Pollen Tube Behavior in Different Mandarin Hybrids

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Abstract. Seedlessness is an important trait in the evaluation of commercial mandarin for fresh consumption. However, in the last decade, the presence of seeds in fruit of cultivars considered as seedless has become a problem in different citrus-growing areas because the commercial value is depreciated. Seeds have appeared concomitantly with the introduction of new cultivars that appear to be cross-compatible. To overcome this problem, different strategies have been explored, but a definitive answer is still elusive. The search for alternatives contrasts with how little is known about the basis of the problem: the pollen-pistil incompatibility reaction in mandarin, and the intercompatibility relationship between different cultivars. In this work, we characterized the pollen-pistil incompatibility in the two commonly grown mandarin cultivars Fortune and Nova, and evaluated the intercompatibility relationship between six cultivars with different genetic origins; these cultivars included Fortune (*Citrus clementina* Hort. *ex* Tan. × *Citrus reticulata* Blanco), Nova [(*Citrus paradisi* Macf. × *C. reticulata*) × *C. clementina*), Comune Clementine (*C. clementina*), Avana apireno (*C. reticulata*), Primosole (*Citrus unshiu* Marcov. × *C. reticulata*), and Simeto (*C. unshiu* × *Citrus deliciosa* Ten.). Following the controlled hand pollination in the field, we evaluated pollen tube performance after self- and cross-pollination, as well as intercultivar compatibility by observing pollen tube growth. The results show the self-incompatibility of these cultivars with the pollen tube having been arrested in the style, which explains their seedless condition when planted in solid blocks. The study of intercompatibility indicates a different pollen tube behavior depending on the genotype, as well as on the cross combinations. These results provide a basis to evaluate self- and intercompatibility in citrus, and the effect of close planting of some cultivars.

Citrus L. species are grown in tropical and subtropical climates with a wide distribution and production. Mediterra-nean countries supply about 70% of the total world's fresh fruit export, and 11% of the transformed products (Imbert, 2005). Seedlessness is a very important feature in the evaluation of commercial mandarins for fresh consumption because seeded fruit depreciate the value of the crop (Pons et al., 1995a; Vardi et al., 2008). However, in all citrus-producing countries, in the last few years, seeds are appearing in cultivars that were previously seedless (Chao et al., 2005; Vardi et al., 2008). The presence of seeds in seedless cultivars is related to the introduction of new genotypes that are sexually intercompat-ible. Results in the field suggest that particular cultivars often produce a high number of seeds (Chao, 2005). However, the basis of this fact remains unknown.

The problem started in the 1970s, when several new cultivars (including hybrids) were released to widen the crop calendar. Several broadly grown seedless hybrids were consti-tuted, such as 'Fortune', 'Nova', 'Ortanique' tangor [*C. sinensis* (L.) Osbeck × *C. reticulata*], and 'Ellendale' tangor (*C. sinensis* × *C. reticulata*), and were then introduced in a number of countries. In Italy, several hybrids with interesting character-istics were constituted by crossing different citrus species, such as 'Primosole' and 'Simeto' (Tribulato and La Rosa, 1993). These cultivars show seeds in their fruit when cultivated near other sexually compatible cultivars.

To avoid seed formation, several strategies have been explored (Vardi et al., 2008). Open pollination factors such as pollen flow (Chao et al., 2005) or the pollination efficiencies of wind and insects, and the possible use of insect repellents (Pons et al., 1995b, 1996) have been studied. Also, a pollen germi-nation inhibitor was used, and good results were obtained, in which there was an increase in the percentage of seedless fruit in field conditions (Mesejo et al., 2006). On the other hand, breeding strategies have been recently reviewed (Vardi et al., 2008); these strategies included attempts for the introduction of cytoplasmic male sterility of 'Satsuma' mandarin (Guo et al., 2004); ploidy manipulation for increasing sterility (Grosser et al., 2000; Grosser and Gmitter, 2005; Navarro et al., 2004; Reforgiato Recupero et al., 2005); induced mutation (Spiegel-Roy and Vardi, 1981), and transgenic approaches (Li et al., 2002). However, a definitive answer is still elusive. The search for alternatives contrasts with the paucity of data on the basis of the problems, which include the pollen-pistil incompatibility and the intercompatibility relationships between the cultivars.

