Sex Plant Reprod (1996) 9:153–160 © Springer-Verlag 1996

ORIGINAL PAPER

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Dynamics of pollen tube growth under different competition regimes

Received: 15 November 1995 / Revision accepted: 7 February 1996

Abstract Pollen tube dynamics following different competition regimes were studied in sweet cherry (*Prunus avium* L.). In the process from pollination to fertilization, a constant reduction in the number of pollen tubes that travel along the style is observed. There could be two main causes of this reduction. One is a physical or physiological constraint consisting of the progressive decrease in the reserves and space available for pollen tube growth along the transmitting tissue of the style, and the other is genetic interaction both among the male gametophytes and between the male gametophytes and the female tissues of the flower. To evaluate the roles that these two forces play in reducing the number of pollen tubes that travel along the style, pistils were subjected to various pollen competition regimes by applying different mixtures of live and dead pollen onto the stigmata. The results obtained were similar when the experiment was repeated with different genotypes over 2 years, both in the laboratory and in the field. The role of stylar constriction is important, but it is not the only cause of pollen tube attrition because with low pollen loads fewer pollen tubes reach the different parts of the style than could fit therein. The fact that under different pollen competition regimes the number of pollen tubes is reduced by the same proportion in each stylar level indicates that genetic interactions play an important role in the control of pollen tube attrition.

Key words Cherry · Gametophytic competition · Pollen competition · Pollen tube growth · *Prunus*

Introduction

In plants and animals alike, postpollination or postcopulation mating success is based primarily on two mechanisms. One is an intrasexual mechanism, the competition among the male gametes in animals (sperm competition) or among the male gametophytes in plants (pollen competition). The other is an intersexual mechanism, consisting in female mate choice that could interact with the male-male competition. These two mechanisms have been intensely studied in numerous animal species but only recently have their implications in plants started to be considered (review, Hormaza and Herrero 1994). Furthermore, due to their sessile nature, active mate choice does not occur in plants; consequently, postpollination mechanisms could be more important in plants than postcopulation mechanisms in animals.

During the gametophytic phase, large numbers of both male and female gametophytes are produced in plants. However, only a small proportion of these achieve fertilization. Although in many species the number of ovules exceeds the number of seeds finally produced (Stephenson 1981; Charlesworth 1989; Herrero 1992b; Rigney 1995), this reductive process is more evident in the male gametophytic generation. In most plant species, the number of pollen grains deposited on the stigma greatly exceeds the number of ovules available for fertilization (Willson and Burley 1983; Snow 1986; Cruzan 1989; Levin 1990; Marshall and Folsom 1991; Plitmann 1993) and, consequently, numerous male gametophytes are lost inside the pistil during the process that extends from pollination to fertilization. Thus, the final outcome of the fertilization process is the result of a series of steps that determines which male gametophyte among all the possible candidates is going to fertilize the embryo sac. Whereas this phenomenon should be far more intense in species that produce single-seeded fruits, most of the studies of pollen competition in plants have been carried out using species that produce multiple-seeded fruits. Additionally, one of the main problems in studying the effects of pollen competition in multiple-seeded fruit species is that the degree of pollen competition can affect the number of seeds per fruit and this could interfere with the results obtained when studying the vigor of the resultant sporophyte (Charlesworth 1988). Furthermore, fruits with a low number of seeds could abscise

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more easily than fruits with a high number of seeds, causing a bias in data from the resulting sporophytic generation (Stephenson and Winsor 1986). Thus, species that present a single ovule per flower could become an interesting system in which to study the mechanisms of pollen competition in plants (Niesenbaum and Casper 1994). This is the case with *Prunus*, where, although initially two ovules differentiate and develop in the single ovary present per flower, one of them degenerates soon after pollination, leaving only one ovule to be fertilized (Pimienta and Polito 1982; Arbeloa and Herrero 1991).

