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Differential germination in flax pollination

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SUMMARY

Counts of pollen germination percentages on stigmas and measurements of pollen tube lengths in the styles of flax were made for various crosses and F_1 back-crosses including reciprocal crosses and selfings. The results were analysed by the diallel method of GRIFFING. Both reciprocal effect and general combining ability were found. Some varieties had better-germinating pollen and styles allowing a more rapid pollen-tube elongation. The F_1 pollen germination showed lower rates on selfing and on back-crosses with the parents. In the same way, the styles of the F_1 discouraged germination and elongation of pollen tubes of both the F_1 pollen and that of the two parents. These effects may be explained by the diversity of action of secondary fertility genes. There are probably several of them, their action varying with number and nature. A cross between two homozygous autogamous varieties disturbs their functioning which may have been established gradually by natural selection. Possible reasons for the observed phenomena are discussed.

The acquisition of autogamy may also have been achieved by mutation at the cistron for the activation of pollen specificities whereas the styles retain their incompatibility reaction. That is the best explanation that can be given for reciprocal effects: the mutated pollen grain will have a more rapid germination and the pollen tubes a faster growth than those of a pollen grain with secondary fertility genes impeding more or less the incompatibility reaction.

Additional key words : *Pollen germination, pollen tube growth rate, autogamy, incompatibility, diallel analysis.*

RÉSUMÉ

Différences dans les vitesses de germination du pollen chez le lin cultivé.

Des comptages de pourcentage de germination de grains de pollen sur les stigmates et des mesures de longueur de tubes polliniques dans les styles ont été réalisés pour différentes combinaisons de croisements de variétés de lin y compris les croisements réciproques et les autofécondations. Ces observations ont également été faites pour deux F_1 et leurs back-cross. Les résultats ont été analysés par la méthode diallèle de GRIFFING. Des différences significatives ont été observées pour l'aptitude générale à la combinaison. Certaines variétés ont un pollen qui germe mieux et des styles favorisant davantage la germination et l'élongation des tubes polliniques. Le pollen des F_1 germe nettement moins bien en autofécondation et sur les styles des deux parents. De même, les styles des F_1 défavorisent la germination et l'élongation des tubes polliniques que ce soit pour l'autopollen ou le pollen des deux parents. Ces différences peuvent s'expliquer par l'action de gènes secondaires de fertilité contrariant le fonctionnement du gène d'incompatibilité. Leur mode d'action doit dépendre de leur nombre et de leur nature. Un croisement entre deux variétés homozygotes autogames perturbe leur fonctionnement qui a dû se constituer progressivement par sélection naturelle au cours des générations.

L'acquisition de l'autogamie a pu se faire également par mutation du cistron d'activation des spécificités du grain de pollen, les styles conservant leur réaction d'incompatibilité. C'est la meilleure explication qu'on puisse donner aux différences entre croisements réciproques: le pollen muté germera plus vite que celui qui a des gènes secondaires de fertilité contrariant plus ou moins le fonctionnement du gène d'incompatibilité.

Mots clés additionnels : *Germination du pollen, autogamie, incompatibilité, analyse diallèle.*

I. INTRODUCTION

Abnormal segregations for flower colours in cultivated flax (*Linum usitatissimum* L.) have been observed for a long time (TAMMES, 1914). Competition between female gametes has been proved by KAPPERT (1935) in his studies on the shortage of anthocyanin devoid genotypes in F_2 segregations of flax. Later studies on other flower colour genes by PLONKA (1971) showed alterations of segregation proportions due to differential fecundation by male gametes. All

results lead to the assumption that the deviations from mendelian proportions in crosses between blue and white flowers of the n^c type are due to the linkage of the n^c gene with a supposed weakened incompatibility gene. Linkages between an incompatibility locus and marker genes have been proved only recently. A linkage with a peroxidase isozyme locus in rye has been found by WRICKE & WEHLING (1985). As many species of *Linum* are heterostylous and as the pollen grains contain three nuclei, the incompatibility mechanism is assumed to be of the sporophytic type.

