead pollen was mixed with well germinating pollen of clone V 505 (germination percentage = 68·88 %), the germination percentage of the mixture (55·26 %) was higher than the average germination percentage of two separate pollen samples germinating in isolation. The result can be explained by postulating that fresh pollen induced germination in seemingly dead pollen, or factors provided by the non-germinating pollen might have enhanced germination in pollen from clone V 505. We consider these possibilities below.

Pollen population effect

Positive interactions or pollen mentor effects *in vivo* have been reported between normally self-incompatible pollen and compatible pollen growing in the same style (Visser, 1983; Bertin & Sullivan, 1988). It has been suggested that the excess pollen serves as mentor pollen, stimulating the performance of the rest (Visser & Marcucci, 1983; Visser, Sniezko & Marcucci, 1988). It is not clear whether the pollen population effect *in vitro* and pollen mentor effects *in vivo* are the same phenomenon. It has been suggested that the pollen mentor effects might be due to the favourable changes in the style when many pollen grains are present (Mulcahy & Mulcahy, 1983; Cruzan, 1986), whereas the pollen population effect *in vitro* must result from factors coming from pollen grains.

As the pollen population effect reported in this study could be overcome by adding aqueous pollen extract to the incubation medium, the presence of germination stimulating substances in pollen grains is indicated. We found that the activity of the pollen extract was not destroyed by autoclaving, which indicates that pollen growth factors are unikely to be proteins or compounds having a functionally important protein component. According to our results such substances are not synthesized only during germination but already exist in the pollen grain before germination. Moreover, they are also present in dead pollen grains that have lost the ability to germinate.

Brewbaker & Kwack (1963) have postulated that the pollen growth factor is the calcium ion, interacting with K, Na and Mg ions, which enhance the binding of calcium to the cell wall. In other studies (e.g. Hepler, 1997) it has been shown that calcium has an important role in guiding pollen tube growth. Given that calcium is essential for pollen tube growth in many species and that pollen grains are low in calcium content (Brewbaker & Kwack, 1963), it seems likely that the pollen population effect is only a passive response to increasing calcium content of the germination medium when many pollen grains are present.

Conclusions

From our results it seems likely that pollen from two donors can affect directly each other's germinabilities in a positive way in *Betula pendula*. However, the biochemical and molecular mechanisms involved are still unknown. If the interactions between pollen grains from different donors could be explained by substances diffusing from pollen grains, the substances might be more specific than simple ions. To determine whether substances leaching from pollen grains have specific effects on the germinabilities of pollen grains from different donors, further studies will have to be made using genetically different pollen donors and pollen extracts from genetically different pollen.

Considering the pollen competition hypothesis, the 'altruistic behaviour' of pollen from some birch clones is surprising, for from an evolutionary point of view, if there were inhibitory interactions between different genotypes during pollen germination, these appear to be more advantageous. Positive interactions between different genotypes will lead to more intensive competition between pollen grains. If there is positive correlation between the intensity of pollen competition and the performance of progeny, as many research results actually indicate (e.g. Stephenson, Winsor & Schlichting, 1988; Richardson & Stephenson, 1992; Janse & Verhaegh, 1993; Quesada, Winsor & Stephenson, 1993; Björkman, 1995), the more intense competition between pollen grains would be an advantage to the 'female' component of reproduction. By contrast, intense competition might not be beneficial to the male element of reproduction, which makes this kind of evolutionary explanation for positive pollen–pollen interactions improbable.

The role of different pollen interactions might be important in plastic house seed orchards, where mass pollinations between different clones are carried out. The pollen pool on the stigmas is composed of pollen from several clones, which might affect each other's fertilization success. Positive pollen–pollen interactions, as well as interactions mediated by a recipient, not only make it possible to make use of poorly germinating pollen in plant breeding, but also make the studying of pollen competition even more complicated. Direct *in vivo* pollen tube growth rate measurements with single donors do not necessarily tell the whole truth about pollen competition. A useful approach for studying pollen competition would be to employ mixed-donor pollen loads and genetic markers to determine *in vivo* whether donors differ in the number of offspring they sire (see e.g. Marshall & Ellstrand (1986) and Snow & Spira (1996)).

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