Whenever the fate of pollen tubes has been observed along the progamic phase, it appears to follow a fixed pattern of reduction since the initial number of pollen tubes is progressively reduced along the style. This reduction has been reported in many different plant species (Ockendon and Gates 1975; Sedgley 1976; Herrero and Dickinson 1980; Pimienta et al. 1983; Kenrick and Knox 1985; Kahn and DeMason 1986; Cruzan 1986, 1989; Hossaert and Valéro 1988; Herscovitch and Martin 1990; Scribailo and Barret 1991; Herrero 1992b). Although this pattern of reduction appears to be fixed, very little is known about how it is regulated. In some species the anatomy of the style appears to function as a regulator because its funnel shape provides a progressive reduction of space and reserves (Cruzan 1986; Herrero 1992b). It has been proposed (Herrero 1992b) that this reduction plays an important role in the decrease in the number of pollen tubes. On the other hand, the progamic phase seems to be well suited to propitiate male-female interaction (Cheung 1995) and, as a result of this interaction, the growth of some male gametophytes could be arrested. This interaction and tube attrition has been extensively studied in the self-incompatibility reaction between closely related individuals (Lewis 1994), in interspecific incompatibility between non-related individuals (de Nettancourt 1977), and in the elimination of defective genotypes (Chasan and Walbot 1993). However, it is still not clear to what extent pollen-pistil interactions may play a role in determining which male gametophytes achieve fertilization in compatible matings. Indirect evidence is accumulating suggesting that fertilization is not always a random process (Mulcahy and Mulcahy 1987) and that mate choice may effectively occur in plants (Marshall and Folsom 1991).

In order to evaluate to what extent pollen tube attrition is a passive reflection of the decrease in the space and reserves available along the pistil or an active pollen-pistil or pollen-pollen genetic interaction, we used different pollination densities and monitored pollen tube dynamics along the style in cherry (*Prunus avium* L.). If pollen tube number reduction is exclusively a reflection of space and reserves, then, regardless of the initial number of pollen grains deposited on the stigma, each pistilar level will be able to support the growth of a certain number of pollen tubes. Alternatively, if genetic interactions play an important role and only some of the male gametophytes are well suited for a particular mating, then the proportion of tube attrition should be constant along the pistil regardless of the number of pollen grains deposited on the stigma, and, thus, different pollen tube numbers will be recorded at each pistilar level depending on the initial size of the pollen load.

Materials and methods

The experiment was repeated over 2 years, with four different genotypes. The first year, the experiment was performed in the laboratory with cut flowers over florist's foam, crossing the cultivars Van×Ambrunes. The second year, a similar experiment was conducted in the field, leaving the flowers in the tree and crossing the cultivars Vignola×Vic. The trees were maintained at the SIA-DGA experimental orchards located at the Campus de Aula Dei in Zaragoza, Spain. Flowers were collected at the balloon stage 1 day prior to anthesis, and the anthers were removed from the flowers and placed on white paper at room temperature. After 24 h the anthers dehisced and pollen was collected by sieving through an 80-μm screen. The collected pollen was used to pollinate flowers that had been emasculated at the balloon stage the previous day by removing the anthers and petals to make them unattractive to insects (Free 1964). The treatment consisted in the application of different loads of viable pollen to the stigmata. This was achieved by mixing, in different proportions, known amounts of live and dead pollen. Dead pollen was obtained by placing the pollen in the oven at 90°C overnight. Five different pollen loads were applied to the stigmata: 4:0, 3:1, 1:1, 1:3 and 0:4 live:dead by weight. Five pistils per treatment for the Van×Ambrunes cross and ten for the Vignola×Vic cross were fixed in FAA 1:1:18 (formalin:acetic acid:70% ethanol). The pistils were fixed when pollen tube growth had already reached the base of the style, 3 days after pollination in the Van×Ambrunes cross (made in the laboratory) and 4 days after pollination in the Vignola×Vic cross (made in the field). Pollen tube growth along the style was monitored on squash preparations of pistils washed in distilled water, boiled in 5% sodium sulphite to soften the tissues (Jefferies and Belcher 1974) and stained with 0.1% aniline blue in 0.1 N K_3PO_4 (Linskens and Esser 1957). Preparations were examined with an Ortolux II microscope equipped with UV epifluorescence, using a BP 355–425 excitation filter and an LP 460 barrier filter. For each cross and treatment, the total number of dead and live pollen grains was recorded at the stigma with a hand counter by scanning the entire stigmatic surface. To evaluate the rate of pollen tube attrition, the number of germinating grains was recorded at the stigma and the number of pollen tubes present was recorded at the transmitting tissue entrance, at one quarter, one half and three quarter of the way down the style and at the ovary entrance. Data were subjected to analysis of variance, and the Student-Newman-Keuls test was used to compare treatment effects.