In citrus, although no proper genetic studies have been done, the control of incompatibility is thought to be gametophytic; that is, based on the behaviors of the pollen tubes, because the incompatibility reaction occurs after pollen has covered some distance through the gynoecium (Nettancourt, 1977, 1997; Sedgley and Griffin, 1989; Soost, 1965). The studies on 'Orlando' tangelo (*C. paradisi* × *C. reticulata*) in which compatible and incompatible self-pollinations were compared showed that incompatible pollen tubes were short and spiraling, with an irregular callose deposition (Kahn and DeMason, 1986, 1988). A morphological appearance was recorded in the gametophytic systems that had the pollen tube arrested in the style (Soost, 1965).

Self-incompatibility is also a critical economic problem in several commercial mandarin without high parthenocarpic abilities (Eti and Stosser, 1992). However, information is lacking on the pollen-pistil incompatibility in the species that constitutes the new mandarin hybrids, except for 'Comune Clementine', which is known to be self-incompatible because pollen tubes arrest in the style (Ton and Krezdorn, 1966). Seedless mandarin and clementine cultivars are empirically expected to be self-incompatible based on the fact that they produce seedless fruit when planted in solid blocks. In the present study, the pollen-pistil incompatibility is characterized, and the self- and intercultivar compatibilities in six seedless cultivars are evaluated.

Materials and Methods

Plant material. Trees, older than 10 years, of six cultivars grown at the "Primosole" experimental farm of Catania University (Catania, Italy) were used for this study. The self-incompatible cultivar Comune Clementine was used as a known reference. Five other important cultivars were tested, including the seedless mandarin Avana apireno; two American hybrids that are diffused worldwide, Fortune and Nova; and two Italian hybrids with promising diffusion, Primosole and Simeto. All the trees used for this study were healthy and subjected to standard cultural practices.

Pollen collection and pollination procedures. To obtain fresh pollen, 40 flowers were collected during the peak bloom just before anthesis from three trees of each cultivar (Comune Clementine, Avana apireno, Nova, Fortune, Simeto, and Primosole), and their petals and pistils were removed. The anthers were left to dehisce for 24 h at room temperature at about 25 °C, and the fresh pollen was immediately used for pollination.

Several groups of flowers were emasculated in the trees of the abovementioned genotypes, hand-pollinated, and bagged in cotton tissue.

Self-incompatibility characterization and intercompatibility relationships. To characterize the self-incompatibility reaction, 1 d before and on the day of anthesis, we self-pollinated and crossed flowers of 'Nova' and 'Fortune' mandarins. Then, sampling sequentially, 10 pistils were collected every 2 d, for up to 14 d (i.e., seven times), and were fixed in a FAA solution (5 mL of formalin:5 mL of glacial acetic acid:90 mL of 70% ethanol, v/v/v). The pistils were maintained at 4 °C for microscopic observation.

To evaluate the possible self-incompatibility and intercompatibility of six cultivars, the cultivars were self-pollinated and intercrossed in all possible combinations, 1 d before anthesis,

following the same emasculation and pollination procedure as described earlier. From each cross, 10 pollinated flowers were collected at 10 d after crossing and self-pollinating, similarly fixed in FAA solution, and stored at 4 °C for microscope observation.

Microscopic preparations. Pollen tubes were monitored on the squash preparations following the modification by Linskens and Esser (1957) and Jefferies (1975). Pistils fixed in FAA solution were washed with water three times (1 h for each wash) and left in a 5% sodium sulfite solution overnight. Pistils were softened in a 5% sodium sulfite solution in a microwave for 45 s. Before the squash, the ovary was cut from the stigma-style, and both parts were further cut longitudinally and split into two parts. Following the staining procedure and squashing with 0.1% aniline blue in 0.1 N K_3PO_4, the preparations were observed by a fluorescence microscope using I3 filter excitation 450 to 490 nm (DM 2500; Leica Microsystems, Wetzlar, Germany). A pollen grain was considered to be germinated when the pollen tube length was larger than the pollen grain diameter. Pollen tube growth in a single style was recorded as the percentage of total length covered by the longest tube. Also, the number of pollen tubes reaching the base of the style was recorded. The number of pollen tubes at the base of the style for each pollen donor and for each female recipient was subjected to analysis of variance (ANOVA) and Duncan's multiple range tests using Statistica 6.0 (StatSoft, Tulsa, OK).

