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Pollination, pollen tube growth and fertilization in *Chaenomeles japonica* (Japanese quince)

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Abstract

The fertilization system in Japanese quince, *Chaenomeles japonica* (Thunb.) Lindl. (Maloideae, Rosaceae) was studied by fluorescence microscopy. In a compatible combination, pollen grains germinated fast and pollen tubes grew rapidly, through the stylar tissue. Most of pollen tubes reached the base of the ovary within 2–6 days. In the ovary, pollen tube growth was considerably retarded, and fertilization of ovules started at the earliest 3 days after pollination. In an incompatible combination only few pollen grains germinated, and pollen tubes grew slowly and were often completely arrested in the style. The pollen tubes reached the ovary in 6–7 days. Fertilization did not take place until 7–9 days after pollination, and the percentage of fertilized ovules was very low. The same results were obtained when selfing self-incompatible genotypes. However, a small percentage of pollen tubes reached the ovary within 3–4 days in some genotypes including self-pollination (as in a compatible cross-pollination), but fertilization was not observed or considerably delayed compared to cross-pollination. *C. japonica* thus shows the characteristics of a gametophytic self-incompatibility system.

The viable period of the embryo sac was 6–7 days in most of the investigated genotypes, and the effective period of pollination was estimated to 5–7 days. Bagging was not necessary to avoid open pollination, provided that the flowers had been emasculated previously. Emasculation or bagging did not decrease fruit set and could, therefore, be used in breeding.

Keywords: Rosaceae; *Chaenomeles*; Pollination; Fertilization; Incompatibility; Fluorescence microscopy

1. Introduction

Chaenomeles japonica (Japanese quince), a diploid species ($2n = 34$) belonging to Maloideae, Rosaceae (Phipps et al., 1990), is a minor fruit crop in Latvia and Lithuania

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(Rumpunen et al., 1998). The dwarf shrubs are propagated by seeds and are phenotypically variable. This makes cultivation difficult and reduces profits, mainly because several fruit characters vary, including time of ripening. Furthermore, considerable genetic variability has recently been revealed by RAPDs and isozymes in this species (Bartish et al., 1999, 2000; Garkava et al., 2000). In order to improve the crop through development of new varieties, basic knowledge on the fertilization system was needed.

The flowers of *C. japonica* are classified as perfect and homomorphic, with a pistil consisting of five partly fused styles, somewhat longer than the surrounding anthers (Weber, 1964). Nevertheless, when screening collections and breeding populations, it was noticed that almost every plant also had several ''imperfect'' flowers with stunted, possibly sterile, pistils. The stigma is of the wet type, group III, following the classification of Heslop-Harrison and Shivanna (1977). Pollinating vectors are honey-bees and bumble-bees which are attracted to the nectar-rich, reddish orange flowers, thus efficiently pollinating the presumably out-crossing plants (Weber, 1964).

It is well established that fruit set and yield are strongly dependent on genotype and genotype interactions for many important fruit crops within the family of Rosaceae, such as apples, pears, cherries, apricots and almonds. In these well-known crops, reproduction is governed by a gametophytic self-incompatibility system associated with stylar RNases (Boskovic and Tobutt, 1996; Boskovic et al., 1997; Broothaerts et al., 1995; Burgos et al., 1998; Janssens et al., 1995; Sassa et al., 1992, 1994, 1997; Tao et al., 1997, 1999). At least in apples these RNases are expressed in the pistil, along the pollen tube growth path (Certal et al., 1999). To investigate the mating system in *C. japonica*, and to test our hypothesis of gametophytic self-incompatibility, we studied pollen viability in vitro, pollen germination on the stigma, pollen tube growth in the pistil, embryo sac viability, fertilization, the effective pollination period (EPP), the period of flowering, the percentage of imperfect flowers, functionality of flowers with stunted pistils and fruit set after self-pollination. Finally, we investigated the effect of emasculation and bagging on fruit set.

2. Materials and methods

2.1. Plant material

The *C. japonica* plants studied were raised from seeds and grown at Balsgård-Department of Horticultural Plant Breeding, in the south of Sweden (genotypes labeled B and NV) and at Dobele, State Horticultural Plant Breeding Experimental Station, in the west of Latvia (genotypes labeled D).

