IN VITRO POLLEN GERMINATION EXPERIMENTS IN *ALNUS* AND THEIR RELEVANCE FOR MATING SYSTEM ANALYSIS

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ABSTRACT

Germination experiments with *Alnus* pollen from a large number of trees were carried out *in vitro* in different years using at least two different germination media in each year. The results show enormous variability in germination percentages. Each of three sources, pollen donor, medium, and physiological state of pollen (in different years), contributed to the variation in germination percentages. Extending over all genotypes and media, physiological state showed the most consistent effect of pollen germination. While media also produced consistent effects over populations, considerable interaction occurred between media and individual genotypes (within and between populations). In addition, genotypes interacted with physiological states in germination. Utilizing the evident analogue between germination medium and stigma, the potential role of the studied factors in the mating system of *Alnus* is outlined.

Key words: *Alnus glutinosa, Alnus incana, in vitro* pollen germination, genotype × environment interaction, mating system.

INTRODUCTION

In population genetic studies, the generative reproduction cycle is of central importance, since it guarantees genetic continuity over generations and, at the same time, organizes the formation of the new genetic structures in the progeny generations. Yet, because of the multitude of mechanisms involved in this cycle, experimental studies usually produce highly integrative results which make it very difficult to assess the significance of the single mechanisms. In order to clarify the position of the present pollen germination experiments in this context, the most important phases of the generative reproduction cycle and their determination will therefore be recollected beforehand.

The reproduction cycle starts with meiosis and gamete formation and ends with the formation of zygotes. With respect to the factors that are relevant for mating systems, this period can be divided into two or three phases: (i) During the *pre-pollination phase* there may be differences in fertility (individuals differ in quantity and/or viability of gametes produced in one or both sexes), and selection among gametes produced by the same parent may take place on the haplophase level; especially pollen is very likely to be subject to considerable such selection. Selection at this stage is predominantly controlled by the interaction of the pollen's or pollen parent's genotype with its specific environment. The pre-pollination phase contributes to the mating success. (ii) During the post-pollination phase between pollination and fusion of gametes, *i.e.* in the *progamic phase*, pollen germination, pollen tube growth and final fertilization require intensive physiological activity, and therefore selection among pollinating pollen grains is almost inevitable. The situation compared to the pre-pollination phase, however, is much more complex, as it provides opportunities for additional biotic interactions: The female stigma and style constitute the biochemical environment for pollen germination, pollen tube growth and germ cell fusion. Even different pollen types present on the same stigma or growing in the same style may represent relevant environments for each other, as was shown by studies of pollen "interference" (BERTIN & SULLIVAN 1988, MARSHALL *et al.* 1996) or pollen "allelopathy" (KANCHAN & JAYACHANDRA 1980; JIMÉNEZ *et al.* 1983, MURPHY & AARSSEN 1989). In general knowledge about this kind of pollen–pollen interaction seems to be very limited. The progamic phase contributes to both mating success and combination of genetic information. (iii) During the *postzygotic phase*, the development from zygotes to viable embryos provides possibilities for diplophase selection. This selection acts mainly via differential rates of abortion during embryo development. Strictly speaking, this phase has to be considered as part of the vegetative development of a diploid individual. In experiments, however, the zygotic state is usually not accessible to observation, so that seeds or even seedlings must be accepted as defining the individual mating events entering mating system analysis.

In contrast to the vegetative phases, the generative phases are characterized by mechanisms that reorganize

genetic information on two levels. The meiotic level, where recombination processes create a gamete population of haploid genotypes from the diploid parental population (beginning of phase (i)), and at the level of pair formation, which effectively combines gametes pairwise into zygotes (end of phase (ii)).

Concepts such as panmixia, positive or negative assortment and their associated well-known models typically refer to this level (phase (ii)).

The forces determining mating success within the generative reproduction cycle act on both levels and must therefore be considered as constituent parts of the mating system. The purely combinatorial effects of mating pair formation, *i.e.* freed from differential mating success, are confined to phase (ii), but are again almost impossible to study directly in experiments. The enormous selective (via differential mating success) and combinatorial potential of mating systems must be considered to play an important role for the adaptation and evolution of populations and species (for a discussion see STEINER and GREGORIUS 1997) and thus necessitates detailed studies and modelling of the basic components of these systems.

As mentioned repeatedly, it is very difficult and sometimes even impossible in experimental studies to separate the effects realized in the different reproductive phases. Pollen germination constitutes a stage of the reprocduction cycle that may affect both mating success and the combinatorial mechanisms of pair formation. If pollen germination experiments in vitro are carried out with the same samples used in pollination experiments in vivo, they provide a method to characterize the pollen in its condition at the time of pollination, *i.e.* at the transition from phase (i) to phase (ii), because the physiological conditions of the pollen are the same for both kinds of experiments, if the time between in vitro test and in vivo experiment is only short and the storage conditions are conservative.

If there are good reasons to assume that the germination tests reflect the germination and fertilization ability/capacity in nature, the germination percentages can even be used as a mating reference (GREGORIUS 1989) to formulate hypotheses about relative mating success under mixed pollination conditions. If the derivation of hypotheses in this sense does not seem to be justified, the germination percentages can at least give an impression of the selection potential during the pre-pollination phase. Selection here refers to selection within a single pollen donor's pollen production (which requires methods of further differentiation of germination percentages within the donor's pollen) as well as to selection between pollen from different donors (variability of germination percentages between pollen donors).

Pollen germination experiments can be performed by using conspecific flowers as the most natural substrate or by using artificial germination substrates. Besides the possible technical advantages of artificial substrates, the latter provide the precondition for testing effects on differential pollen germination which are primarily due to the pollen type. Even if enough female flowers of a single genotype were studied under controlled conditions in a greenhouse, developmental and physiological differences would remain to exist even within a single plant. These differences could interact with the pollen behavior, making it more difficult to characterize the pollen individually. Therefore an artificial germination medium is the only method that allows to apply a maximum environmental homogeneity to the different pollen tested, the pollen themselves being the only explanations for germination differences on the same medium and the media being the only explanation for germination differences of the same pollen type on different media. Another advantage of artificial germination media is the well-defined biochemical composition of a very limited number of substances, so that the possibilities for complex biochemical interactions as they are expected on stigmas are also restricted.

The present experiments are part of controlled pollination experiments which were aimed to study the mechanisms of the mating system which affect the maintenance of genetic polymorphisms at different levels, including the possibility of genotype \times environment interactions as determinants of the mating system. The results on pollen germination presented below are intended to shed light on the potential effects of selection acting on this phase prior to the specific effects of combination. The implied consequences for mating success will be evaluated with respect to their significance for the characterization of the mating system in *Alnus*.