Results

Pollen tube behavior following self- and cross-pollinations. No clear differences were observed in the stigmas of the compatible and self-incompatible crosses (Fig. 1, A and B). However, evident differences were observed in the styles between both types of crosses. The compatible combinations showed pollen tubes with thin walls that grew straight without twisting and formed regular-sized callose plugs at regular intervals (Fig. 1C), which reached the base of the style (Fig. 1E). However, in self-incompatible combinations in the style, less pollen tubes grew (Fig. 1D) and they were arrested, showing altered morphologies with irregular depositions of callose in the tube walls, and heavy callose depositions at the pollen tube tips (Fig. 1, D and F). In cross-pollinations, there was a reduction in the number of pollen tubes growing along the style, and in the upper part of the style, there were more pollen tubes than in the lower part; only a few reached the ovary (data not shown).

Pollen tube growth was sequentially examined in 'Nova' and 'Fortune' following the self- and cross-pollinations. Pollen tube growth differences between crossed and self-pollinated flowers were evident 2 d after pollination. Four days after pollination, the pollen tubes in self-pollinated flowers were arrested in development in the first half of the style, while in crossed flowers, pollen tubes continued to grow until they reached the base of the style (Fig. 2A). Ten days after cross-pollination, pollen tubes reached the base of the style in most flowers (Fig. 2B); this started to occur in a high proportion of the flowers at 6 d after pollination in 'Nova' and at 8 d after pollination in 'Fortune'. None of the self-pollinated flowers showed pollen tubes at the base of the style. The number of pollen tubes that reached the base of the style was different between both crosses; while an average of 10 pollen tubes reached the base

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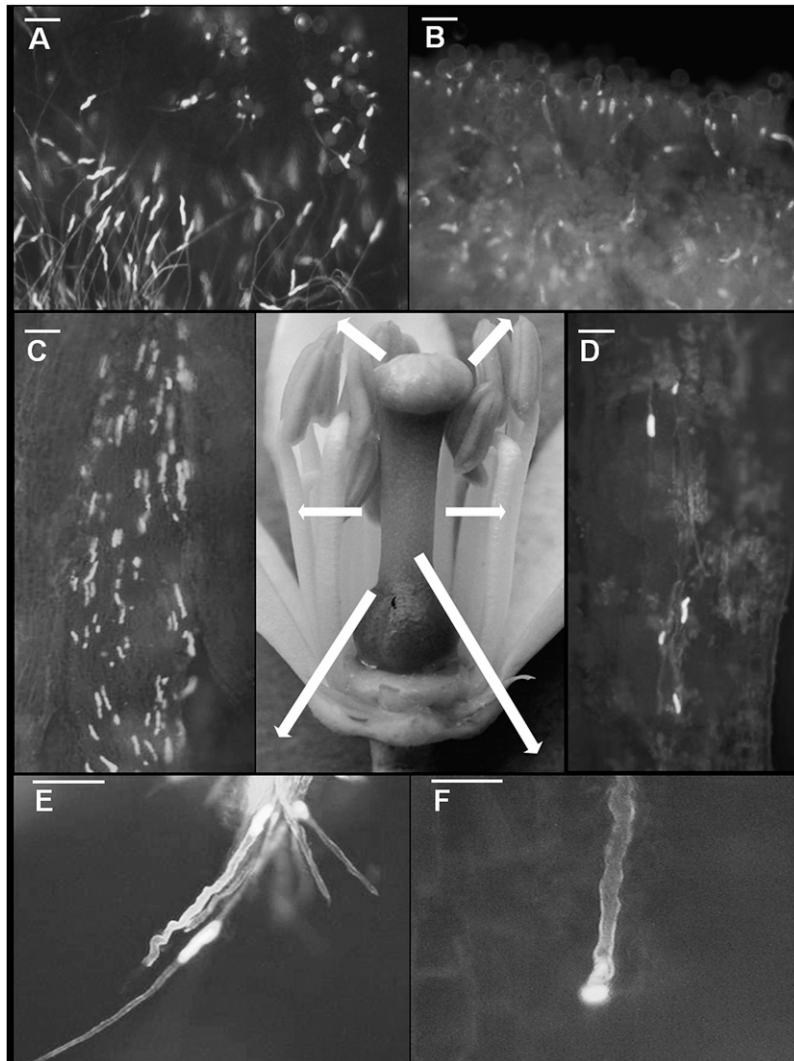J. Amer. Soc. Hort. Sci. 134(6):583–588. 2009.