Table 1 Means (±SD) of the number of pollen grains deposited on the stigma and of the percentage of germination with the different treatments. Each mean is based on measurements from five and ten pistils for the Van×Ambrunes and Vignola×Vic crosses, respectively. Values within columns followed by the same letter are not significantly different according to the Student-Newman-Keuls test (P=0.05)

Proportion live:dead	Vignola×Vic		Van×Ambrunes	
	Number of grains	% germination	Number of grains	% germination
4:0	319±66 a	61±5 a	245±69 ab	64±9 a
3:1	408±101 b	53±7 b	264±46 ab	55±15 ab
1:1	300±69 a	48±6 c	302±78 a	43±9 bc
1:3	298±56 a	35±4 d	223±84 b	38±11 c
0:4	372±17 b	0 d	116±13 c	0 d

Results

Pollen left in the oven at 90°C overnight was efficiently killed, and no germination was recorded in either cross with the 0:4 live:dead treatment. However, when dead pollen was mixed with live pollen, pollen germination of the mixture was higher than expected considering the composition of the pollen mixtures (Table 1). In spite of this fact, the use of mixtures of live and dead pollen had a clear effect on pollen germination on the stigma, and the germination percentages obtained with the five different pollen loads applied were significantly different between pollen loads and not significantly different between the two crosses (Fig. 1).

In spite of careful application of pollen trying to evenly coat each stigma, the total number of pollen grains deposited varied among stigmata (CV=37.91 and 24.12 for the Van×Ambrunes and Vignola×Vic

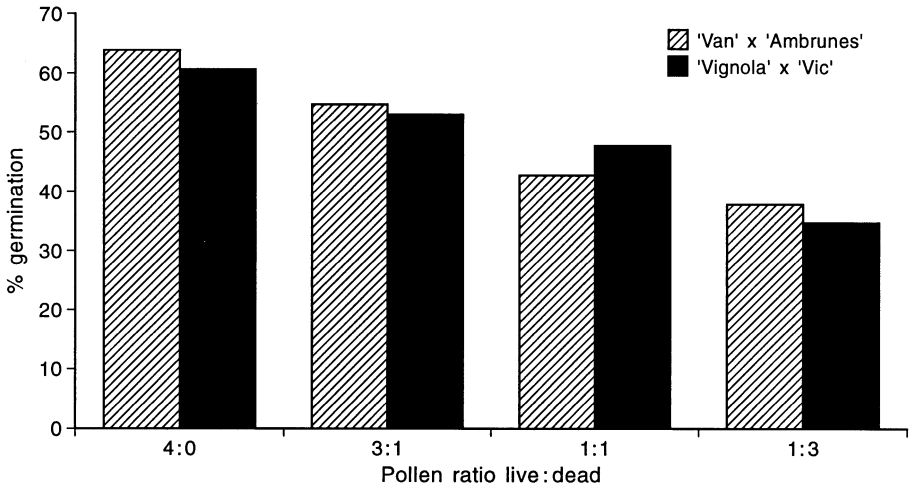
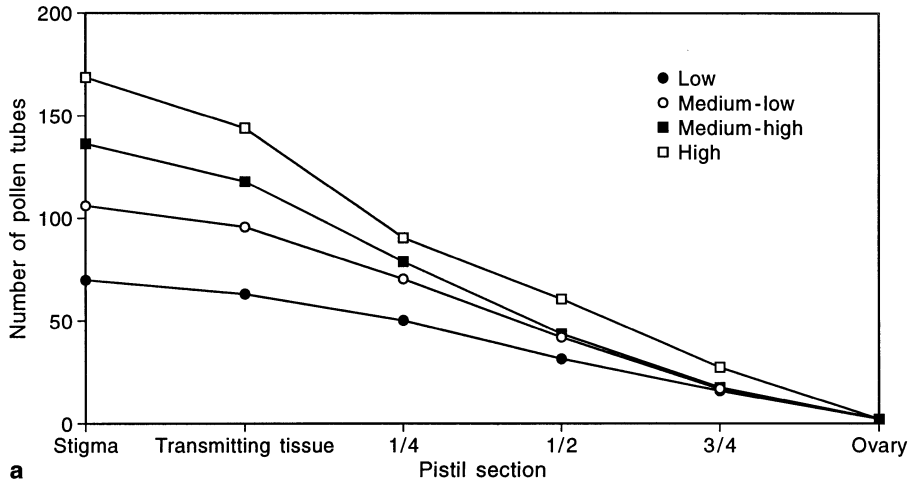