MATERIAL AND METHODS

Tree species – The species used for these experiments is *Alnus glutinosa* (L.) Gaertn., black alder, and, to a very small extent, also *Alnus incana* (L.) Moench., grey alder. Both tree species are common in central Europe, *A. glutinosa* being more frequent and of higher importance for forestry and timber production than *A. incana*. The reproduction biology of both species is similar: The trees are wind-pollinated and monoecious, the female and male inflorescences being well separated. Flowering occurs before leaf flush in very early spring (or even end of winter) more or less simultaneously for

both sexes. As a consequence, the weather conditions are very variable and highly unpredictable during the flowering period, which distinguishes *Alnus* from many other plant species. Temperature differences, for example, range from freezing and snowing to more than 10 °C and sunshine. Since this range may be realized between years as well as within a single year, various forms of selection can be implied. *A. glutinosa* and *A. incana* are reported to be self-incompatible (JOHNSSON 1951, WEISS 1964, HAGMAN 1970, 1975), although some other authors do not confirm this statement (HEITMÜLLER 1957, ROHMEDER & SCHÖNBACH 1959 [P. 71], HOLZER 1961; WEISGERBER 1974).

Individual ramets from a clonal seed orchard were used as *A. glutinosa* pollen parents, as well as some trees of a planted forest stand and some roadside trees of unknown origin. For *A. incana* no seed orchard material is available, because this species is not subject to legal regulation in forestry, so that trees of a forest stand were used. The trees used are summarized in Table 2.

Pollen sampling – To collect pollen, branches of the different trees were cut about two weeks before the expected beginning of flowering. (As the pollen was to be used in field pollination experiments and the pollen samples must be kept separate, the branches had to be cut some time before flowering.) The cut branches were kept in isolation and put in water and stored under "spring conditions" (about 21 °C, 12h light, high humidity) to induce flowering. Within hours to days, the male catkins elongated to about double length, the yellow anthers became visible and the pollen could be collected. Pollen was then kept under dry, dark and cold (5–10 °C) conditions for several days until use.

The experiments were performed in the years 1993, 1994 and 1996, no more than several days up to some weeks after pollen collection and only a few days before starting the pollination experiments in the field. Only newly collected pollen was used in all years to avoid confusion with effects of pollen age on germination ability.

In 1994 additional germination experiments were performed with pollen mixtures consisting of pollen from two different parental trees in known weight proportions.

Germination media – Four different methods (A, B, C, D, described in detail in Table 1) were used to promote pollen germination. Two media (A and B) were taken from the literature (ESCHRICH 1976 and LINARES 1985, the latter of which also worked for *Alnus glutinosa*). As a pretreatment before applying medium A the pollen must be exposed to warm temperatures (about 25–30 °C) in a moist chamber for 12 to 24 hours under conditions of saturated humidity in order to enable hydration before contact with the medium. This pretreatment alone has proven to be sufficient to promote pollen germination and was therefore used as a separate germination method C in 1996. One medium (D) is a modification of B with 50 % of the original concentrations. The use of the media in the different years is summarized in Table 2.

Evaluation of germination – After the pollen was exposed for about 24 hours to the germination medium, it was examined under the microscope. 100 grains per pollen parent and medium were counted along a randomly chosen microscope transect. Among these 100 grains all grains showing germination were counted. A pollen grain was classified as germinated if at least the beginning of a developing pollen tube could be seen emerging from one of the pores.

Data analysis – To test for homogeneity of germination percentages in different samples, two tests were performed: the Pearson χ^2 test for homogeneity and, for pairs of samples, an exact test based on confidence values (or p-values, *i.e.* the sum of all probabilities of events equally or less probable than the observed pair of samples calculated for the product of two binomial distributions using the maximum likelihood estimate of

Table 1. Germination media

A[1]	B[2]	C	D[3]
0.01% H_3BO_3	0.01 % H_3BO_3	moist chamber	0.005 % H_3BO_3
0.01 % $CaCl_2$	0.30 % Ca_2NO_3	with saturated	0.15 % Ca_2NO_3
20 % succrose	7 % saccharose	humidity only,	3.5 % saccharose
1% agar	1 % agar	no chemicals!	0.5 % agar

All percentages of chemicals are w/v in bidestilled water
[1] after LINARES (1985), the mixture has to be boilied for 20 min and then poured into Petri dishes
[2] after ESCHRICH (1976, p. 141f)
[3] all concentrations of 50% medium B

Table 2. Survey of the pollen germination experiments and their results (germination percentage, confidence, differentiation

Year	1993				1994				1996					
Pollen source	Medium				Medium				Medium					
	A	B	δ	conf	A	B	δ	conf	A	B	C	D	δ	conf
Forest stand trees														
H 01	32	31	1.0	87.9										
H 02 a	7	9	2.0	60.2										
H 02 b	17	23	6.0	28.9										
H 06 a	65	33	32.0	**0.0**										
H 06 b	45	33	12.0	8.2										
H 08	38	27	11.0	9.7										
H 12	25	16	9.0	11.5										
H 13 a	15	20	5.0	35.2										
H 13 b	19	13	6.0	24.7										
H 16	6	12	6.0	13.8										
H 24	29	40	11.0	10.2										
H 25	55	41	14.0	**4.8**										
H 27	35	22	13.0	**4.2**										
H 31	32	40	8.0	23.9										
H 33 a	23	17	6.0	28.9										
H 33 b	39	–	–	–										
H 34	13	15	2.0	68.4										
H 38	11	11	0.0	100.0										
H 39	9	14	5.0	26.8										
H 41	28	22	6.0	32.7										
Delta (%)	9.3	13.3												
Conf. (%)	**0.0**	**0.0**												
Arithmetical mean	27.2	23.1		**0.4**										
Pollen mixtures														
M01 (Sf1–246 + S1–219)					5.0[1]	–	–	–						
M02 (Th6-517 + Me8-630)					2.0[1]	8	6.0	**1.2**						
M03 (Wb16-571 + Ds4-512)					3.5[1]	10	6.5	**2.2**						
M05 (Th4-698 + Wb16-571)					29.0[1]	12	17.0	**0.1**						
M07 (Th5-571+ ATW 4)					10.5[1]	6	4.5	19.9						
M09 (Wb16-571 + S1-219)					8.5[1]	10	1.5	66.9						
M11 (ATW 2 + Ds 4-512)					9.5[1]	6	3.5	30.1						
M13 (ATW 2 + R 8-500)					11.0[1]	4	7.0	**4.2**						
M15 (WE 2 + ATW 2)					19.5[1]	–	–	–						
Delta (%)					6.7	2.7								
Conf. (%)					**0.0**	36.7								
Arithmetical mean					10.9	8.0		**0.2**						

[1] sample size $N = 200$, in all other cases $N = 100$; confidences <5 % are indicated in boldface and underlining

Table continued on next page

the common binomial parameter, GILLET pers. comm., also see *e.g.* GREGORIUS [1996]). A confidence of less than 5 % is considered as sufficient evidence for rejection of the hypothesis.