Fig. 1. Pollen tube growth of mandarin hybrids in compatible ('Nova' × 'Fortune') and incompatible ('Nova' self-pollinated) combinations. In cross pollination of 'Nova' × 'Fortune' pollen grains germinated on the stigma (**A**), elongated in the style (**C**), and reached the base of the style (**E**). In self-pollination of 'Nova' pollen grains germinated on the stigma (**B**), only few grew down in the style (**D**), and stopped before reaching the base of the style (**F**); bar = 50 μm.

grain germination aptitude in relation to the stigma and pollen genotypes, although the pollen donor appeared to be the major determinant. The germination rates of 'Simeto', 'Comune Clementine', and 'Avana apireno' ranged between 250 and 500 germinated pollen grains per stigma, while in 'Fortune', a higher number of pollen grains germinated (500–750 pollen grains per stigma; data not shown).

Following pollen germination, the pollen tubes reached the stylar canals, through which they were oriented toward the ovary. In the style, clear differences were observed between compatible and incompatible crosses. In the compatible combinations, 25 to 100 pollen tubes were observed in the upper part of the style, while fewer pollen tubes were observed following the self-pollination. In general terms, the pistils of 'Fortune', 'Nova', 'Primosole', and 'Simeto' appeared to support greater numbers of pollen germinations and pollen tube growths in the upper part of the style than 'Comune Clementine' and 'Avana apireno'. Ten days after pollination, except for the crosses of 'Primosole' × 'Comune Clementine' and 'Primosole' × 'Avana apireno', the pollen tubes reached the base of the styles in most of the flowers following cross-pollination. However, they were arrested in the first half of the style following self-pollination, except for 'Avana apireno' (Table 2).

Data on the percentage of flowers with pollen tubes at the base of their styles (Table 3) confirm the self-incompatibility of all genotypes, except for 'Avana apireno', and show that some cultivars have more effective pollen than others; for example, the pollen of 'Fortune' reached the base of the styles in all of the flowers from all of the genotypes. Differences were also observed in the number of pollen tubes that reached the base of the style (Table 4); some combinations showed a low or null number of pollen tubes at the base of the style, as in 'Primosole' pollinated by 'Comune Clementine' or 'Avana apireno'. However, the reciprocity of the crosses was not necessarily the same.

The pollen performance showed significant differences depending on the pollen donor genotype. Thus, pollen of 'Fortune' and 'Comune Clementine' resulted in high numbers of pollen tubes that reached the base of the style, while a few were encountered when 'Simeto' was the pollinator. On the other hand, no significant differences were recorded in the pollen performance between different pistil genotypes, except for 'Fortune', which also had more pollen tubes at the base of the style (Table 4).

of the style in 'Nova' × 'Fortune', only one to two pollen tubes were found in this area in the 'Fortune' × 'Nova' cross, and this number did not increase with time (Fig. 2C).

To evaluate if the incompatibility reaction was already acquired before anthesis, 'Fortune' and 'Nova' were self-pollinated 1 d before and on the day of anthesis (Table 1). At anthesis, the self-incompatibility reaction was clear and the pollen tubes were arrested in the first third of the style. On the contrary, when flowers were self-pollinated 1 d before anthesis, the pollen tubes grew straight to the ovary. Thus, the acquisition of the incompatibility reaction appears to occur particularly late, just before anthesis.

THE INTERCOMPATIBILITY RELATIONSHIP BETWEEN CULTIVARS. Once the pollen tube behavior, following self-pollinations and crossed pollinations, was established, the intercompatibility relationship was examined between the cultivars. For this purpose, six cultivars were crossed in all possible combinations. Pollen germination rates ranged between 250 and 750 pollen grains per stigma. The results showed a different pollen

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Discussion

The results herein show a clear presence of self-incompatibility in mandarins and provide a basis for the study of the
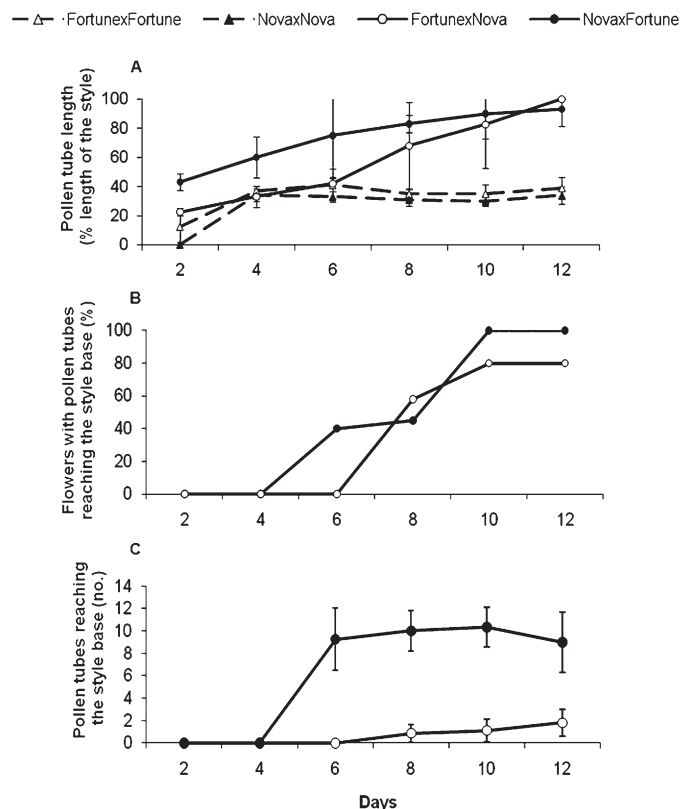