2.2. Pollen viability in vitro

To study pollen germination in vitro, anthers were sampled in the field, at late balloon stage, from 17 genotypes of *C. japonica* during 2 years (1994–1995), and from 12 additional genotypes during 3 years (1994–1996). The anthers were dried in an incubator at 20–22 °C until dehiscence, which usually took about 24 h. The germination tests were

then immediately performed. The optimum experimental condition to study pollen viability in vitro had previously been determined (results not presented). The germination substrate consisted of 1% agar and 15% sucrose dissolved in boiling water at pH 5.6. The substrate was poured into 90 nm Petri dishes, and pollen were distributed on the surface of the cooled, but still somewhat fluid, substrate. The Petri dishes were then kept at 23–25 °C. A pollen grain was considered germinated when the tube had grown to a length of approximately twice the diameter of the pollen grain. Counts of germinated pollen grains were made under a light microscope after 3 h. Counts were postponed till 6 h for poorly germinating material. For each genotype, three replicates with approximately 100 pollen grains were counted.

2.3. Pollen germination, pollen tube growth and fertilization

Pollen germination and pollen tube growth were studied by fluorescence microscopy: (I) in 21 genotypes, which had either been self-pollinated or cross-pollinated with a mixture of pollen from several genotypes, (II) in a genotype B1 that was pollinated with three other genotypes B2–B4, and (III) in flowers with stunted pistils. In all studies, about 70–80 perfect flowers of each genotype were emasculated at loose balloon stage and enclosed in isolation bags of woven fabric (Agryl^{TM}) to prevent pollination by honey-bees and bumble-bees. For obtaining pollen, flowers were sampled at late balloon stage, separately for each genotype, and put under an electric bulb to allow anther dehiscence. The pollen was then collected in a test tube and stored at 4–6 °C in a desiccator until pollination. A mixture of pollen from the same set of 21 genotypes was prepared for cross-pollination in experiment I. Controlled self- and cross-pollination were made when the stigma was wet and thus receptive which in the prevailing weather typically took 24–48 h from emasculation. A high load of pollen on the surface of the stigma may result in increased pollen germination and tube growth in the style (Marcucci and Visser, 1987). Therefore, care was taken to ensure as large pollen loads as possible by thoroughly dipping the pistils in a small test tube with dried pollen (rather than applying the pollen by a brush).

Samples were prepared for fluorescence microscopy following the methods successively developed by Martin (1959), Kho and Baër (1970), Preil (1970) and Anvari and Stösser (1978). From each genotype, 7–10 pistils were sampled every 24 h, from the first to the fifth, seventh or ninth day after pollination. The pistils were put in fixative FAA (80%ethanol:37%formaldehyde:100%acetic acid, in proportions 8:1:1) for 24 h, rinsed in tap-water for 4 h, softened for 8–10 h in 8 N NaOH and rinsed again for 4–6 h in tap-water and distilled water. The pistils were then stained in 0.1% water-soluble aniline blue and 0.1 N K_3PO_4 for 12–24 h. Instant samples were prepared in a droplet of the same staining solution. The samples could safely be maintained in the staining solution for 2–4 weeks at +4 °C, which made the precise time for evaluating the samples less critical and thus the analysis of the samples more efficient. For each genotype, the ovary was separated from the base of the style during the sample preparation procedure. The samples were then covered with cover slips and softly squashed. In each of the five styles, pollen tube growth was followed to the ovules under microscope (Leitz) and photographed (Kodak Ectachrome Elite, 100ASA).

2.4. Embryo sac viability and effective pollination period

An ovule was considered viable based on its fluorescence (Anvari and Stösser, 1978; Dys, 1984). The effective pollination period (EPP) was calculated as the viability period of the egg apparatus minus the time necessary for the pollen tube to reach the egg cell (Williams, 1970).

2.5. Optimum pollination period

To establish the optimum pollination period of *C. japonica* in the field, 10 branches of each of two genotypes (D1 and D2) were isolated with two layers woven fabric 1 day before estimated anthesis. The flower clusters were thinned, leaving two to three flower buds of similar size in each cluster to avoid drop of fruit embryos and fruits. During 10 days, the flowers in one bag after the other were successively cross-pollinated by hand, with mixed pollen collected from eight other genotypes. In total 50–80 pistils were pollinated in each bag. The fruitlets were counted after 1 month, as were also later the resulting ripe fruits.