Fig. 1 Means of pollen germination percentages with four different live:dead pollen mixtures for the two crosses



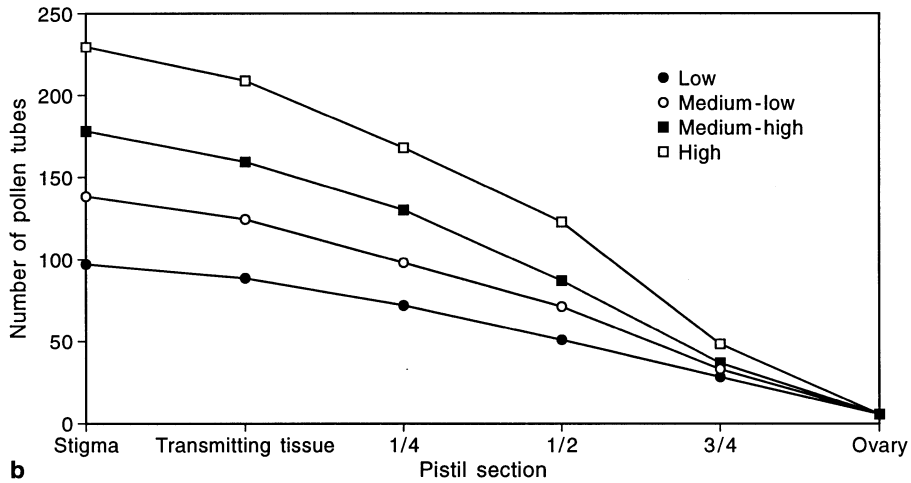


Fig. 2a, b Means of the number of pollen tubes in each level of the style. **a** Van×Ambrunes; **b** Vignola×Vic

Table 2 Means (±SD) of the number and percentage of pollen tubes at different levels of the pistil 72 h after pollination for the Van×Ambrunes cross. Percentages are based on 100 in the stigma. Each mean is based on measurements from five pistils. Values within columns followed by the same letter are not significantly different according to the Student-Newman-Keuls test (P=0.05)

Treatments		Pistil level					
		Stigma	Transmitting tissue	1/4 style	1/2 style	3/4 style	Ovary
Low	number	70±15 a	63±14 a	50±13 a	32±6 a	17±5 a	3±1 a
	%	100±22 a	91±20 a	71±18 a	46±9 a	24±7 a	5±2 a
Medium-low	number	106±12 b	96±10 b	70±9 b	42±3 b	19±4 a	2±1 a
	%	100±11 a	90±10 a	66±8 a	39±3 ab	18±4 ab	2±1 b
Medium-high	number	137±8 c	119±10 c	79±17 cb	45±12 cb	19±7 a	3±1 a
	%	100± 6 a	87±7 a	58±12 a	33±9 b	14±5 b	2±0 b
High	number	169±12 d	144±12 d	90±13 c	61±12 c	28±11 a	2±1 a
	%	100±7 a	85±7 a	53±8 a	36±7 ab	16±6 ab	1±0 b

Table 3 Means (±SD) of the number and percentage of pollen tubes at different levels of the pistil 96 h after pollination for the Vignola×Vic cross. Percentages are based on 100 in the stigma. Each mean is based on measurements from ten pistils. Values within columns followed by the same letter are not significantly different according to the Student-Newman-Keuls test (P=0.05)

Treatments		Pistil level					
		Stigma	Transmitting tissue	1/4 style	1/2 style	3/4 style	Ovary
Low	Number	97±14 a	89±15 a	72±15 a	51±12 a	28±6 a	6±2 a
	%	100±15 a	91±15 a	73±16 a	52±12 a	29±6 a	6±2 a
Medium-low	Number	139±15 b	125±16 b	98±15 b	72±15 b	33±5 ab	6±1 a
	%	100±11 a	90±11 a	70±11 a	52±11 a	23±3 b	4±1 b
Medium-high	Number	180±15 c	161±20 c	130±2abc	88±14 c	36±5 b	6±2 a
	%	100±8 a	89±11 a	72±14 a	49±8 a	20±3 c	3±1 c
High	Number	231±36 d	210±33 d	168±24 d	123±22 d	48±13 c	5±2 a
	%	100±15 a	91±14 a	73±11 a	53±10 a	21±6 bc	2±1 d

crosses, respectively) and, consequently, among the different pollen treatments (Table 1). Therefore, in order to evaluate pollen tube attrition independently of the number of pollen grains deposited onto the stigma, for each cross treatments were placed into one of four different classes depending on the number of germinating pollen grains on the stigma. The four classes were called high, medium-high, medium-low and low competition.

