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# The mentor pollen phenomenon in poplars: a new concept

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**Summary.** The mentor effect has been investigated in poplars. Attempts to overcome interspecific incompatibility are analysed by pollen germination and pollen tube behaviour in situ, both for compatible and incompatible crosses. We have demonstrated that following the mixed pollination, the two pollen sets interact at different levels of the progamic phase. A hypothetical model is proposed which describes mentor effect as the result of interactions of antagonist and cynergic forces applying on compatible pollen and tubes. These forces promote pollen tube growth both on the female partner surface and within the tissues.

**Key words:** Mentor effect – Interspecific incompatibility – *Populus* – Male-male interaction

## Introduction

Since the "mentor effect" was fortunately discovered (Michurin 1948 cited in Stettler and Ager 1984), it has been investigated by breeders as a possible tool to overcome incompatibility barriers both in self- or interspecific incompatibility. The technique consists of a mixed pollination involving both compatible mentor pollen and viable incompatible pollen. Mentor pollen is rendered genetically ineffective (i.e. blockage of fertilizing capacity) by several different treatments, including the action of ionizing radiations or repeated cycles of freezing and thawing (see reviews: Stettler and Ager 1984; Knox et al. 1987).

Numerous hypotheses of this phenomenon have been proposed (Knox et al. 1987). Unfortunately, even if some of them are particularly attractive, they are generally characterized by their lack of significant experimental supporting data. Moreover, mentor techniques sometimes completely failed: expected hybrid progeny has sometimes never been obtained (Sastri and Shivanna 1976; Taylor et al. 1980).

The choice of our experimental material has been determined by the abundant literature on poplars hybridization by mentor techniques (see review Knox et al. 1987). Our model involves *Populus nigra* (taxonomic section *Aigeiros*) and *Populus alba* (section *Leuce*) known to be strictly incompatible (Gaget et al. 1984). Our data, from comparative analyses of in situ pollen tube behaviour, are discussed according to new concepts on male-female interactions. The pollen grain is considered as the carrier of specific molecular information interacting with a read-out system localized in the pistil (Knox 1984; Dumas et al. 1984).

## Materials and methods

### *Plant material*

*Populus alba* (*Leuce* section) and *Populus nigra* (*Aigeiros* section) cuttings were obtained from the INRA Forestry Research Station of Orleans (France) in the winter of 1984 and stored in a cold room before use.

Flowering of male clones (*P. alba* and *P. nigra*) was obtained by forcing twigs in tap water at room temperature. Pollen was collected, dehydrated overnight on silicagel and stored in a freezer at −18 °C. *P. nigra* cuttings were used as the female parent because of their ability for rooting. They were cultivated in a greenhouse. Only five catkins were maintained on each branch in order to prevent nutritional competition between developing seeds.

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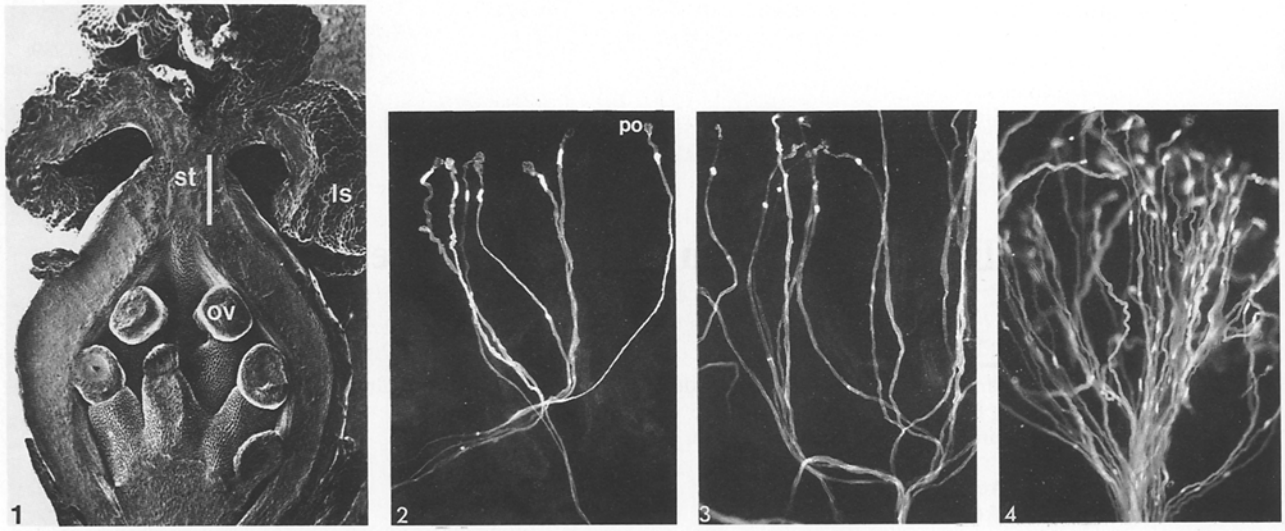