The heterostylous primroses, also of the sporophytic type, show an example of conversion to homostylous self-fertility. PIPER *et al.* (1984) observed better a fertility in a self-fertile homostyle variant of *Primula vulgaris* in terms of seeds per plant. Cultivated flax is usually autogamous but various degrees of allogamy are observed. It is probable that adaptation from allogamy to autogamy followed the general scheme proposed by LEWIS (1960) i.e. a mutation of activation of pollen specificities, the styles maintaining their incompatibility reaction. But, to explain the occurrence of partial allogamy and the lack of complete autosterility, another device for autogamy is supposed to exist, namely the development of secondary fertility genes thwarting the incompatibility reaction.

Using marker genes in pollinations with half and half pollen mixtures, enabling the types of pollen which achieve fecundation to be recognized in the offspring, PLONKA *et al.* (1968) showed a strong departure from the 0.5 proportion. The proportion of the n^c gene was much reduced when the pollen mixture ($n^c + N^c$) was deposited on the styles of the n^c parent. On the styles of the other parent (having the allele N^c), the proportion of this marker was a little lower than 0.5 but not significantly. In backcrosses on the other hand, the strong departure occurred with both parents (PLONKA, 1971, table 9). These observations fit well with the hypothesis of a weakened sporophytic incompatibility gene where the specificities of the two parents are transmitted together in the pollen grains of the F_1 . They are activated only in the pollen grains bearing a weakened but not mutated incompatibility gene. Since the styles always maintain their ability to react, the pollen with activated specificities (the n^c pollen) germinates more slowly than the other (the N^c pollen) on both parents.

The rate of natural hybridization generally lies between 1 and 5 %. Nevertheless it is possible to breed lines descended from crosses where the proportion of natural hybrids is much higher by selecting at each generation the offspring showing more hybridization. In a current experiment which uses lines homozygous for two or three recessive genes for flower colour, more than 50 % of natural hybrids have been found (when the plants were allowed to flower in a nursery).

The purpose of these experiments is to show that there are really differences in the germination delay of pollen and in the growing speed of pollen tubes according to various cross-combinations.

The practical aim of this work is the possibility of working out a method of F_1 seed production by selecting lines which will be almost allogamous but remaining self-fertile if submitted to selfing. If mixed, these lines would intercross and flower-colour markers should give the proportion of heterozygous F_1 plants.

II. MATERIAL AND METHODS

As the experiments were undertaken during the months from September to December, the plants were grown in a heated glasshouse supplied with a double light source with high output. Each floodlight was equipped with a sodium vapour lamp of high pressure giving a maximum emission in the red-orange wave-

length and a mercury vapour and metallic iodide lamp allowing an enrichment in the blue. The electric power consumed by each source was about 400 W. According to BOYER & DE PARCEVAUX (1975), the double light source is more efficient in terms of PAR (photosynthetically active radiation) emission than either incandescent, fluorescent or mercury halide lamps; the ratio of efficacy being of about 1.5. A glasshouse without electric lighting, receives in winter, one tenth of the light of the natural conditions of growth in spring and in summer. With this double light source, the ratio is reduced to 1/4.5. The auxiliary lighting was given 2 hours in the evening and from midnight to nine o'clock in the morning. Being under far from natural conditions, the plants were weakened during the months of November and December. To reduce etiolation, the night temperature was maintained at 7 °C. The plants were grown in tanks filled with river sand mixed with fine gravel and fed with a nutrient solution.

Flax flowers are very short-lived and because the pollen grains begin to germinate after 10 mn, the handling time must be reduced as much as possible. Pollinations were made on detached flowers. Of the 5 styles of a flower, 4 were excised and about 10 pollen grains were deposited on the remaining stigma with the help of a binocular magnifying-glass and then counted. After the timed germination period, the flower was immersed for 12 to 24 hours in modified Carnoy's fixative made of 3 parts of alcohol, 2 of chloroform and 1 of acetic acid. In order to observe the pollen tubes at different levels in the style, 12, 15 and 18 mn germination periods were allowed to elapse before fixation. The figures given in the tables are the means of 4 styles belonging to 4 different flowers. The germination period chosen was of 15 mn. The germination rates ranged between 15 and 40 % at the time of fixation, thus 2 to 4 pollen tubes were present in each style, since about 10 pollen grains were deposited on the stigma which was retained in the flower. The lengths of the pollen tubes, in μm , are the means of 6 to 15 tubes, approximately, measured on 4 flowers involving about 40 pollen grains. The temperature of the laboratory was about 18-20 °C.