2.6. Fruit set at self-pollination

To estimate fruit set after self-pollination in the field, 39 genotypes were bagged a few days before anthesis. The flower clusters were thinned, leaving two to three flower buds of similar size in each cluster. At anthesis, the remaining flowers were self-pollinated by gently rubbing one flower against the other, thus ensuring that its own pollen reached the stigma. This procedure was repeated 2 days later. Fruit set was counted after approximately 3 months.

2.7. Flowering period and stunted pistils

The flowering period and the number of stunted pistils were studied for five genotypes during three successive years. Open flowers were removed and counted, from the very first to the very last day that a flower could be found. The number of flowers with stunted pistils was noted separately. When analyzing the data, flowering was considered to have started when 5% of the total number of flowers had opened. Likewise, flowering was considered to have finished when 95% of the flowers had opened. This made it possible to accurately calculate the onset, duration and end of flowering as well as the date for 50% flowering.

2.8. Emasculation and bagging

Effects of emasculation and bagging on fruit set of *C. japonica* were studied in the field for 13 genotypes. For this purpose, genotypes with a sufficiently high number of flower buds were selected a few days before anthesis. The flower clusters were thinned, leaving two to three flower buds of similar size in each cluster. On average, 40 flowers per genotype were used in each of four treatments: (I) open pollination, mainly by bumble-bees, (II)

controlled cross-pollination by hand in isolation bags, (III) emasculation without bagging, and (IV) emasculation and controlled cross-pollination by hand in isolation bags.

3. Results

3.1. Pollen viability in vitro

When evaluating pollen germination of 12 genotypes during 3 years, the genotype, year and genotype × year interaction were all highly significant ($p = 0.0001$) with corresponding F-values of the same order. Thus, different genotypes reacted differently to environmental changes. The genotype average of pollen germination were 52, 36 and 41% for the 3 years, respectively, while the individual genotype estimates varied from 2 to 85%. However, some genotypes were less variable than others and thus should be preferred as pollen donors.

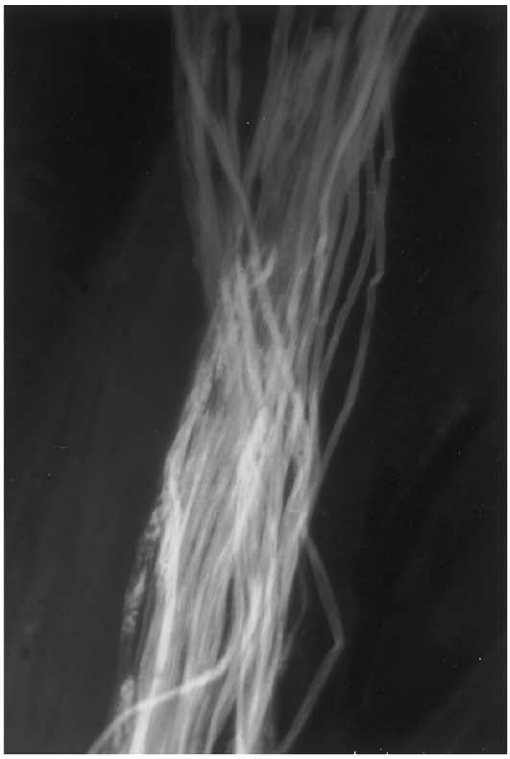
The pollen could be stored in a desiccator at 4–6 °C for at least 1 year with only a slight loss of viability as tested in vitro (results not presented).