**Fig. 1–4. 1.** Organization of a female flower of *Populus nigra* (×80); *ls* – stigmatic lobes; *st* – stylodium; *ov* – ovule. **2.** Illustration of class $\sum$ (×200). **3.** Illustration of class + (×200). **4.** Illustration of class + + (×200)

*Controlled pollinations*

Pollen quality was estimated according to the procedure described by Heslop-Harrison et al. (1984), based on plasma membrane integrity and the presence of active cytoplasmic esterases (FCR test). Pollen grains were scored as "viable" when showing a bright yellow fluorescence with a 365 nm light. Before they reached receptivity, female catkins were carefully protected with pollination bags in order to prevent stray pollination. Pollination was performed using a bulb, blowing a standard amount of pollen (100 mg) onto the bag. For each cross, 10 female cuttings, i.e. 50 catkins were pollinated.

*Cytological observations*

Female flower characteristics have been investigated using scanning electron microscopy (SEM Jeol 35 CF) following a procedure described previously (Villar et al. 1987).

In situ pollen tube observations were done 24 h after pollination, with two catkins picked at random among the 50 catkins. These two catkins were fixed, cleared and stained with aniline blue according to the technique of Linskens reviewed in Dumas and Knox (1983). Squashed female flowers were observed using UV light of a Reichert Zetopan microscope (excitation filter no. 11, barrier filter no. 2).

For each cross we noted: (1) the presence or absence of pollen germination on the stigmatic surface, (2) the presence of pollen tubes at the stylodium level (Fig. 1).

Ten flowers were systematically investigated for pollen germination or pollen tube density in the stylodium tissues. These observations allowed us to score each flower in one of the four following classes:

+ +: when a female flower showed the maximum density of pollen tubes as observed in compatible cross;
0: when no pollen germination or no pollen tubes were noticed;
+: for the intermediate density (between + + and 0);
$\Sigma$: when female flowers showed a very few number of pollen tubes (between 0 and 10).

These classes are illustrated in Figs. 2–4.

*Compatible pollen treatments*

Four treatments were used as listed in Table 1: (1) Alternative period of freezing and thawing (modified from Pandey 1978). Pollen was treated by 14 cycles either −196 °C/+40 °C (rapid freezing) or −196 °C/+20 °C (slow freezing, 3 °C/mn, with the aid of an Air Liquide programmable freezer, Minicool). (2) Ionizing radiations (Stettler 1968). Pollen irradiation was performed by a cesium source ($Cs^{137}$) in the Nuclear center of Cadarache, at a dose of 100 Krad at 4 °C. (3) Pollen extracts. *P. nigra* pollen was homogenated in distilled water, centrifuged and the supernatant was freeze-dried overnight (simplified from Knox et al. 1972b). (4) Dead pollen. A sample of pollen had been abandoned at room temperature for 1 month. It showed 100% of FCR-pollen.