Five varieties were used: two fiber flaxes, 'Datcha' and 'Silva', two linseed flaxes, 'Ocean' and 'Nored', and a short and very early variety of the Versailles collection, 'Bombay R 88'. Their pollen diameters are given in table 1. As a rule, cultivated flax is highly

TABLE 1
Diameters (μm) of pollen grains. Numbers observed per size class.
Diamètres (μm) des grains de pollen.
Nombres par classe de dimensions.

	45	45-50	50	Size of sample
R 88	17	29	26	72
F_1 : Ocean \times R 88	7	13	21	41
Ocean	2	14	50	66
Nored	2	8	36	46
Silva	6	13	18	37
Datcha	10	10	1	21
Homogeneity $\chi^2_5 = 63.4 - P 0.01 = 15.1$				

autogamous as the varieties are mostly near homozygosity but 'Silva' and 'Bombay R 88' have a natural hybridization rate often exceeding 5 %.

Two types of measurements were made after germination of pollen grains put on the stigmas : 1) percentages of germinated pollen grains after a definite time, and 2) the length of pollen tubes growing in the styles at the moment of fixation. Before staining with aqueous light green, the styles were submitted to a digestion by pectinase. Taken from GURR (1965), this method derives from PANDEY & HENRY (1959) and has given good results in selfsterility research with *Physalis*. In our stainings, the pollen tubes were entirely visible and allowed measurement with an ocular micrometer. Figure 1 shows two photographs of pollen tubes stained *in situ* in this manner. The styles, fixed in Carnoy fixative, were put directly into a 5 % macerozyme solution adjusted to pH 4 with 1N HCl. Next, the styles were transferred to an acid fuchsin-light green stain for 24 hours. The stain consists of 15 parts of 1 % aqueous light green solution to one part of Darlington & La Cour's acid fuchsin-light green stain. This last consists of 6 ml glycerol, 1 ml fuchsin acid at 1 % and 1 ml light green at 1 % for 54 ml lactic acid.

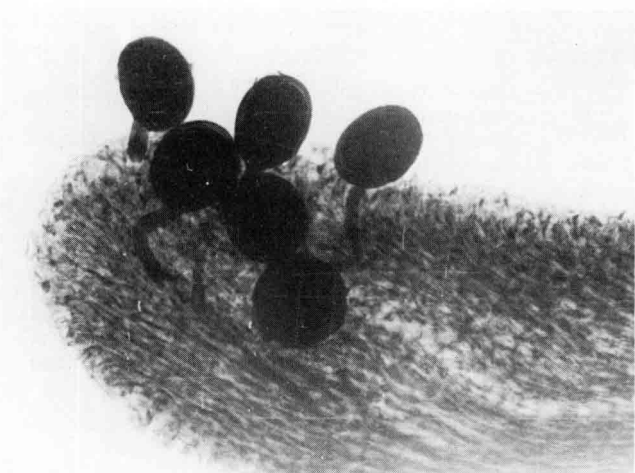
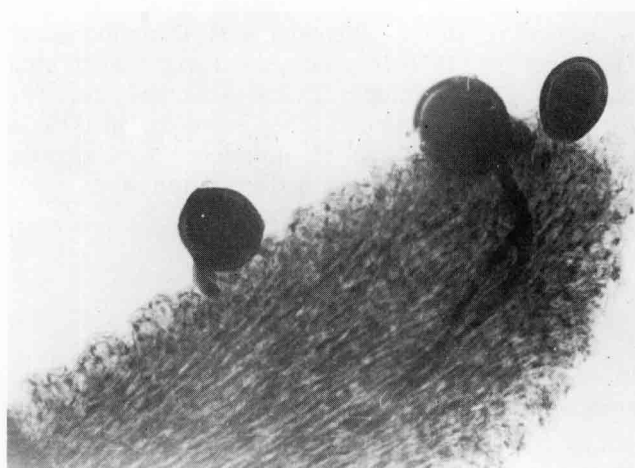


Figure 1.

Pollen tubes stained "in situ" by using pectinase and aqueous light green.

Tubes polliniques colorés in situ en utilisant la pectinase et le vert lumière.