A reduction in the number of pollen tubes growing down the style was recorded in all four treatments that included live pollen. The pattern of reduction was very similar for the two crosses involving different genotypes, in different years and carried out in different places (field and laboratory) (Fig. 2a,b). Regardless of the number of pollen grains germinated on the stigma, only two or three tubes in the Van×Ambrunes and five or six tubes in the Vignola×Vic crosses penetrated the ovary and, eventually, only one pollen tube can fertilize the ovule (Tables 2, 3). The differences in the number of germinating pollen grains on the stigma among the different pollen loads are maintained when the pollen tubes penetrate the transmitting tissue and grow along the first half of the style. However, the differences become progressively less significant in the second half of the pistil, where for each cross the number of pollen tubes becomes more independent of the number of tubes present in the stigma and, eventually, the final pollen tube number in the base of the style is approximately the same for the four treatments in each of the two crosses.

While clear differences are recorded among the different pollen loads in the number of pollen tubes that reach each pistilar level, the percentage of pollen tubes crossing each pistilar section (based on the initial number of pollen grains present at the stigma) is approximately constant along the style regardless of the number of pollen grains present on the stigma (Fig. 3a,b).

Discussion

Modulation of pollen competition with different pollen doses

The results show that a dramatic reduction in the number of pollen tubes as they grow along the style occurs in cherry. These results were similar in the two crosses studied in spite of the different cultivar combinations and of the different conditions in which the experiment was carried out, in the laboratory and in the field. The observations that different numbers of pollen tubes grow in the style depending on the initial number of pollen grains and that the number of pollen tubes is reduced by the same proportion under different pollen load treatments suggest that genetic interactions may play a major role in the regulation of pollen tube attrition.

Although many studies have reported, in other species, the reduction in the number of pollen tubes growing down the style, only in a few cases has the pattern of that