*Crosses*

All crosses are grouped in Table 1.

**Table 1.** Crosses performed with *P. nigra* as female partner. Control and assays. Treated pollen is italicized

| | | |
|---|---|---|
| 01 CC. | Compatible cross | P. nigra × P. nigra |
| 02 IC. | Incompatible cross | P. nigra × P. alba |
| 03 CT.ft.1 | Control pollination freezing and thawing (−196 °C/+20 °) | P. nigra × *P. nigra* |
| 04 ME.ft.1 | Mentor cross freezing and thawing (−196 °C/+20 °C) | P. nigra × (*P. nigra* + P. alba) |
| 05 CT.ft.2 | Control pollination freezing and thawing (−196 °C/+40 °C) | P. nigra × *P. nigra* |
| 06 ME.ft.2 | Mentor cross freezing and thawing (−196 °C/+40 °C) | P. nigra × (*P. nigra* + P. alba) |
| 07 CT.rad | Control pollination gamma radiations | P. nigra × *P. nigra* |
| 08 ME.rad | Mentor cross gamma radiations | P. nigra × (*P. nigra* + P. alba) |
| 09 CT.p. ex. | Control pollination pollen extract | P. nigra × *P. nigra* |
| 10 ME.p.ex. | Mentor cross with pollen extract | P. nigra × (*P. nigra* + P. alba) |
| 11 CT.d.po. | Control pollination dead pollen | P. nigra × *P. nigra* |
| 12 ME.d.po. | Mentor cross with dead pollen | P. nigra × (*P. nigra* + P. alba) |

**Table 2.** Germination on surface. Size of classes + +, +, Σ and 0 for each of the 12 crosses

| Crosses | + + | + | Σ | 0 | $X_j$ |
|---|---|---|---|---|---|
| 01 CC | 8 | 2 | 0 | 0 | 10 |
| 02 IC | 6 | 1 | 3 | 0 | 10 |
| 03 CT.ft.1 | 1 | 4 | 5 | 0 | 10 |
| 04 ME.ft.1 | 8 | 2 | 0 | 0 | 10 |
| 05 CT.ft.2 | 0 | 3 | 7 | 0 | 10 |
| 06 ME.ft.2 | 10 | 0 | 0 | 0 | 10 |
| 07 CT.rad | 8 | 1 | 1 | 0 | 10 |
| 08 ME.rad | 9 | 1 | 0 | 0 | 10 |
| 09 CT.p.ex. | 0 | 0 | 4 | 6 | 10 |
| 10 ME.p.ex. | 9 | 1 | 0 | 0 | 10 |
| 11 CT.d.po. | 0 | 0 | 3 | 7 | 10 |
| 12 ME.d.po. | 7 | 1 | 2 | 0 | 10 |

**Table 3.** Presence of pollen tubes in the stylodium. Size of classes + +, +, Σ and 0 for each of the 12 crosses

| Crosses | + + | + | Σ | 0 | $X_j$ |
|---|---|---|---|---|---|
| 01 CC | 8 | 2 | 0 | 0 | 10 |
| 02 IC | 5 | 0 | 0 | 5 | 10 |
| 03 CT.ft.1 | 0 | 1 | 0 | 9 | 10 |
| 04 ME.ft.1 | 2 | 8 | 0 | 0 | 10 |
| 05 CT.ft.2 | 0 | 0 | 1 | 9 | 10 |
| 06 ME.ft.2 | 10 | 0 | 0 | 0 | 10 |
| 07 CT.rad | 6 | 1 | 1 | 2 | 10 |
| 08 ME.rad | 2 | 1 | 2 | 5 | 10 |
| 09 CT.p.ex. | 0 | 0 | 0 | 10 | 10 |
| 10 ME.p.ex. | 5 | 0 | 1 | 4 | 10 |
| 11 CT.d.po. | 0 | 0 | 0 | 10 | 10 |
| 12 ME.d.po. | 5 | 0 | 1 | 4 | 10 |

Pollen mixes were prepared by thoroughly mixing *P. alba* pollen with mentor *P. nigra* pollen in a ratio of 1:1 by weight. For each cross, control pollination was performed in which treated mentor pollen or pollen extract was used alone.