3.2. Pollen germination and pollen tube growth through the style

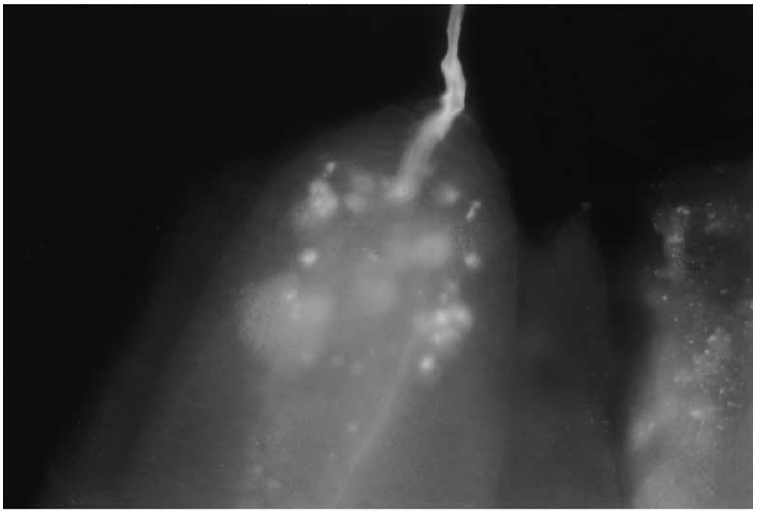
Pollen germination started immediately after pollination. A compatible combination was characterized by a rapid germination, a high number of germinating pollen grains, and after 24 h most of the pollen grains had germinated. At the same time, in an incompatible combination only a small number of the pollen grains had germinated.

In experiment I, where 21 genotypes were cross-pollinated with mixed pollen, differences in germination and pollen tube growth were noticed. Compatible pollen developed tubes that, in a dense cluster, grew rapidly down the style (Fig. 1A). At 24 h, pollen tube tips were found in the style from 1/4 to 1/2 of the length (Fig. 2). Only few tubes penetrated the ovary at this time, and the growth of pollen tubes was much slower in the ovary tissue than in the style. Some of the pollen tubes reached the base of the style within 2 days, but fertilization of ovules (Fig. 1B) was first noticed 3 days after pollination. Incompatible pollen developed tubes that grew slower, were often arrested in the style and had heavy depositions of callose along and at the end of the tube (Fig. 1C). The same reaction was observed after self-pollination. In this case, a small number of the pollen tubes reached the base of the ovary, usually not until 6–7 days after pollination. Fertilization occurred late (after 7–9 days), and the percentage of presumed fertilized ovules (tips of pollen tubes entering micropyle) was very low (about 5%). However, also after self-pollination, a small number of the pollen tubes reached the base of the style already in 2–3 days in some genotypes, but fertilization (if any) was always delayed compared to cross-pollination (Fig. 3).

In experiment II, in which genotype B1 was self-pollinated and cross-pollinated, respectively, the fastest pollen tubes reached the ovary after 2–3 days depending on combination (Table 1). However, fertilization did not take place until 5–7 days after cross-pollination. After self-pollination, fertilization did not take place during the period of sampling.

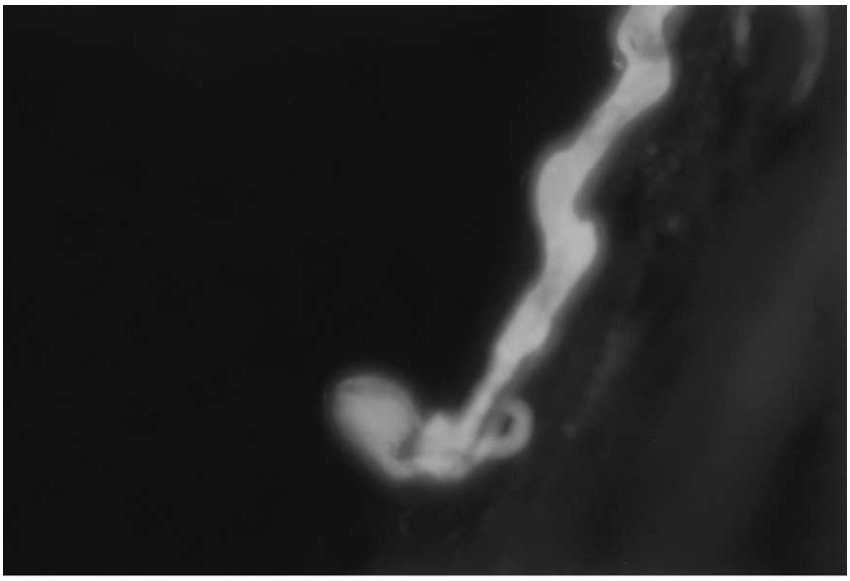


(a)



(b)

Fig. 1. (a) Growth of compatible pollen tubes through the style (275×), (b) a pollen tube that has reached the ovule with presumed fertilization taking place (675×), and (c) the typical inhibited growth of an incompatible pollen tube in *C. japonica* (675×).