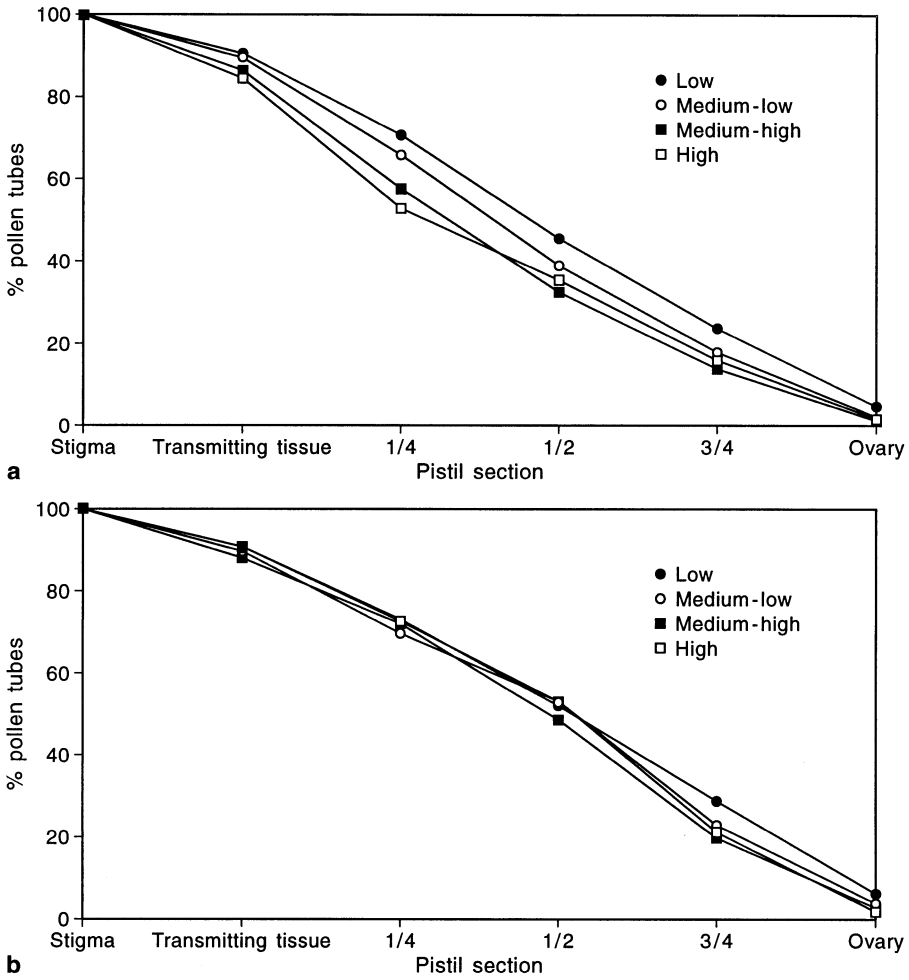


Fig. 3a, b Means of the percentage of pollen tubes at each level of the pistil calculated by dividing the number of pollen tubes at each section by the number of germinating pollen grains on the stigma. **a** Van× Ambrunes; **b** Vignola×Vic

reduction been studied. The results obtained show a high variability among species. In some species, the main bottleneck seems to be in the upper portion of the style and few additional pollen tubes stop growing between mid-style and ovary. That is the case in, for example, *Brassica oleracea* (Ockendon and Gates 1975), *Persea americana* (Sedgley 1976), *Petunia hybrida* (Herrero and Dickinson 1980), *Nicotiana glauca* (Cruzan 1986), *Pontederia sagittata* (Scribailo and Barrett 1991), and *Cucurbita pepo* (Winsor and Stephenson 1995). Similarly, Sayers and Murphy (1966) report that in *Medicago sativa* the main determinant of pollen tube number reduction was the failure of pollen tubes to penetrate the stigma. An extreme case occurs in *Triticum durum*, where it has been reported that only a single pollen tube is capable of penetrating the stigma (Rudramuniyappa and Panchaksharappa 1974). In other species, however, a high rate of pollen tube attrition has also been observed in the lower part of the pistil. That is the case in *Erythronium grandiflorum* (Cruzan 1989), *Grevillea banksii* (Herscovitch and Martin 1990) and *Acacia retinodes* (Kenrick and Knox 1985). Our results suggest that the selection pressure in cherry appears to occur along the entire style length.

The use of different pollen loads may be a useful tool to study pollen tube attrition. The use of mixtures of live and dead pollen has proven to be a useful method to modulate the size of the pollen load applied onto the stigma. Various methods have been used previously to modulate pollen load size, for example, dilution of viable pollen with talc (Lee and Bazzaz 1982) or with Lycopodium powder (Janse and Verhaegh 1993); use of an insect pin (Cruzan 1986) or a stainless-steel rod (Schlichting et al. 1990); covering differing proportions of the stigmatic surface with pollen (Bertin 1990); varying the number of touches to the stigma (Winsor and Stephenson 1995); visually counting pollen grains and placing a specific number of grains on the stigma (Palmer and Zimmerman 1994). However, all these methods can be cumbersome and require either a species with large stigmata or the use of a binocular microscope in species with small stigmata. Thus, the approach we followed to modulate the intensity of pollen competition consisted of the application of different mixtures of live and dead pollen to the stigmata. The pollen was killed by heat treatment, though methanol-killed pollen had previously been used as a mentor pollen to overcome self-incompatibility (Dayton 1974) and for studies on pollen competition (Visser and Verhaegh 1988). The method we applied is easy to use and allows the application of any pollen load desired, since it involves mixing the appropriate weights

of live and dead pollen prior to pollination. However, while clear differences can be recorded between high and low pollen loads, the method is not precise enough to compare close pollen loads. This is due to the variability recorded among different stigmata in the number of pollen grains applied. One factor that could account for that variability is variation in the stigmatic areas among flowers. Another factor could be the higher than expected germination percentages observed in the mixtures with dead pollen. While a mentor effect has been suggested for dead pollen stimulating the germination of other pollen grains (Janse and Verhaegh 1993), no clear explanation can be given for this, and these differences could be caused by some washing off of dead pollen during the fixation process. Nevertheless, those two obstacles will be less important when the differences in the amount of viable pollen among the treatments are large. Furthermore, this pollination procedure can be applied to a wide range of plant species, and a large number of pollinations can be performed by an inexperienced worker. This can be very useful for modulating the intensity of pollen competition in the field in order to study its influence on the resulting offspring.