To quantify the variability of germination the measure δ of differentiation developed by GREGORIUS and ROBERDS (1986) was used.

For graphical illustrations the concept of ARF (analysis of response functions) according to GREGORIUS (1977) and GREGORIUS and NAMKOONG (1986) was applied. In Figures 1, 2, and 3, values obtained for one observed object under varying (environmental)

Table 2 (continued)

Year	1993				1994				1996					
Pollen source	Medium		δ	conf	Medium		δ	conf	Medium				δ	conf
	A	B			A	B			A	B	C	D		
Road side trees														
ATW 2	21	9	12.0	**1.7**	0.5[1]	7	6.5	**0.1**	7	12	18	30	9.7	**0.0**
ATW 4	37	14	23.0	**0.0**	2.5[1]	2	0.5	78.7	26	16	28	18	6.7	10.9
Seed orchard trees														
Bu1-464					7.5[1]	1	6.5	1.8						
Me7-098					27.5[1]	4	23.5	0.0						
Me7-546					6.0[1]	2	4.0	12.2						
Me8-108					22.5[1]	4	18.5	0.0						
Me8-630					12.0[1]	–	–	–						
Me9-544					11.5[1]	–	–	–						
Th3-219					32.5[1]	–	–	–						
Th4-707					5.5[1]	16	10.5	0.3						
Th4-320					13.0[1]	46	33.0	0.0						
Th2-698					24.0[1]	12	12.0	1.4						
Ma3-329	39	41	2.0	77.3										
R3-379	66	57	9.0	19.1										
S1-219	51	35	16.0	2.2	14.0[1]	4	10.0	0.8						
Ds4-512	57	59	2.0	77.4	14.5[1]	–	–	–	55	23	59	3.5[1]	29.0	0.0
R8-500	61	45	16.0	2.3	15.0[1]	11	4.0	34.2	31	22	54	8	18.3	0.0
Sf1-246					36.0[1]	9	27.0	0.0	12	13	21	23	6.3	9.0
Th5-571					13.5[1]	9	4.5	25.8	24	50	14	20	15.3	0.0
Th5-603					5.5[1]	1	4.5	6.1	32	26	20	12	8.7	0.6
Wb16-571					4.0	3	1.0	70.0	32	12	51	17	18.0	0.0
Sf1-330									30	20	18	11	7.0	0.9
Delta (%)[2]	15.1	17.8			8.2	7.2			10.4	8.7	17.4	7.2		
Conf. (%)[2]	0.0	0.0			0.0	0.0			0.0	0.0	0.0	0.0		
Arithmetical mean[2]	47.4	37.1		0.0	14.1	8.7		0.0	27.7	21.6	31.4	15.8		0.0
***Alnus incana* trees**														
WE 1					14.0[1]	24	10.0	3.1	23	24	18.5	22	2.3	66.5
WE 3					24.5[1]	15	9.5	5.8	43	4	50	9	15.7	0.0
WE 2	51	37	14.0	4.6	27.5[1]	14	13.5	0.9	15	42	32	12	26.5	0.0
WE 4	44	47	3.0	67.0	14.0[1]	2	12.0	0.1	57	24	57	40	16.7	0.0
WE 6	60	27	33.0	0.0										
Delta (%)	8.3	10.0			8.0	7.8			20.7	13.0	18.8	13.7		
Conf. (%)	7.6	1.4			0.0	0.0			0.0	0.0	0.0	0.0		
Arithmetical mean	51.7	37.0		0.0	20.0	13.8		0.8	34.5	23.5	39.4	20.8		0.0

[1] sample size $N = 200$, in all other cases $N = 100$;
[2] road side trees + seed orchard trees
confidences, <5 % are indicated in boldface and underlining

conditions are connected to each other by lines, which shall not imply the existence of intermediate values but simply allows for better comparisons of the objects considered and especially their changes of ranking.

Groups of pollen types were formed and analyzed separately according to a genetic and ecological classification of the pollen parents: Pollen mixtures were treated as one group and the *Alnus incana* trees as a second group. The *Alnus glutinosa* trees were divided into two groups, one consisting of individuals of a single forest stand that are separated by rather small distances leading to the expectation of considerable

competition, the other group consisting of trees from seed orchards or from a roadside with very limited intraspecific and interspecific competition and well-developed crowns. These ecological conditions were correlated with flowering intensity, the nearly solitary trees flowering more intensively than those in the relatively narrow forest stand.

RESULTS

The germination percentages for each single experiment are given in Table 2 as well as the confidence values c for the homogeneity tests and the differentiation values δ, all expressed in percent. The δ's in the columns measure the germination variability of the pollen source (first column) across the different media in the respective year. The c-values in the neighbouring columns express the statistical confidence in the hypothesis that no differences in germination percentage exist among the different media and that the deviations from equality can be explained by sampling effects only. Confidence values <5 % are indicated in Table 2 by boldface and underlining.

In the first two lines below each group of trees, δ and c are given for the whole group of trees for each single medium. They describe the germination variability of different pollen sources (genotypes) under identical environmental conditions. In the last line below each group of trees the respective overall germination percentage is given. Germination percentages of groups are defined by equal contributions of all of their subgroups. Thus, group values result by taking the unweighted arithmetic mean over the respective subgroups. The confidence in this last line (for the hypothesis that the arithmetic means are equal) is calculated under consideration of the total sample sizes of the groups.

Medium effects – One of the most surprising results is possibly that pollen is able to germinate and start pollen tube growth to a considerable degree on a clean glass surface in a simple moist chamber without addition of any specific substances for nutrition or growth promotion. This does not mean, however, that pollen germination is very easy to encourage in *Alnus* because even on the previously tried and tested substrates germination rarely exceeds 50 %. The differentiation of germination caused by different media comprises a broad range of δ from 0 % to 33 % for single pollen sources.

The confidence values for many single pollen parents and especially those for the pooled data of the tree groups clearly show the importance of the germination medium: Out of 68 samples which were tested for at least two media, 35 showed significant deviations from the hypothesis of homogenous germination behavior on the different media. The picture is even more clear for the pooled data: The confidences for all 8 groups in Table 2 are extremely low, the forest stand group of 1993 showing the maximum value of c = 1.5 %. Also if the homogeneity tests of the 1996 results were restricted to media A and B, the confidence values for both groups are also very low (0.1 % and 0.3 %). In all groups the average germination percentage on medium A is significantly higher than on medium B, but this need not be true for single genotypes: Among the 25 significant cases of A-B tests contained in Table 2, 19 showed superiority of A and 6 superiority of B (if among the 1996 results only A and B were tested for homogeneity, 4 pollen parents shows superiority of A and 2 superiority of B). Within groups (*e.g.* all three 1994 groups) there may occur pollen sources showing significant deviations in favor of medium A, others in favor of medium B, and others without any significance.