A. Adjusting the method

A test of the germination time taken by the pollen between its being put on the stigmas and immersion in Carnoy fixative was made (table 2). Too low a percentage of pollen grains had begun to germinate after 12 mn, with a laboratory temperature of 18 to 20 °C. Indeed, the mean of the 4 varieties, 'Ocean', 'Nored', 'Datcha' and 'Silva', was only 18 % with a mean length of tube of 47 μ m. The data in table 2 show that the best germination time before fixation was about 15 mn. The mean percentage of germinated pollen grains was then about 21 % with a mean pollen tube length of 117 μ m. At the end of 18 mn, the mean percentage of germination was 40 % and the tubes measured, on average, 156 μ m. They were then too long and became entangled, rendering measurement difficult. Therefore all our data were taken from styles where the pollen was given 15 mn to germinate.

The slower progress of the pollen tubes of the variety 'Silva' after selfing was highly significant with respect to the interaction time \times variety. Not all the pollen grains germinated at the same time and there always remained some grains which did not germinate even if a longer time was allowed before fixation. Those which germinated earlier had longer tubes, and the question arises whether earliness alone suffices to give an advantage in pollen-tube competition. That is why we always noted the percentage germination before measuring the length of the pollen tubes.

B. Statistical method of analysis

Each type of experiment comprised all possible cross-pollinations between the lines involved including reciprocal crossings and selfpollinations. Therefore, the data lent themselves to analysis by the diallel method of GRIFFING (1956), using a fixed-effects model: the conclusions then referred only to the parents in the experiment. The n parents of each experiment gave n^2 data units, i.e. n^2 measures of pollen grain germination percentages and pollen tube length.

Each experiment was repeated at 3 different dates considered as blocks. A normal analysis of variance was done for each measurement to test the overall effect of the crossing combination and the date effect. Further analysis was carried on only if there was a significant effect. Three types of effects were then investigated :

1. *General combining ability* to see if a parent gave on the whole a better percentage of germination or a more rapid growth of the pollen tubes in the styles.

2. *Specific combining ability* to see if a parent reacted differently according to the partner of the cross combination including selfpollination.

3. *Reciprocal effects* to see if there was a difference according to the direction of the cross, the variety being used as male or female parent.

When one of these overall effects was significant, individual tests of significance were made.

As the date effect was often significant, a normal analysis of variance was carried out to test the

TABLE 4
Cross combinations between parents and F_1 .
Combinaisons de croisements entre 4 variétés et F_1 .

Pollen	Style	1° F ₁ 'Ocean' × 'R 88' - 1976						2° F ₁ 'Nored' × 'R 88' - 1976					
		September 21 %	September 21 μm	December 6 %	December 6 μm	December 10 %	December 10 μm	November 23 %	November 23 μm	November 25 %	November 25 μm	November 29 %	November 29 μm
R 88	R 88	38.8	81.8	46.9	112.1	29.6	55.5	31.9	79.6	49.6	157.5	49.0	96.0
	Oce/Nor	41.5	136.3	47.5	126.2	35.7	66.5	54.3	149.5	57.4	176.4	21.9	85.3
	F ₁	31.0	108.7	17.9	100.6	24.0	51.7	35.6	49.8	37.4	54.6	36.8	82.8
Oce/Nor	R 88	34.6	142.0	35.2	82.8	42.5	89.7	24.3	60.4	68.8	139.1	33.2	68.2
	Oce/Nor	28.7	81.4	41.0	76.0	31.4	76.5	35.6	97.7	34.4	146.6	8.1	63.2
	F ₁	26.0	110.4	22.8	32.2	6.5	69.9	43.2	55.4	39.8	94.3	21.1	73.6
F ₁	R 88	9.3	97.7	17.5	80.5	17.3	35.6	11.5	11.5	27.9	59.8	22.7	47.6
	Oce/Nor	18.3	75.5	31.2	56.8	8.5	63.2	23.5	42.4	11.5	20.1	16.4	30.2
	F ₁	8.3	46.0	26.3	51.7	6.8	80.5	0.0	0.0	5.7	71.9	9.9	17.2
Coefficients of variation													
percentages 26.1, μm 30.4						percentages 40.6, μm 34.6							
General combining ability for pollen germination percentages													
R 88 16.87, Ocean 11.52, F ₁ – 28.39						R 88 25.3, Nored 5.1, F ₁ – 30.4							
level of significance ± 7.94						level of significance ± 13.8							
General combining ability of the lengths of pollen tubes													
Interaction date × style						R 88 49.57, Nored 42.69, F ₁ – 92.25							
						percentages F = 4.31* P 0.05 = 3.84							