*Data processing*

Four notes were attributed to each of the 12 crosses corresponding to the number of flowers classed + +, +, Σ and 0 (see Table 2 for "germination on surface" and Table 3 for "presence of tubes in the stylodium").

Qualitative comparisons of these data have been realized by means of $2\hat{\imath}$ criterium of information (Sokal and Rohlf 1969). The estimation of the minimum discrimination information $\hat{\imath}$ is suitable for evaluating the resemblance between two samples $x$ $y$. $2\hat{\imath}$ converges asymptotically to Chi-square distribution in the null hypothesis (homogeneity of samples) with $i$-1 *df*.

The calculations needed to apply the test are divided in different $n$ log $n$ elements, such as:

$$\hat{\imath} = \Sigma_i (x_i \log_e x_i + y_i \log_e y_i) + N \log_e N - \Sigma_i (x_i + y_i) \log_e (x_i + y_i) - N_1 \log_e N_1 - N_2 \log_e N_2$$

$x_i$ = size of sample $x$ for class $i$
$y_i$ = size of sample $y$ for class $i$
$N_1$ = number of observations for sample $x$ (=10)
$N_2$ = number of observations for sample $y$ (=10)
$N = N_1 + N_2 = 20$.

## Results

Frequencies of the four classes for each of the 12 crosses are indicated in Tables 2 and 3.

Homogeneity between the 12 crosses is tested at two levels: (1) according to the data on the presence of pollen germination on the stigmatic surface (Table 4), (2) according to the data on the presence of pollen tubes in the stylodium (Table 5).

In both tables, the five mentor crosses (ME) are compared to compatible and incompatible controls (CC and

**Table 4.** Test of homogeneity of the crosses according to the data on the germination on the stigmatic surface. Homogeneity between two crosses is tested by comparison of $2\hat{\imath}$ value to Chi-square values. (The $2\hat{\imath}$ values are not indicated to clarify the table)

| | ME.ft.1 | ME.ft.2 | ME.rad | ME.p.ex. | Me.dpo | CC |
|---|---|---|---|---|---|---|
| 1 CC | NS | NS | NS | NS | NS | – |
| 2 IC | NS | * | NS | NS | NS | NS |
| 3 CT.ft.1 | ** | – | – | – | – | ** |
| 4 CT.ft.2 | – | ** | – | – | – | ** |
| 5 CT.rad | – | – | NS | – | – | NS |
| 6 CT.p.ex | – | – | – | ** | – | ** |
| 7 CT.d.po | – | – | – | – | ** | ** |

NS – non significant
* – significant at 5% level
** – significant at 10% level

**Table 5.** Test of homogeneity of the crosses according to the data on the presence of pollen tubes in the stylodium. Homogeneity between two crosses is tested by comparison of $2\hat{\imath}$ value to Chi-square values. (The $2\hat{\imath}$ values are not indicated to clarify the table)

| | ME.ft.1 | ME.ft.2 | ME.rad | ME.p.ex. | Me.dpo | CC |
|---|---|---|---|---|---|---|
| 1 CC | ** | NS | ** | * | * | – |
| 2 IC | ** | * | NS | NS | NS | ** |
| 3 CT.ft.1 | ** | – | – | – | – | ** |
| 4 CT.ft.2 | – | ** | – | – | – | ** |
| 5 CT.rad | – | – | NS | – | – | NS |
| 6 CT.p.ex | – | – | – | ** | – | ** |
| 7 CT.d.po | – | – | – | – | ** | ** |

NS – non significant
* – significant at 5% level
** – significant at 10% level

IC) and to their control crosses (treated mentor pollen alone: CT) to analyse efficiency of the mentor techniques. Moreover, control crosses (CT) are compared to compatible control (CC) to analyse the effects of the treatments.