To summarize the effects of the medium it can be stated that they obviously exist at considerable size, but that they are not consistent und thus n1ot predictable, neither quantity nor the direction of superiority: pollen donors differ as to their preferred germination medium.

Paternal effects – Paternal effects on germination are defined by differences in germination percentages among pollen donors (single trees or pairs of trees in the case of pollen mixtures) under identical conditions. Paternal effects combine genotypic effects and effects of physiological state of the pollen, where the latter result from environmental modifications during pollen development among pollen donors. The low confidence values in Table 2 verify highly significant differences in pollen germination within most groups of pollen donors on the same medium and in the same year. Among the 20 combinations of groups, years and media, only 2 show confidences higher than 5 %, with the group of pollen mixtures showing a rather uniform (δ = 2,7 %, c = 36.7 %) germination behavior on medium B, and the 1993 grey alder group on medium A being slightly above (c = 7.6 %) the significance limit, while all other confidences are below the 5 % limit. 17 c-values are even below 0.1 %.

Although the c-values are very low, the differentiation caused by the pollen parent differences within the groups is comparatively limited, ranging from 2.7 % to 20.7 % among the groups. As can be intuitively expected, the group of the lowest differentiation shows the highest confidence.

Besides the differentiating medium effects, the paternal effects on germination behavior could thus also be shown to be of considerable importance. The

Table 3. Comparison of pollen germination in different years

Pollen source	Medium A								Medium B							
	Germination (%)			Statistics					Germination (%)			Statistics				
	1993	1994[1]	1996	δ	conf (3 yr.)	conf. 93/96	conf. 94/96	conf. 93/94	1993	1994	1996	δ	conf (3 yr.)	conf. 93/96	conf. 94/96	conf. 93/94
ATW 2	21	0.5	7	11.5	**0.0**	**0.4**	**0.1**	**0.0**	9	7.0	12	2.7	47.3	48.9	22.8	60.2
ATW 4	37	2.5	26	19.3	**0.0**	9.4	**0.0**	**0.0**	14	2.0	16	8.7	**0.2**	69.2	**0.1**	**0.2**
Ds4–512	58	14.5	55	28.0	**0.0**	66.9	**0.0**	**0.0**	59	–	23	26.0	–	**0.0**	–	–
R8–500	61	15.0	31	25.3	**0.0**	**0.0**	**0.1**	**0.0**	45	11.0	22	19.0	**0.0**	**0.1**	**3.6**	**0.0**
WE 2	51	27.5	15	19.8	**0.0**	**0.0**	**1.6**	**0.0**	37	14.0	42	17.0	**0.0**	47.0	**0.0**	**0.0**
WE 4	44	14.0	57	24.3	**0.0**	6.6	**0.0**	**0.0**	47	2.0	24	22.7	**0.0**	**0.1**	**0.0**	**0.0**
Mean	45.2	24.7	31.8		**0.0**	**0.0**	**0.0**	**0.0**	35.2	7.2	23.2		**0.0**	**0.0**	**0.0**	**0.0**

[1] sample size N = 200, in all other cases N = 100

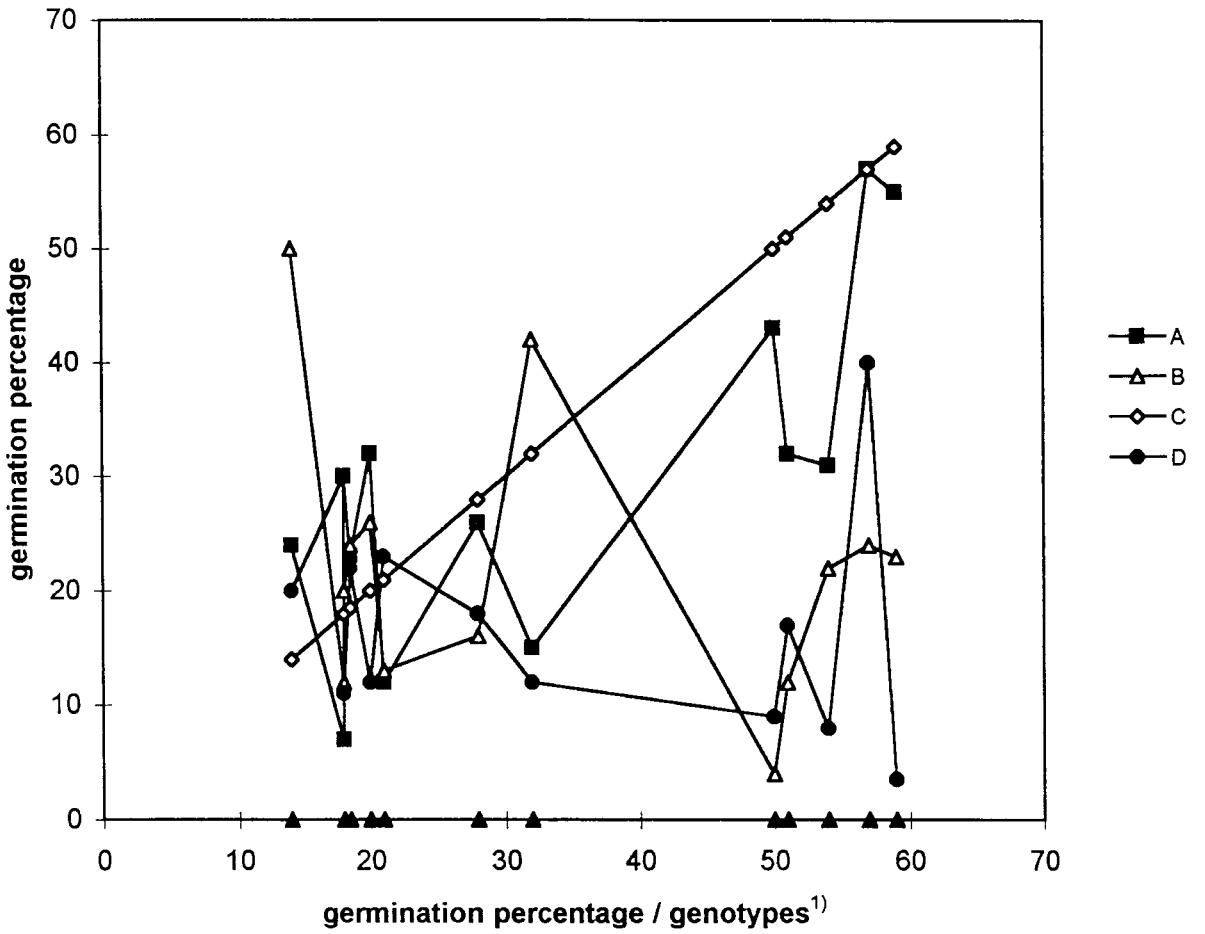

Figure 1. Response functions of four media in 1996.
[1] Note: The position of genotypes on the *x*-axis is indicated by filled triangles in the following order: 1. Th5-571; 2. = 3. ATW2 + Sf1-333; 4. WE 1; 5. Th5-603; 6. Sf1-246; 7. ATW 4; 8. WE 2; 9. WE 3; 10. Wb16-571; 11. R8-500; 12. WE 4; 13. DS4-512.

question of consistency of paternal effects will be treated in more detail after the following section.