Causes for pollen tube attrition

Although this pattern of reduction in the male gametophyte population appears to be consistent among very different plant species, the mechanisms that regulate it are still little known. Two main forces could determine such a pattern. One force could be the differences in pollen competitive ability. The other could be a modulation of these differences by the pistil. This modulation would comprise both physical and physiological constraints and a genetic pollen-pistil interaction. A physical limitation could be caused by a constriction of the width of the transmitting tissue of the style (Modlibowska 1942; Cruzan 1986; Herrero 1992b). A physiological limitation could be caused by a restriction in the nutrient supply since, in binucleate pollen, after an initial period of autotrophic growth, pollen tube growth continues heterotrophically (Herrero and Dickinson 1981; Mulcahy and Mulcahy 1983) and the large amount of nutrients required to produce the cell wall are taken from the style tissue (Herrero and Dickinson 1979). This trophic dependence allows the pistil to influence pollen tube growth rate (Herrero and Arbeloa 1989) and dynamics (Herrero 1992b). If stylar space and reserves were the main limiting factors for pollen tube growth each area of the style would be able to support the growth of a determinate number of tubes. However, the number of pollen tubes recorded here for a particular area depends on the size of the load of viable pollen applied. Thus, with a small pollen load fewer pollen tubes reach each stylar area than could fit in that area. Since the rate of pollen tube attrition is more or less constant along the pistil, it seems that superimposed on the physical or physiological constraints presented by the pistil is a genetic interaction between the pollen tubes and/or between those tubes and the style that is contributing in a substantial way to the determination of the attrition pattern. However, in the lower part of the pistil, the number of growing pollen tubes starts to become independent of the initial number of germinating pollen grains on the stigma. The fact that in this area the number of pollen tubes becomes very similar among the four treatments would support the hypothesis that physical or physiological female-controlled attrition is the limiting factor in the lower section of the pistil. As a result of the overlap of these two mechanisms, a very small number of pollen tubes are allowed to enter the ovary in cherry and, eventually, only one pollen tube will fertilize the ovule.

The fact that a determined genetic pollen-pistil interaction is superimposed over a physical and/or physiological pistilar restriction to pollen tube growth along most of the length of the style gives stability to the system. If the selection pressure were uniquely dependent on the number of individuals present at the stigma, this system would be exposed to changes in pollination conditions and, consequently, highly vulnerable to random forces (Haldane 1932). On the other hand, the fact that the genetic interaction seems to be more conspicuous in the upper half of the style than in the lower half is a characteristic shared with the self-incompatibility reaction (Herrero and Dickinson 1980), a different but well-defined genetic interaction that also takes place during the reproductive process in plants. Thus, fertilization does not depend uniquely on passive physical or physiological constraints by the pistil, but also on the genetic interactions among the male gametophytes and between the male gametophytes and the female tissues. Again the two types of interaction are probably superimposed, and although clear differences have been reported between different male genotypes (Snow and Spira 1991), it has also been suggested (Cruzan 1993) that differences among pollen donors after pollinations can be due to stylar inhibition of pollen tube growth rather than to differences in pollen vigor. This interaction between the male gametophytes and the female tissues of the flower could explain why genetic variation for male gametophytic fitness has been maintained in plant populations when we could expect plants not to show much variation in pollen tube growth rates if better quality pollen always produces more and more vigorous progeny (Charlesworth et al. 1987; Walsh and Charlesworth 1992).

While it appears clear that a genetic interaction occurs along the progamic phase, the question remains about the effect of the physical constriction of the pistil. A possible answer could be that this constriction would superimpose a selective force for vigor over a genetic interaction. Results with different species have shown a correlation between higher pollen competitive ability and offspring fitness (Mulcahy 1971; Marshall and Whittaker 1989), and that differences in vigor in the offspring can be modulated by modifying the pollen doses and thus the intensity of pollen competition (Hawthorn et al. 1956; Mulcahy et al. 1978; Bertin 1990).

It appears that two main forces may be involved in the process of pollen tube number reduction in the cherry pistil. A genetic interaction appears to play a major role. Superimposed on this interaction, there is a physical constraint involving a reduction in the diameter of the transmitting tissue and in nutrient availability along the style. These two mechanisms converge in the idea that the progamic phase, from pollination to fertilization, seems to be well adapted to encourage interaction among the male gametophytes and between the male gametophytes and the female tissues of the flower. These interactions take place, both in time and space, along the different structures that the pollen tubes transverse on their way toward the ovule (Herrero 1992a). Further work on the mechanisms that regulate this genetic interaction will enlighten our understanding on how plants select among their possible mates in spite of their sessile nature.

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