*Presence of pollen germination on the stigmatic surface (Table 4)*

Lines 1 and 2: the whole mentor crosses do not differ from compatible control. ME.ft.2 is the only cross that significantly differs (at 5% level) form incompatible control. IC and CC are not significantly different.

Lines 3–7: CT. rad is the only cross that is not significantly different from ME.rad and CC. Other control crosses, including CT.ft.1 and Ct.ft.2 differ from the compatible control and the mentor crosses.

*Presence of pollen tubes in the stylodium (Table 5)*

Lines 1 and 2: ME.ft.2 is the only cross non-significantly different from CC. Moreover, ME.ft.1 is different from both compatible and incompatible control. According to the frequencies of its four classes (Table 3), ME.ft.1 has an intermediate behaviour between CC and IC. Other mentor crosses do not behave differently from incompatible control. IC and CC are significantly different.

Lines 3–7: CT.rad is the only cross that is not significantly different from ME.rad and CC. Other control crosses differ from the compatible control and the mentor crosses.

## Discussion

*Interspecific cross P. nigra × P. alba*

Our work has been initiated on the basis of a sporophytic incompatibility phenomenon (Knox et al. 1972a; Hamilton 1976). This phenotype of incompatibility is characterized by a surface rejection, pollen tubes being arrested at the surface and failing to penetrate the stigmatic tissues. In our own experiments, we have shown that the compatible and incompatible cross cannot be differentiated according to the data on pollen germination on the stigmatic surface. Moreover, although incompatible pollen tubes never reach the ovules, incompatible pollen is able to produce a tube growing deeply in female tissues (Table 3). In this interspecific cross, *P. nigra* × *P. alba*, the phenotype of the incompatibility rejection is typical of gametophytically controlled systems. However, incompatibility levels are numerous in *Populus* sp. flowers, rejection occurring in the style but also in the ovule be-

fore or after fertilization (Melchior and Seitz 1968; Guries and Stettler 1976; Stettler et al. 1980; Gaget et al. 1984).

*Interspecific crosses with mentor pollen*

Our systematic observations of pollen tube behaviour allow us to estimate: (1) the impact of treatments on the mentor pollen-growing capacity; (2) the efficiency of the pollen mixes in promoting the incompatible pollen tube growth.

Consequently, we propose an integrative hypothesis for the mentor effect.

*Effects of mentor pollen treatments*

Freezing and thawing greatly reduced pollen germination on the stigmatic surface and the presence of the pollen tubes in the stylodium. Rapid or slow freezing have the same effect on pollen germination. The precise cellular site of injury of this treatment is yet unknown. But the plasma membrane has been described as the target of freezing injury in plants (Yoshida 1984). Alterations of the plasma membrane structure (changes in lipid-protein interactions or protein conformation) may partly explain the decrease in viability as tested by the FCR test. We suppose that the plasma membrane of treated pollen undergoes changes in membrane permeability which are at the origin of a greater passive diffusion of pollen compounds.

On the other hand, irradiation does not seem to affect pollen germination at the female surface and pollen tubes presence at the stylodium. Our results are in agreement with earlier studies in *Populus* species (Stettler and Guries 1976) and in other angiosperms (Brewbaker and Emery 1962; Den Nijs and Oost 1980). Speranza et al. (1982) also noted that high doses of radiation did not prevent *Malus domestica* pollen from germinating, but sterilized it by immobilising the generative cell and the vegetative nucleus in the upper pollen grain. This assumes that the target of ionizing radiation is the nuclei, ultimately the DNA of the cells (Knox et al. 1987).

In the case of control crosses involving dead pollen or pollen extracts, pollen germination is obviously reduced on the stigmatic surface and no pollen tube is observed in the stylodium.