Effects of years – The medium has already been considered as an important environmental factor for pollen germination, but other important environmental factors already act during pollen development before germination. These factors must be considered to result in different physiological states possibly leading to different germination percentages of the same pollen donor among different years. As not all pollen parents could be tested in each of the three years, the germination values for different years cannot be compared directly. Nevertheless, the poor 1994 germination is remarkable, the maximum of 6 arithmetic means being only 20.0 % and thus still below the minimum (21.6 %) of the 10 arithmetic means of 1993 and 1996 on media A and B (see Table 2).

Pollen from 6 trees (4 *A. glutinosa* and 2 *A. incana*) pollen was used in all three years, thus providing the possibility to study the effects of different years on different pollen donors. The results of these trees are listed and tested for homogeneity in Table 3. In 10 out of 11 cases there are highly significant (below the 0.1 % level) deviations in germination success among the three years. This emphasizes the importance of the pollen's physiological state (depending on environmental conditions acting during pollen development and differing between years) for pollen performance. This inhomogeneity between years refers to both of the tested media and seems to be largely due to the low germination rates in 1994, but homogeneity tests for all pairs of years (also included in Table 3) also demonstrate the high amount of inhomogeneity between all years: For each medium A and B the pooled data have extremely low confidences, and even the single tests reveal only very low homogeneity of pairs of values. The data presented in Table 3 are also graphically presented in Fig. 3a and 3b, where for each genotype those germination percentages are encircled which show no significant difference between years. It can be seen that on both media A and B in most cases the germination percentages differ significantly between years, thus indicating highly differentiating effects of the physiological state of pollen or pollen donors on germination behavior.

Consistency of effects – The medium effects have already been described as inconsistent in a previous section. For example, medium A can be superior to medium B for one pollen type while the reverse holds true for another pollen type in the same year (*inconsistency of medium effects over genotypes*). The 1996 data from four substrates are also suitable to demonstrate graphically this inconsistency of medium effects over genotypes. Fig. 1 gives a graphical illustration of the medium performance for 13 genotypes. It is constructed as recommended for the analysis of response functions (ARF, see GREGORIUS [1977] and GREGORIUS & NAMKOONG [1986]) with medium C chosen as reference. It can be clearly seen that none of the four media enhances germination consistently as compared to any other medium, and this shows as intersections between all response lines. In addition, the range of variation of germination percentages among genotypes is almost the same on all media. Every medium supplies for at least one genotype the minimum germination value of all media and at least once the maximum value. There also exists another form of inconsistency of medium effects which is characterized by the fact that the superiority-inferiority relations of different media may change even for the same pollen parent in different years (*inconsistency of medium effects over years*). Table 4 summarizes the comparison of media A and B for all parent genotypes tested for both media in at least two years. Among the 13 trees only two show consistent medium properties over years (superiority of A for S1-219 in both years tested and absence of significant differences for Th5-603 in both years tested). For all other genotypes the medium effects significantly interact with the effects of physiological state (year), where ATW 2 represents the most extreme case in that it shows significantly better germination on medium A than on medium B in 1993, reversed situation in 1994, and absence of significant difference in 1996.

Table 4. Comparison of pollen germination on media A and B for identical genotypes in different years

Pollen source	1993	1994	1996
ATW 2	A	B	0
ATW 4	A	0	0
R8–500	A	0	0
WE 2	A	A	B
WE 4	0	A	A
D 4–512	0		A
S1–219	A	A	
Sf1–246		A	0
Th5–571		0	B
Th5–603		0	0
Wb16–571		0	A
WE 1		B	0
WE 3		0	A

A, B indicate significant superiority of medium A or B, resp., 0 indicates absence of significant difference

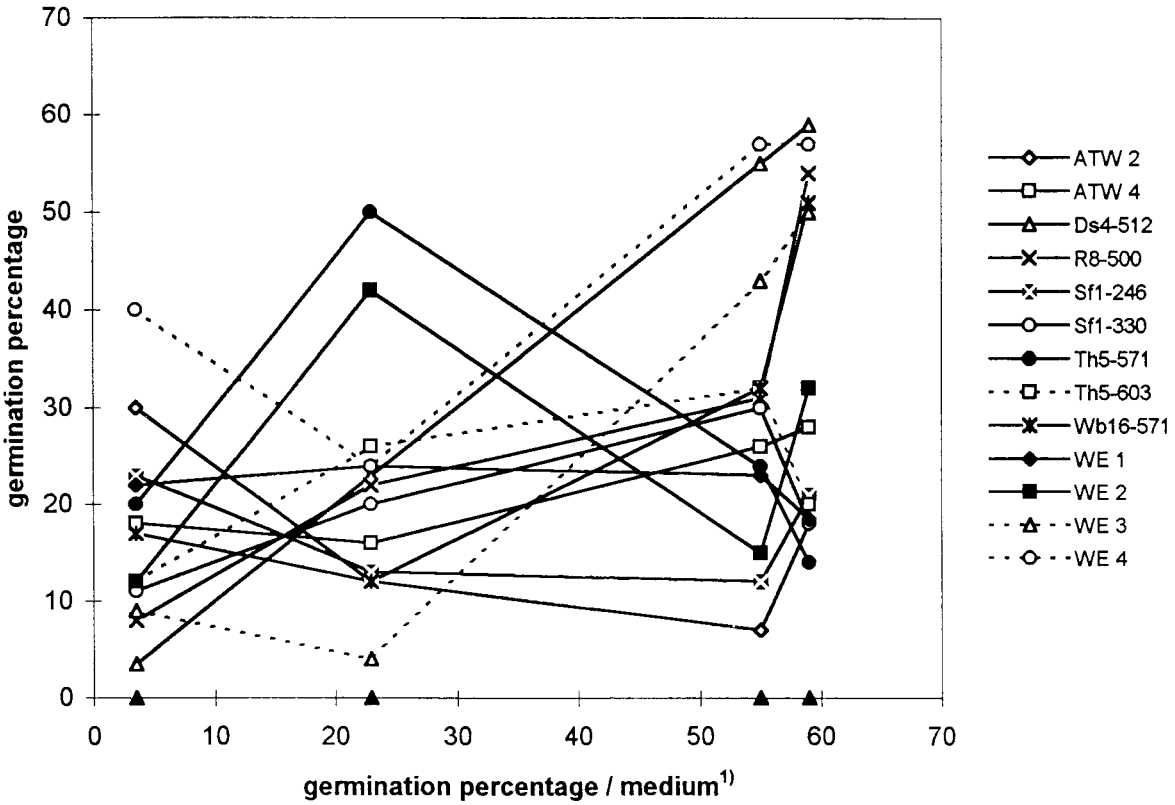

Figure 2. Response functions of 13 genotypes for 4 media.