Fig. 2. Kinetics of pollen tube growth in the styles of cross- and self-pollinated flowers of 'Fortune' and 'Nova' mandarin hybrids. Style length covered by the pollen tube (**A**), flowers with pollen tubes reaching the style base (**B**), pollen tubes reaching the style base (**C**).

Table 1. Pollen tube behavior in 'Fortune' and 'Nova' mandarin self-pollinated 1 d before (–1) and on the day of anthesis (0). Style length covered by the pollen tube, flowers with pollen tubes reaching the style base (B), pollen tubes reaching the style base.

	Pollen tube length (% length of style)		Flowers with pollen tubes reaching the style base (%)		Pollen tubes reaching the style base (no.)	
Flower stage	–1	0	–1	0	–1	0
'Fortune'	100	35	100	0	11	0
'Nova'	100	30	100	0	7	0

intercompatibility relationships between the cultivars. Sequential examination of pollen tube growth shows that self-incompatibility is clear and manifested by an arrest of the pollen tubes in the first half of the style.

In citrus species, the incompatibility reaction has been reported to occur along different phases of pollen tube growth through the gynoecium (Nettancourt, 1977, 1997; Sedgley and Griffin, 1989; Soost, 1965). Ton and Krezdorn (1966) observed different degrees of incompatibility in clementine varieties, and it has been proposed that self-incompatibility reactions, between the pollen tubes and the style, can be classified by the degree of self-incompatibility of each portion of the style (Ngo et al., 2001; Yamamoto and Tominaga, 2002). In some incompatible conditions, the pollen tubes find the primary arrest site in the stigma, such as in the self-pollinated clementine and 'Orlando' tangelo (Ton and Krezdorn, 1966), or 'Monica' mandarin and Satsuma (Gómez et al., 2004). On the other hand, Gómez et al. (2004) and Sage et al. (1994) proposed that in mandarin, the site of inhibition occurs at the base of the style, in the ovary or ovules. Precluded penetration of pollen tubes in the ovule was suggested by Ton and Krezdorn (1966), who hypothesized that the ovary is the major area of inhibition. Really, the picture is far from clear, and conflicting reports in the literature regarding different pollen tube arrest sites may be related to the hybrid origin of most citrus varieties.

Differences in the site where the pollen tube arrests could also be related to the differences in the stages of pistil maturation. Results herein show that in 'Nova' and 'Fortune', the acquisition of the self-incompatibility system is surprisingly late because the pollinations that were performed just 1 d before anthesis resulted in no pollen tube arrests in the style. The acquisition of the incompatibility reaction occurs at a particular stage of the flower bud development in petunia [*Petunia hybrida* Hort. (Herrero and Dickinson, 1980)], and bud pollination has been used by plant breeders to overcome the self-incompatibility barrier (Hiscock and Dickinson, 1993); this fact, together with the fact that in other species, warm temperatures close to flowering time result in flowers opening with underdeveloped pistils (Rodrigo and Herrero, 2002), suggests that the differences in the pistil developments at anthesis could also account for the differences in the sites of arrest of the pollen tubes. While this premature flowering has not been previously investigated in the context of the incompatibility reaction, it might be worth it to explore whether this could occur in citrus species and thus contribute to explaining the conflicting reports on the incompatibility reaction arrest site and also to so far unexplained seediness. The incompatibility reaction has also been reported to be weak in old flowers (Williams and Maier, 1977).

Table 2. Percentage of style length covered by the pollen tubes in six mandarin cultivars 10 d after self- and cross-pollination. Bold numbers indicate self-pollinated combinations.