*Effect of mixes of pollen*

According to the data *on the stigmatic surface,* most pollen mixes behave the same way as the compatible and incompatible control. Nevertheless, mentor crosses with freeze-thawed pollen behave differently than incompatible controls. The demonstration of this positive effect is illustrated in Table 6. Experimental frequencies of ME.ft.2, deduced from Table 2, are compared with theoretical frequencies (CT.ft.2 + IC). If one considers that the two sets of pollen are independent, not interacting one with another, then theoretical frequencies should be equal to experimental frequencies. However, they are different (Table 6). We think that this difference is the result of interactions occurring at the female partner surface between the two types of pollen. This represents the first component of the "Mentor Effect Factors" during the succession of events following the mixed pollination, called "surface Mentor Effect Factors" (sMEF). Our work consequently points out that even if pollen tube rejection occurs deeply in female tissue, mentor pollen promotes pollen tube germination at the surface. This emphasizes the first level of interaction between the two pollen sets at the stigmatic surface.

According to the data *at the stylodium level,* mentor techniques with freeze-thawed pollen are the most efficient. Other treatments do not lead to effective enhancement of pollen tube presence in the stylodium (ME.rad, ME.pex., ME.dpo.).

The effectiveness of such a mentor cross, for example ME.ft.2, is illustrated in Table 7. The great difference between theoretical and experimental frequencies clearly demonstrates that another Mentor Effect Factor might be implicated at this level. These dMEF (deep Mentor Effect Factors) are likely to result from interactions between compatible and incompatible pollen tubes within female tissues. Numerous hypotheses are given in the literature to explain the action of mentor pollen on the stigmatic surface (see review, Knox et al. 1987). Two hypotheses can be advanced to explain the mechanisms of action of the mentor pollen tube: (1) compatible pollen tubes may provide substances to incompatible ones promoting their growth (Pandey 1978). Our recent works on the physiology of pollen tube growth in vivo has clearly demonstrated the involvement of a pollinic enzyme ($\beta$-galactosidase) in the heterotrophic nutrition of the pollen tube (Villar 1987; M. Villar et al. in preparation). Such a system, active in mentor pollen tube (Gaget 1988), could provide growth substances to the surrounding incompatible pollen tubes. (2) Compatible tubes may also provide recognition signals to incompatible ones allowing incompatible tube acceptance by the female partner (Knox 1984; Linskens 1986; Gaude and Dumas 1987).

These pollen tube signals could be at the origin of the activation of the ovary (see review in Mulcahy and Mulcahy 1986). In poplars, these pollinic signals may stimulate physiological processes, such as the extent of female receptivity and retention of catkins (Stettler and Guries 1976).

Our observations of pollen tube have been completed by observation of pollen tubes within the ovary. Unfortunately, the presence of tannins which fluoresce with UV in dark red and the thickness of pollen tubes at the ovary level allowed us to note only the presence or ab-

**Table 6.** Comparison of experimental frequencies and theoretical frequencies in the case of ME.ft.2, according to the data on germination on surface. Size of classes are translated in frequencies. Experimental frequencies of ME.ft.2 are compared to the mean of experimental frequencies of the two sets of pollen used in equal proportion, that is IC+TC.ft.2

| | | ++ | + | Σ | 0 |
|---|---|---|---|---|---|
| Experimental frequencies of crosses | IC: | 0.6 | 0.1 | 0.3 | 0 |
| | CT.ft.2: | 0 | 0.3 | 0.7 | 0 |
| Theoretical frequencies | IC+CT.ft.2: | 0.3 | 0.2 | 0.5 | 0 |
| Experimental frequencies | cross ME.ft.2: | 1 | 0 | 0 | 0 |