(c)

Fig. 1. (*Continued*).

In experiment III, in which flowers with stunted pistils (reduced style and partially undeveloped stigmas) were pollinated with a pollen mixture, only a few pollen grains germinated. The pollen tubes grew to about 1/6 to 1/4 of the style. The apical part of the pollen tube then swelled, split, or was arrested. Thus, fertilization was impossible.

The percentage of imperfect flowers was both dependent on genotype and environment (Table 2). As much as 94% of the total number of flowers were imperfect in one genotype.

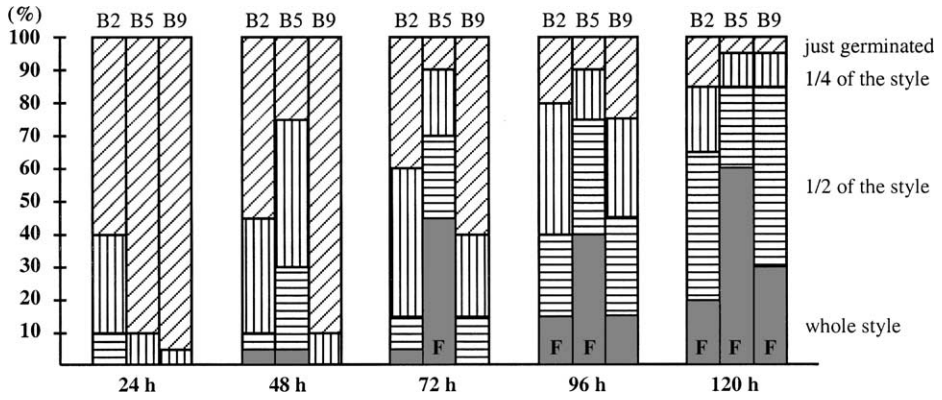


Fig. 2. Growth of pollen tubes in the style at 24, 48, 72, 96 and 120 h following cross-pollination for three genotypes (B2, B5, B9) of *C. japonica*. A bold **F** in the figure indicates that fertilization has taken place.

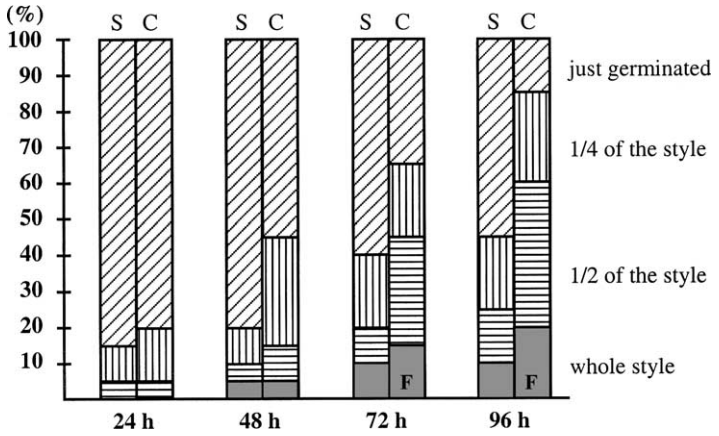


Fig. 3. Growth of pollen tubes in the style at 24, 48, 72, and 96 h following self-(S) and cross-(C) pollination of a genotype (B6) of *C. japonica*. A bold **F** in the figure indicates that fertilization has taken place.

3.3. Fruit set at self-pollination

In 1995, 17% of the genotypes studied showed some degree of self-fertility (in these genotypes the fruit set was 1–11%) while in 1996 the corresponding percentage was 9% (in these genotypes the fruit set was 1–5%). Thus, about 80–90% of investigated genotypes was completely self-incompatible.