Female/Male	Style length covered by pollen tube (%)					
	'Fortune'	'Nova'	'Simeto'	'Primosole'	'Comune Clementine'	'Avana apireno'
'Fortune'	**39**	97	100	100	100	100
'Nova'	100	**34**	97	87	95	97
'Simeto'	94	92	**34**	75	78	90
'Primosole'	86	92	88	**41**	47	25
'Comune Clementine'	100	86	91	91	**33**	100
'Avana apireno'	100	97	94	74	82	**100**

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Table 3. Percentage of flowers with pollen tubes reaching the style base in six mandarin cultivars 10 d after pollination. Bold numbers indicate self-pollinated combinations.

Female/Male	Flowers with pollen tubes reaching the style base (%)					
	'Fortune'	'Nova'	'Simeto'	'Primosole'	'Comune Clementine'	'Avana apireno'
'Fortune'	**0**	60	100	100	100	80
'Nova'	100	**0**	30	80	80	90
'Simeto'	100	80	**0**	100	70	40
'Primosole'	100	60	30	**0**	0	0
'Comune Clementine'	100	30	40	80	**0**	90
'Avana apireno'	100	90	90	50	70	**70**

Table 4. Number of pollen tubes reaching the style base in six mandarin cultivars 10 d after pollination. Bold numbers indicate self-pollinated combinations. Mean line shows average of pollen tubes for each pollen donor, mean column shows average of pollen tubes for each female recipient.

Female/Male	Pollen tubes reaching the style base (no.)						
	'Fortune'	'Nova'	'Simeto'	'Primosole'	'Comune Clementine'	'Avana apireno'	Mean^z
'Fortune'	**0**	2	3	9	18	4	8.9 b
'Nova'	10	**0**	1	7	3	10	5.1 a
'Simeto'	9	4	**0**	4	4	1	4.3 a
'Primosole'	8	2	1	**0**	0	0	2.6 a
'Comune Clementine'	7	1	1	3	**0**	9	2.7 a
'Avana apireno'	5	6	3	2	3	**2**	3.4 a
Mean^z	7.6 e	2.8 ab	1.8 a	3.7 abc	5.7 de	4.8 cd	

^{z}Mean separation via Duncan's multiple range test at $P < 0.05$.

Five of the six cultivars examined behave as self-incompatible, and the sites of pollen tube arrest were very similar in all of them in spite of the fact that they have a very different genetic origin. This homogeneous self-incompatibility response, with a different species background, can be explained by the prevailing view that the incompatibility system predated speciation (McClure et al., 1989; Sutherland et al., 2008; Xue et al., 1996).

Differences in the number of pollen tubes reaching the base of the style were encountered in the different crosses examined. The pollen donor cultivar induced a significant difference on the subsequent pollen performance, and the number of pollen tubes that reached the base of the style was higher when the pollens of 'Fortune' and 'Comune Clementine' were used. The pistil could also play a part in modulating pollen performance; 'Fortune' had more pollen tubes at the base of the style, and, interestingly, no pollen tubes reached this area in 'Primosole' when pollinated with 'Comune Clementine' or 'Avana apireno'. This could be due to an interincompatibility relationship. However, because the reciprocal crosses did not yield a similar response, this behavior probably respond to differences in the pollen-pistil interaction and should be cautiously taken. Crosses failing to get pollen tubes at the base of the style would be interesting from a nonseed production perspective. On the other hand, some differences that were encountered in pollen performance cannot be attributed just to the pollen or the pistil genotypes. Differences in pollen performance, depending on the pollen and the pistil genotypes, have been previously reported in cherry (*Prunus avium* L.); but in this species, the main factors appear to be the pollen-pistil interaction and the interaction with the prevailing environmental conditions, mainly temperature (Hedhly et al., 2005). The effect of these three factors, pollen, pistil, and the interaction between them, convert to the point that differences were encountered for a same pollen pistil combination in response to the reversing male/female roles. This lack of reciprocity has been previously reported in citrus species for pollen tube behavior (Ngo et al., 2001), but also when seed production was examined (Papadakis et al., 2009).

The pollinator genotype has also been shown to induce a different number of seeds in kiwi [*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson] (González et al., 1998). In citrus, a relationship between the pollen origin and seed number has been reported (Bono et al., 2000), and it may be worth it to evaluate, to what extent, the pollen tube number at the base of the style could predict the affinities between the cultivars and the risks of obtaining seeded fruit under close planting. So far, these results set a baseline that clarifies the pollen-pistil incompatibility in mandarins and opens up a way to evaluate the intercompatibility relationships.

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