**Table 7.** Comparison of experimental frequencies and theoretical frequencies in the case of ME.ft.2, according to the data on the presence of pollen tubes in the stylodium. Sizes of classes are translated in frequencies. Experimental frequencies of ME.ft.2 are compared to the mean of experimental frequencies of the two sets of pollen used in equal proportion, that is IC+CT.ft.2

| | | ++ | + | Σ | 0 |
|---|---|---|---|---|---|
| Experimental frequencies of crosses | IC: | 0.5 | 0 | 0 | 0.5 |
| | CT.ft.2: | 0 | 0 | 0.1 | 0.9 |
| Theoretical frequencies | IC+CT.ft.2: | 0.25 | 0 | 0.05 | 0.7 |
| Experimental frequencies | cross ME.ft.2: | 1 | 0 | 0 | 0 |

sence of pollen tubes for each cross. Observations are correlated with presence or absence of seed set. Compatible control and mentor effect by freeze-thawed pollen showed pollen tube in the ovary and produced seeds (about 300 seeds per branch for compatible crosses and 260 per branch for mentor crosses with freezed and thawed pollen). Identity of seedlings obtained will be discussed elsewhere, nevertheless morphological and biochemical analyses of the progenies showed that plantlets did not occur from hybridization but likely from parthenogenetic development of embryo sacs.

## Conclusion

From this study it appears that the mentor effect occurs at different levels of the progamic phase by promoting incompatible pollen tube growth at the female surface and at the stylodium level. Moreover, these experiments constitute the first evidence of the prime importance of the presence of compatible pollen tubes for mentor effect effectiveness. The mentor effect effectiveness is supposed to depend on several Mentor Effect Factors. The nature of these MEF implicated in male-male interaction during mixed pollinations is yet unknown. MEF might be compared with specific information signals of pollen grain wall (as described by Dumas et al. 1984). Our data may indicate that these MEF can also be present in the cell wall of the extended pollen tube. Part of them could be compared with factors involved in parthenocarpic development. This phenomenon has been frequently mentioned after mixed pollination (Stettler 1968; Ramulu et al. 1979; Pandey 1983; Georges et al. 1984).

The major contribution of the experiments described in this paper is the absolute demonstration of the unceasing rate of interactions occurring between mentor pollen and incompatible pollen during the physiological processes of pollen germination and pollen tube growth down female tissues. In order to clarify the network of male-male interactions that we pointed out during prefertilization events, we propose an integrative model representing the different levels of interaction: pollen-pollen interactions at the surface and tube-tube interactions in deep female tissues (Fig. 5).

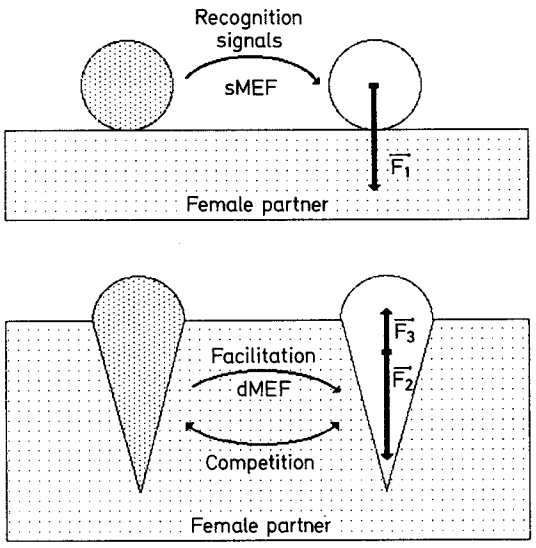


**Fig. 5.** Mentor effect. Integrative model. Two levels of male-male interactions are represented: stigmatic surface and stylar tissues. $F_1$ and $F_2$ represent the promoting effect of compatible pollen on incompatible pollen. $F_3$ represents the competition force between tubes. Mentor effect efficiency is described as the result of several forces which apply on incompatible pollen. $ME = F_1 + F_2 + F_3$. ▨ compatible pollen □ incompatible pollen

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