Table 1
Percentage of fertilized ovules (mean of 5–7 samples) detected by fluorescence microscopy when pollinating a genotype (B1) of *C. japonica* with its own pollen or with pollen from each of three genotypes (B2–B4)

Combination	Day after pollination							
	1	2	3	4	5	6	7	8
B1 × B1	0	0	0	0	0	0	0	0
B1 × B2	0	0	0	0	0	0	4	31
B1 × B3	0	0	0	0	10	45	47	46
B1 × B4	0	0	0	0	0	8	39	42

Table 2
Percentage of imperfect flowers for five genotypes of *C. japonica* observed during 3 years

Genotype	Imperfect flowers (%)			
	1998	1999	2000	Average
NV14–11	46.4	36.0	67.3	49.9
NV15–25	75.6	73.9	94.0	81.2
NV18–63	85.9	78.5	75.8	80.1
NV21–2	19.5	25.5	45.6	30.2
NV24–2	12.0	12.5	90.5	38.3
Mean	47.9	45.3	74.7	55.9

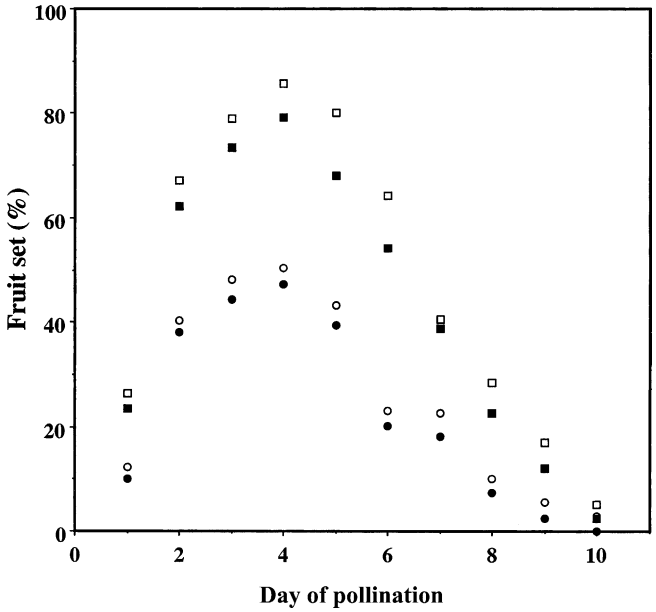


Fig. 4. Fruit set (% of pollinated flowers) of two genotypes, D1 (squares (□)) and D2 (circles (○)), following cross-pollination by hand. Fruitlets were counted after 1 month (open symbols) and the resulting fruits at ripening (filled symbols). Day 0 is the day of emasculation and day 1 is the day of anthesis and pollination.

3.4. Embryo sac viability and EPP

The embryo sac was ripe 2–4 days after anthesis and viable until 7–9 days after anthesis, whereas fastest pollen tube growth to the embryo sac was 2–3 days in a compatible combination. Thus, the EPP could be estimated to 5–7 days in *C. japonica*, depending on genotype.

3.5. Optimum pollination period

By controlled pollination, the highest fruit set was obtained when pollination took place 2–6 days after anthesis (Fig. 4).

3.6. Flowering period

The flowering period of *C. japonica* was long (10–31 days) and depended both on genotype and environment (Fig. 5). Average flowering period differed by 10 days among years (range: 13–23 days), and by 6 days among genotypes (range: 15–21 days).

3.7. Emasculation and bagging

Emasculation of flowers resulted in very low fruit set, as did pollination with their own pollen of isolated flowers (Table 3). Open-pollination and cross-pollination, respectively,

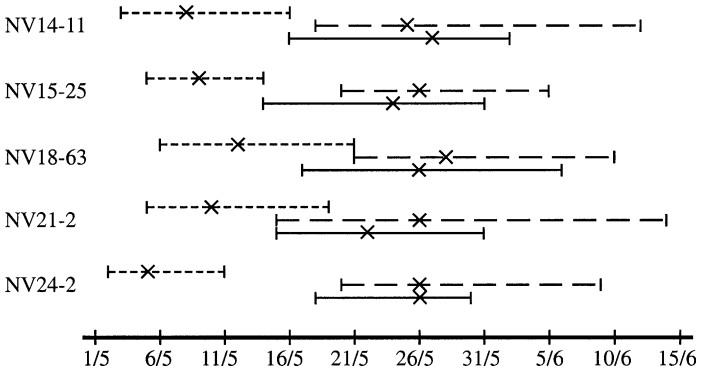


Fig. 5. Flowering period for five genotypes of *C. japonica* during 3 years (1998: — —, 1999: – –, 2000: - -). A cross (×) marks the date for 50% flowering while the start and the end were counted at 5 and 95% flowering, respectively. In the year 2000, flowering took place extremely early.