[1] Note: The position of media on the *x*-axis is indicated by filled triangles in the following order: 1. D; 2. B; 3. A; 4. C.

Concerning the inconsistency of *paternal effects over media*, a graphical illustration is given in Fig. 2 using the same data set as in Fig. 1 (13 genotypes on 4 media in 1996). The numerous intersections among response lines again indicate an inconsistency of genotypic effects over germination media. There are of course genotypes (*e.g.* WE 4) showing better pollen germination on all 4 media than some other genotypes (in the case of WE 4 for example R8-500, Sf1-330 or Th5-603), but no genotype can be said to be superior or inferior to all other genotypes on all media.

The *inconsistency of paternal effects over years* can be seen from Table 3 or from the corresponding graphics in Fig. 3c and 3d. They indicate that no complete consistency exists but that the number of intersections is relatively small. Many pairs of genotypes do not show any intersection and some of the existing intersections are not statistically significant in the sense that adjacent years show no significant differences between the genotypes (confidences not included in Table 3).

The most striking results concerning inconsistency of genotypes are, however, provided by repeats of a pollen parent in the same year on the same medium. This aspect will be treated in one of the following sections, as it primarily concerns the question as to what extent parental genotype or environmental influences govern pollen germination.

The nearly complete *consistency of the effects of physiological states (years) over genotypes for each medium* can be seen again from Table 3 and the associated Fig. 3a and 3b. For the 6 trees tested, the 1993 germination was never significantly lower than germination in the other two years. The 1994 values were – with one exception (WE 2 on medium A) – never higher than those of the other years. Germination taken over all genotypes in each year reveals highly significant differences among years with the same ranking of years on both media. The *inconsistency of the effects of years over media for every genotype medium performance* can be seen from Table 4, where no specific superiority-inferiority pattern can be recognized among the 3 years.

Environmental component of paternal effects – In the previous section the physiological state of pollen (differing between years) has been shown to express the most consistent effect on pollen germination over media and over pollen donors. It must be assumed that the environmental differences between years are at least in part also expressed within years in the form of

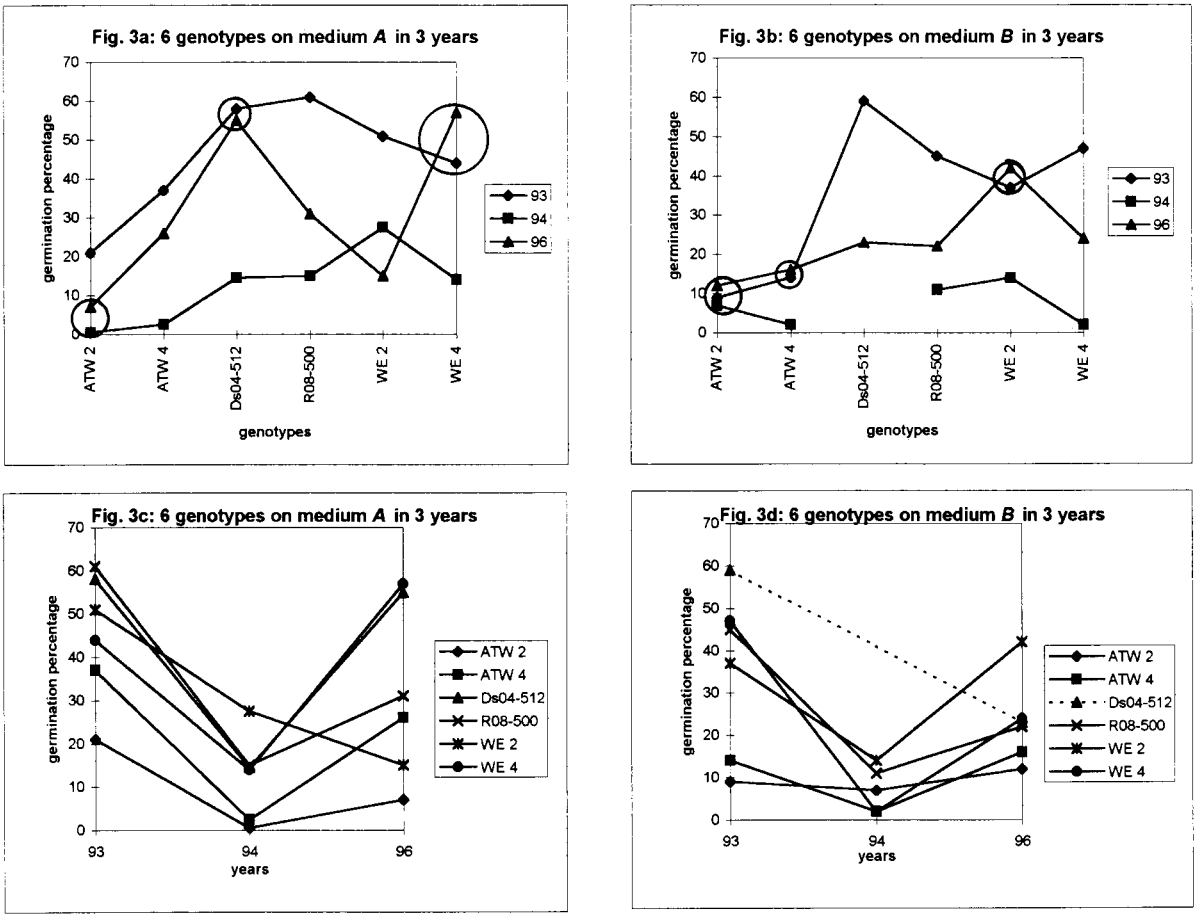

Figure 3a–d. Norms of reaction of 6 pollen source genotypes in different years and on different media. Circuits in Fig. 3a and 3b indicate the absence of significant differences between the encircled values (data from table 3).

Table 5. Confidences of homogeneity tests for germination percentages of 11 pairs of ramets on different media.