III. RESULTS

Table 1 gives the frequencies of the pollen diameters of these varieties and also that of the F_1 'Ocean' × 'R 88'. 'Ocean', which has the biggest seeds and also the largest flowers, gives the biggest pollen grains with a diameter of about 50 μm. The correlation between size of flowers and size of pollen is not high, since 'Datcha' which has flowers of the same size as 'Bombay R 88' clearly has smaller pollen grains. The diameter of a pollen tube is about 12 μm and the distance from ovules to stigmata is approximately 5,000 μm for fiber flaxes and 7,500 μm for linseed varieties with large flowers of the Moroccan type.

Correlation between germination percentage and length of pollen tubes

The time taken by the pollen to germinate varied. Even 20 mn after putting the pollen on the stigmata, although some grains were already empty, a quarter had not yet sent out a tube. A pollen tube which has begun to elongate earlier is necessarily longer, assuming a constant speed of elongation.

The correlation coefficient r computed on the 114 pairs of observations in tables 2, 3, and 4 is highly significant with a value of + 0.558. The determination coefficient $r^2 = 0.312$ shows that 31.2 % of the variance of the pollen tube lengths can be assigned to its linear regression on the percentage germination. An attempt to reduce the error variance by using the analysis of covariance was not successful.

1. Cross combinations between four varieties (table 3)

The analysis of variance shows a significant genotypic effect for the percentage of germination but not for the pollen tube length probably on account of a greater coefficient of variation which amounts to 27.5 % for the percentages and 48.3 % for the lengths. The date effect on the other hand, whereas not significant for the percentages, is highly significant for the length with timing of pollinations going from September 28 to October 19 to November 8. The slowing down of the growth of the tubes is more marked for the second date (table 3). It is known that flax plants do not grow well when the day length is diminishing in autumn, and the proportion of successful hand crossings in the glass-house then diminishes drastically.

There is a significant difference in general combining ability for pollen germination percentages: 'Datcha' has a pollen which germinates better and there is a better germination in its styles too (table 3).

There are also significant reciprocal effects in the crosses 1 (table 3, 'Ocean' × 'Nored' and reverse) and 5. By contrast, there seems to be no reciprocal effect for the crosses 2 and 3. The difference in reciprocal effect between crosses 3 and 5 is slightly greater than the level of significance. Such effects have already been observed in other experiments based on fertilizations with pollen mixtures (PLONKA *et al.*, 1968).

No significant differences are observed in specific combining ability. If the coefficient of variation had been lower, the worse pollen germination of 'Silva' on selfing would perhaps have been significant.

There was a significant date × pollen interaction for both the percentage and the length due to a lowering of

germination and growth of the pollen of 'Nored' on October 19 (table 3).

The interaction date \times style, for the tube length only was also found in 'Nored'. This variety appears to be more sensitive to the reduction of the day length between October 19 and November 8 which resulted in a slowing down of the mean elongation speed of the tubes of pollen of the 3 varieties in the cross combinations.

2. Cross combinations between parents and F_1

The analysis of variance for overall genotypic effect gives highly significant differences for the germination percentages with both F_1 , 'Ocean' \times 'R 88' and 'Nored' \times 'R 88'. The pollen tube lengths show significant differences only with the second F_1 .

As with the cross combinations between 4 varieties, there is a marked date effect which is always significant except for percentages of the second F_1 . The germination rate and the pollen tube length diminished as the days drew in. The sunshine level the day before the flowers were cut off may also have an influence as might be the case for the data of the F_1 'Nored' \times 'R 88'. There was much better pollen tube growth on November 25 (the second date) attributable perhaps to a much longer insolation the day before: 0.5 versus 0.1 for the first date and 0 for the third (1 = maximum insolation, 0 = no insolation at all).