Table 3
Effects of emasculation and isolation on fruit set of 13 genotypes of *C. japonica*[a]

Treatment	Fruit set (%)
Emasculated and open pollinated	2.6 a
Isolated and selfed	1.3 a
Emasculated, isolated and cross-pollinated	55.5 b
Open pollinated	48.6 b

[a] PLSD ($p = 0.05$) = 12.8.

of emasculated and isolated flowers resulted in similar levels of fruit set. Thus, emasculation (and isolation) per se did not reduce the potential fruit set, and at the same time made the flowers unattractive to pollinating insects.

4. Discussion

4.1. Pollen viability

The genotype average of germinated pollen (36–52%) for *C. japonica* is similar to percentages previously reported for diploid apples (Stott, 1972), also with clear fluctuations between years. The highest percentage noticed in our study for one genotype (87%) is just somewhat lower compared to the very high value reported for some apple varieties (over 95%). Addition of boron to the substrate would perhaps have improved germination percentages as has previously been reported for many rosaceaous species (Calzoni et al., 1979; Visser, 1955; Voyiatzi, 1995). However, although our simple sugar–agar substrate may not be optimal, it seems to have been sufficiently efficient to evaluate genotype differences of pollen germination of *C. japonica*. No disintegrating pollen grains were noticed, and high germination percentages were obtained for some genotypes.

4.2. Pollen tube growth through the style

Pollen tube growth was considered normal for a gametophytic self-incompatibility system. While the majority of pollen tubes grew undisturbed, some pollen tubes branched already in the style and some pollen tubes swelled, bent back, and grew in the opposite direction when a mix of pollen from different genotypes was used for pollination. This phenomenon is possibly explained by differences in compatibility of the pollen donors and the recipient genotype. Studies of Stott (1972) and Seilheimer and Stösser (1982) in apples showed that pollen tubes reached the base of the style in 4–5 days for a compatible combination, but not until after 8–12 days for an incompatible combination. For apples, fertilization occurred 5–7 days after pollination, depending on genotype and season (Williams, 1970). Yamashita et al. (1990) found that in pears, with compatibility the pollen tubes reached the ovary 3 days after pollination, but with incompatibility they were completely arrested not far from the stigma. This is similar to the pattern found here in *C. japonica* where fertilization in some cases, for a compatible combination, started as early as 3 days after pollination. The fact that no fertilization was obtained until day 5, when one genotype was cross-pollinated with each of three other genotypes (Table 1), shows a strong genotypic interaction that should be considered when new varieties are selected. Compared to 3 days, this is a considerable delay that may influence fruit set, especially if temperature is low during flowering. However, since pollen tube growth is strongly dependent on temperature, genotype comparisons would become more reliable if made in a controlled environment (Petropoulou and Alston, 1998).

Fertilization was not observed when flowers with stunted pistils were pollinated. The reason for the very high number of imperfect flowers found in some genotypes of *C. japonica* (Table 2) is still not known. Such flowers were found on almost every genotype to a lesser or larger extent. Possibly, unfavorable temperatures trigger the development of imperfect flowers, but our results demonstrate that genotype differences are also involved. Yet, in each genotype, the number of perfect flowers present should allow a high yield if *C. japonica* is similar to apple, where about 10% of the flowers are sufficient for normal fruit set. We do not know why the number of imperfect flowers increased in the year 2000. It could, for instance, not be explained by drastic differences in winter temperatures between the years.

It was noticed that female sterility could be assessed morphologically by ovary thickness, just like in almonds (Socias i Company and Felipe, 1987). A small, unswollen ovary indicated a stunted pistil. This observation made it easy to identify perfect flowers, worthwhile to emasculate.