Pollen source	Medium A		Medium B		Medium C		Medium D		Year
	Confidence	Superior ramet	Confidence	Superior ramet	Confidence	Superior ramet	Confidence	Superior ramet	
H 02	**3.0**	b	**0.7**	b					1993
H 06	**0.4**	a	100.0						1993
H 13	45.1		18.2						1993
H 33	**1.4**	b	–						1993
Me7–	**0.0**	–098	40.7						1994
Me8–	**2.9**	–108	–						1994
Th3–	**0.0**	–219	–						1994
Th4–	**1.6**	–698	**0.0**	–320					1994
Th5–	**3.6**	–603	**0.9**	–603					1994
Th5–	14.0		**0.0**	–571	18.1		12.3		1996
Sf1–	**0.0**	–330	18.2		54.0		**2.4**	–246	1996

small-scale temporal and spatial variation of environ mental conditions, *e.g.* micro-climate, which are relevant for modifications of physiological parameters affecting pollen germination. To gain an impression about the importance of this kind of within-year physiological differentiation, some of the material listed in

Table 2 is very useful, as it represents pollen sample repeats of genetically identical trees: 4 forest stand trees were sampled twice for pollen with a postponement of 3 to 5 days and kept separate during further investigations in 1993 (indicated by the extensions a and b for the first and second sample, resp.); among the seed orchard material, 6 clones (Me7, Me8, Th3, Th4, Th5 and Sf1) are repeated by pollen collected from two different ramets of each clone and kept separate during further investigation. 5 pairs of ramets were investigated in 1994 and 2 pairs in 1996. The classification of these ramets as belonging to the same clone was tested with isozyme gene markers. If the repeats of identical genotypes in the same year on the same medium show differences in germination, these differences cannot be attributed to effects of genotype, medium or physiological differentiation between years, but they must be attributed to physiological differences between repeats of the same genotype. For each pair of genetically identical pollen donors, the germination behavior on the same medium was tested for homogeneity, and the results are presented in Table 5.

Surprisingly, 14 out of 23 tests revealed significant differences in germination for pollen collected from the same genotype in the same year at nearly the same time and germinated on the same medium. Only one genotype (H 13) shows homogeneity of its two pollen samples on each of the two media tested. Among the forest stand trees of 1993, H 02 and H 33 showed better germination of the pollen collected later, while the reverse is true for H 06. The time of pollen collection therefore does not seem to have a consistent influence on pollen germination. Among the seed orchard trees, clones Th4 and Sf1 show the most remarkable behavior: Ramet 320 of clone Th4 shows significantly better germination on medium B than ramet 698 of the same clone, while the reverse holds true on medium A. For the two ramets of Sf1, ramet 330 shows significantly better germination on medium A than ramet 246, the reverse ranking occurs on medium D, and no significant difference between the two ramets occurs on media B and C. The data imply that environmental modifications within a single year can lead to considerable differences in physiological state even for genetically identical trees.

Pollen mixture behavior – The pollen mixtures examined in 1994 show the lowest differentiation values (δ = 6.7 % on medium A and δ = 2.7 % on medium B). This could be expected by assuming that different pollen types of germination percentages are combined in the mixture, resulting in an intermediate germination behavior.

Since the proportions of the two pollen types are known in each mixture, and since the germination values were determined for each of the pollen types separately, it was possible to test the hypothesis that a mixture's germination percentage equals the weighted mean of the separate germination percentages of the two involved pollen samples. The observations, expectations and confidences are given in Table 6. For medium A the hypothesis is falsified in 4 out of 9 cases, while for medium B all 4 cases are in accordance with the hypothesis. Among the 4 significant deviations from the expectation, some additional form of interaction (allelopathy, interference) between the two pollen types may occur. The interactions observed here at the stage of pollen germination are predominantly of a negative kind in that contact of two different pollen types seems to inhibit pollen germination of at least one type. Only in one case might there be an encouragment for pollen germination in the presence of competitors.

Table 6. Comparison of observed and expected germination percentages of pollen mixtures

Pollen source	Medium A			Medium B		
	observed N = 200	calculated expected	Conf.	observed N = 100	calculated expected	Conf.
M 01	5.0	19.85	**0.0**	–	6.01	–
M 02	2.0	11.92	**0.0**	8	–	–
M 03	3.5	9.54	**1.4**	10	–	–
M 05	29.0	12.23	**0.0**	12	6.70	19.8
M 07	10.5	7.62	31.6	6	5.26	82.0
M 09	8.5	9.08	83.8	10	3.51	6.7
M 11	9.5	6.31	23.7	6	–	–
M 13	11.0	6.87	14.8	4	8.76	16.9
M 15	19.5	20.00	90.1	–	12.05	–
Arith.mean	10.9	11.49	60.4	8.0	7.05	51.8

DISCUSSION

The results of the pollen germination experiments reveal manifold interactions, but also cases of consistency, of the effects of (i) pollen donors (genotypes), (ii) physiological state of pollen when entering the germination experiment (year), and (iii) germination conditions (media). Concerning the last factor, it turned out that for each of the groups of genotypes medium A showed a higher germination percentage than medium B in each year. Yet, this tendency did not extend to each genotype within a group, which emphasizes the existence of genetic variability within groups for preferred germination medium.

For reasons of representation of all combinations, the effects of factor (i) could be demonstrated only for a reduced number of genotypes (Table 3). Among these genotypes, no one shows germination percentages which are consistently the highest over years, and only in one year, 1994, does genotype WE 2 consistently have the highest germination percentage over the media. On the other hand, genotype ATW 2 consistently shows the lowest percentages on medium A and, with the possible exception of the year 1994, also on medium B. In clear contrast with the other factors, factor (ii) shows high consistency in pollen germination both over genotypes and media. In fact, there is only one exception to this pattern, in that genotype WE 2 shows inconsistency on one medium, A, between years 1994 and 1996. This strong effect of the physiological state corresponds to the observation of pronounced environmental variation between years during pollen development.

LINARES' (1985) germination experiments with *A. glutinosa* pollen – Comparing the present results with those obtained by LINARES (1985) for the same tree species and the same germination medium, the large difference in germination success is conspicuous. Linares reports that "it was possible to obtain 60–80 % germination of control pollen, depending on the individual genotype". Unfortunately it cannot be concluded from his statements, whether these are maximum or average values. The maximum value obtained in our experiments was 66 %, the arithmetic means for the different groups ranging from 8.0 % to 51.7 %. As the same biochemical procedure was used, the difference could only be explained by (genotypic) differences in the trees tested or by environmental differences concerning either the conditions in the field in different years or in the greenhouse or lab (compared to LINARES (1985) no additional light was applied to enhance germination). The comparison again reveals the enormous variability of pollen germination in this species, as was also stated by Linares.

The importance of the germination medium was also emphasized by LINARES (1985), who tested several media from the literature without success for *Alnus glutinosa*. This again points to the high sensitivity of germination behavior to environmental conditions.