The general combining ability was significant in both F_1 s for percentage germination, but the differences between pollen tube lengths were significant (highly) only for the second F_1 . The F_1 condition slowed down the germination and the growth of the pollen tubes in the styles (table 4).

The interaction date \times style was significant for the germination percentage with the F_1 'Nored' \times 'R 88' which fell much more in the styles of 'Nored' on November 29 than in those of 'R 88' or the F_1 (table 4). Again, the styles of 'Nored' were more sensitive to the reduction day length and the lack of sunshine.

IV. DISCUSSION

Many of the *Linum* species have an heteromorphic system of incompatibility and some of the wild species are homostylous and self fertile (OCKENDON, 1968). Hence, *L. usitatissimum* is not the sole self fertile species of the genus. Our previous and present results agree with the hypothesis of a sporophytic incompatibility gene which can either mutate for pollen specificities activation or be thwarted by other genes we call secondary fertility genes. In *Trifolium hybridum* L., TOWNSEND (1969) studied an allele which suppressed in the heterozygous state and for a given genotypic environment the action of some S alleles. In the tomato, MARTIN (1968) showed the presence of a sporophytic dominant switch gene necessary for self-incompatibility expression. Likewise, one or several major genes, independent of the S locus, have been described by THOMPSON & TAYLOR (1966, 1971) in *Brassica* and by RONALD & ASCHER (1975) in *Chrysanthemum morifolium*, which act on the expression of self-incompatibility. Other authors (see DE NETTAN-

COURT, 1977) have demonstrated the action of several genes preventing the normal functioning of the self-incompatibility gene. Recently, DANA & ASCHER (1985) evidenced the presence of a discriminating style and a pollen-mediated pseudo-self compatibility phenomenon which modifies the function of the self-incompatibility system. 'The genes responsible may be the sort described by MATHER (1943) in his proposed scheme for the evolution of self-incompatibility' working on *Cichorium intybus* L., DETCHEPARE (1985) comes to the conclusion that the weakening of the self-incompatibility system is to ascribe to modifying alleles.

The action of the environment on the germination percentage of the pollen grains and the elongation speed of the pollen tubes in the styles has also been observed by RICHARDS & THURLING (1973) on *Brassica campestris* L.

In our experiments all varieties showed, on the whole, a more or less pronounced slowdown in the speed of germination and elongation of the pollen tubes as the day drew in, the linseed 'Nored' being the most sensitive to the lack of insolation. The decline in vigour of the fecundation process concerns the function of the styles in promoting the elongation of the pollen tubes as well as that of pollen grain germination. This effect seems to be due to a general physiological cause rather than to a weakening of the action of the secondary fertility genes.

The aim of our investigation has been to check if there were any differences in the speed of germination and elongation between the various crosses. The better germination on the whole of the pollen of 'Datcha' can be explained either by a more pronounced action of the secondary fertility genes in thwarting the functioning of the incompatibility gene or by a mutation preventing activation of the pollen specificities. The occurrence of differences in reciprocal crosses is understandable with the aid of the hypothesis of LEWIS (1960): in mutated strains, the styles maintain their incompatibility reaction while the pollen specificities are not activated. If one of the parents is of this type and the other has reached autogamy by the aid of secondary fertility genes, the pollen of the first type always germinates normally and that of the second is hampered when the S allele of the female parent is incompatible. If the parents of a cross are compatible there should be no differences in reciprocal crosses. That is the case of 'Ocean' \times 'Datcha' and 'Ocean' \times 'Silva'. In the cross 'Nored' \times 'Silva', the two parents must be incompatible for their S alleles.

Differences in reciprocal crosses might also be explained without the mutation hypothesis. They may occur if the secondary fertility genes are more efficient in the pollen of one of the parent. In the pollen mixture experiments with marker genes (PLONKA *et al.*, 1968), the explanation is similar: the proportion of a marked pollen which performed fecundation varied with the female parent fertilized.

The fact that F_1 plants give pollen which germinates worse than average and have also styles which restrain germination may be explained by a perturbation of the functioning of the secondary fertility genes. The establishment of the normal function is carried out by

natural selection during the generations following a crossing. In our plant breeding work on flax and linseed, lines with a high outbreeding system sometimes appear in the first generations after crosses between autogamous varieties.

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