4.3. Embryo sac viability and EPP

The viability of the embryo sac is as important as a high percentage of germinating pollen grains and rapid pollen tube growth, to obtain a high fruit set. We noticed that in *C. japonica*, the embryo sac was viable until about 7–9 days after anthesis. For diploid apples, ovule longevity is approximately the same, about 8 days (Williams, 1970), while ovules of triploid apple varieties have a considerably extended longevity, about 12 days, as a result of the comparatively slow development of the egg apparatus.

EPP is an important measure since flower ineffectiveness may limit cropping rather than a lack of sufficient pollen transfer. For *C. japonica*, EPP is 5–7 days depending on genotype. This is approximately the same as for diploid apple varieties, while for triploids an average EPP of about 7 days is reported (Williams, 1970). In a controlled environment, the length of EPP was 4–6 days at 13 °C for pear, but 2–4 days at 17 °C (Tromp and Borsboom, 1994). Thus, a higher temperature during blossom may reduce the effective pollination period. Other important factors influencing EPP is tendency to biennial cropping and nitrogen availability in the year preceding flowering (Williams, 1970).

4.4. Optimum pollination period

In controlled cross-pollination experiments using mixed pollen on two genotypes, the highest fruit set was obtained when pollination took place 2–6 days after anthesis (Fig. 3). This corresponds very well to the EPP estimated by fluorescence microscopy. The fact that the fruit set also occurred when pollination was made late (days 7–10) may indicate that size of flower bud is not fully correlated to developmental stage of the ovules. Despite that flower buds were thinned, a slight decrease in the number of resulting mature fruits (compared to the number of fruitlets at early stage) was obtained. This may either indicate that we should have further reduced the number of flower buds to prevent drop of fruit embryos or that other factors limited final fruit set.

4.5. Self-pollination in the field

The degree of self-fertility (1–11%) found in a minor part of the genotypes is still too low to enable commercial plantations with only one variety, and furthermore demonstrates the very strong self-incompatibility system prevailing in *C. japonica*. This is similar to apple, where pollination of a large number of diploid varieties with their own pollen resulted in no more than 10% fruit set in any variety (Crane and Lawrence, 1952).

4.6. Flowering period

In the breeding process, it is necessary to develop varieties that flower simultaneously to ensure sufficient cross-pollination. This is, however, not a serious problem for *C. japonica* since its flowering period is extended and normally lasts about 3 weeks (Fig. 5). Thus an overlapping flowering period is easy to achieve for most combinations of genotypes. A few days of mild or even severe frost during flowering are not so harmful for total fruit set in *C. japonica* since its flowering period is long, especially compared to stone fruits but also compared to apples and pears. Thus there are always some flower buds that escape the frost.

4.7. Emasculation and bagging

Field experiments have to rely upon pollination bags to prevent natural pollination by insects or by wind, mechanically forcing flowers on neighboring twigs in contact with each other. However, in applied breeding it is sometimes impractical or very difficult to use pollination bags, especially on thorny shrubs like *C. japonica*. We found that the

emasculated flowers do not attract pollinators, despite the rich amount of nectar secreted at the base of the style, and that there is a very small risk for other unintentional pollination (e.g. by mites or wind). Furthermore, emasculation per se does not reduce fruit set, nor does the use of pollination bags. Emasculation without bagging, therefore, appears to be sufficiently efficient to prevent unintentional pollination of *C. japonica* in applied breeding. In this case, emasculation prevents the flowers from attracting pollinating bumble-bees, but emasculation to prevent self-pollination is in practice not necessary owing to the strong self-incompatibility system that delays or prevents fertilization with own pollen.

It would be interesting to study the stylar RNases in *C. japonica*, since recent reports provide evidence that RNases are associated with gametophytic self-incompatibility in pear (Sassa et al., 1992, 1997), apples (Broothaerts et al., 1995; Sassa et al., 1994) and other fruit crops in Rosaceae. In addition, the allele-specific PCR-based marker system developed by Janssens et al. (1995) and applied by Sakurai et al. (1997) to determine the self-incompatibility alleles of apples would probably be useful also for further studies in the genus *Chaenomeles*. This information would make it possible to select highly compatible varieties early in the breeding process.

5. Conclusion

A strong self-incompatibility system of the homomorphic gametophytic type according to the classification of Newbigin et al. (1993) appears to be present in *C. japonica*. This is likely to contribute to the large phenotypic and genetic variation revealed in the species.

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