Moist chamber results. A very interesting point of discussion is given by the relatively high percentage of germination after exposure to high humidity in a moist chamber only, especially because this method was used as a pretreatment before applying method A. At first it is remarkable that moist chamber treatment alone is sufficient to induce germination especially because some other germination methods from the literature were unable to induce germination as reported by LINARES (1985) without further detail. Pollen hydration might be a necessary precondition for germination not only for the agar medium A used here but possibly also for other media. Furthermore, it seems difficult to explain how the germination percentage could decrease after transfer of pollen from the moist chamber to the medium. In 2 out of 13 pollen parents tested, the results after pretreatment alone are significantly higher than after applying method A. Possible explanations for this observation are: (i) During transfer from the moist chamber to the agar medium some of the already germinating pollen grains lost their pollen tube because of mechanical destruction. However, pollen tubes separated from their grains could be observed only in very rare cases under the microscope. Either the effect of mechanical destruction cannot be very high or separated pollen tubes are very rapidly destroyed (emptied completely) and can be recognized only for a very short period of time. (ii) The transfer from the moist chamber to the medium was carried out very carefully in order to avoid mechanical disturbance as far as possible, but as a consequence this may have resulted in very different chances for germinated and ungerminated pollen to get transferred to the medium, the still ungerminated grains probably being less adhesive to their environment than the already germinating grains. (iii) As the pollen samples used to study germination after moist chamber treatment and after medium treatment are not identical but simply repeated and largely independent samples from the same pollen storage vessel, and as the moist chamber treatments for both procedures were realized also in independent repeats, some unknown modifications in germination conditions might have resulted in different germination conditions in the two moist chamber experiments. This last point again would emphasize the sensitivity of germination to small environmental modifications.

Differences between groups of trees. It is remarkable that in each year and on each medium the *A. incana* group never shows significantly lower germination percentages than each of the *A. glutinosa* groups; in fact in most cases the difference is significant. This might be interpreted as a species specific germination advantage of *Alnus incana* pollen, but it might also be an effect of the good growth and flowering conditions to which the parent trees used in this study had been exposed. Among the *A. glutinosa* groups, it is remarkable that the forest stand group shows very poor germination compared to the solitaire group in 1993, the forest stand trees apparently being more stressed by competition and showing less intensive flowering than the seed orchard trees. This might be considered as a hint that ecological conditions favoring intensive flowering also favor production of good quality pollen with superior germination percentage.

Physiological differentiation of genetically homogeneous pollen sets. A possible explanation for the germination differences could be the extreme physiological sensitivity to presumably small-scale environmental differences. The environment in our experiments consists of two main components: (1) the environment in the field from the time of meiosis in August (or even before, *e.g.* from the time of flower formation during the bud setting process) and (2) the experimental treatment from cutting to pollen shedding in the greenhouse. From observations in the field it is obvious that flowering behavior is very sensitive even to very small modifications of the environment. In the same seed orchard, ramets of one clone show differences in relative abundance of flowers, in male-to-female ratio and in time of flowering. Even within the crown of a single individual it can be observed that under certain conditions the pollen shedding starts earlier in those parts of the crown that are more exposed to the sun than those parts on the shadowed side, although the differences in light/shadow regime are very small because of the complete absence of leaves during the flowering period.

JOHNSON & SKRØPPA (1996) have reported strong evidence for extreme sensitivity of the reproductive process to local environmental conditions during the phase from flowering to ripened seed. It is believed that crucial and probably irreversible genetic regulations concerning physiological and phenological traits are determined during this period (comparable to imprinting phenomena). Haploid phases may be especially sensitive to such environmentally caused genetic switches (because of the unmasked condition of every active gene). Although the environmental sensitivity of the reproductive process seems to be restricted to the female part (at least for the morphological and phenological traits studied in the above-cited paper), pollen also can be considered to be sensitive because of its direct contact with environmental conditions and the absence of any buffering maternal tissue. We can therefore take into account the possibility of very complicated procedures of modification that may lead to highly differentiated sets of pollen, even if they were produced on the same plant within the same flowering period. The pronounced differences which are shown for pairs of clones under identical experimental conditions support this suggestion of high sensitivity to small-scale environmental differences. Moreover, it must be taken into account that the observed paternal effects could be due primarily to environmental modification and only slightly to genotypic differences. Further research would be necessary to gain insight into this problem.

Ranges of variation. Our experimental design allows comparison of variability of germination across years between media for each genotype. It turned out that for all genotypes studied in all three years the degree of variability on medium B is consistently lower than on medium A. One might therefore be tempted to conclude that medium B is less discriminating for differences in physiological state of the pollen. Yet, taking into account the finding that in the majority of cases medium B provides worse conditions for germination than A, the generally lower percentages may per se have a share in the reduced variability.

Relevance for mating system analysis. The germination medium represents the evident analogue of the stigma, as was argued in the introduction. On the basis of this analogue, the present results provide interesting indications as to the potential role of the studied factors in the mating system of *Alnus*. The most conspicuous observation concerns the high degree of consistency in the effects of the physiological state of the pollen. This observation, though measured over years, applies equally to the situation within a single year when considering the typically high variation of climatic conditions during the period of pollen development characteristic of *Alnus*. This is corroborated by the results obtained for pollen samples from the same and from different clonal ramets during the same flowering period, as discussed above. Additional evidence comes from the comparatively low overall germination percentages. Consequently, the consistency of the effect of physiological state of pollen would imply a strong and equally directed preference on all media for physiological state which, by the medium-stigma analogue, suggests a system of random preferential mating (see chapter 2.6 in GREGORIUS 1989) for this trait.

On the other hand, genotypes interact in multiple ways with the physiological state of pollen in germination on both media. From the viewpoint of mating preferences this implies different preference patterns on the two media for genotypes in total as well as in combination with various physiological states. Hence, pronounced assortative mating for genetic traits would prevail (see chapter 2.5 in GREGORIUS 1989) if, again, media could take the place of stigmas. Whether this assortment would be positive or negative cannot be decided by the present experimental design. Random mating would in any case be highly unlikely under this scenario.

These predictions are meaningful only in the absence of pollen type interference on a stigma. In fact, the germination experiments with pollen mixtures yield different results in this respect for the two media. An additional source for assortative mating can thus be expected from the differential capacity of stigma types to enhance interference among pollen types during germination.

Some experimental data for alder species show Hardy-Weinberg proportions (BOUSQUET *et al.* [1987a, b] for *Alnus crispa*; PRAT *et al.* 1992 for *Alnus glutinosa*) which might imply the absence of assortative mating effects. Concerning the effects discussed above, it seems more probable, however, that the variable mating preferences found in our investigation sum up in a way resulting in a loss of simple assortative mating patterns. Limited sensitivity of the applied statistical test may then explain the finding of non-significant deviations from the hypothesis of Hardy-Weinberg proportions.

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