1 Male-female interaction and temperature variation affect pollen performance in
2 *Citrus*
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1 **ABSTRACT**

2
3 Despite the extensive research work characterizing pollen performance in several plant species, less
4 effort has been made to characterize it in some economically important species as *Citrus*, in which
5 the failure of the sexual reproductive process, and subsequent parthenocarpic fruit development and
6 seedlessness are prized characters. In this work we characterized pollen-pistil interaction in the
7 three ancestral true-type *Citrus* species in order to determine the influence of the male and female
8 genotypes, as well as of the temperature regime on pollen performance. Specifically, we
9 characterized the effect of temperature on pollen grain germination *in vitro*, and on pollen tube
10 growth *in vivo* in cut flowers and *in planta* under field temperature conditions. Results obtained
11 showed that temperature variation has a strong effect on pollen germination, and on pollen tube
12 kinetics and on their population size depending on the specific male-female combination. The
13 optimum temperature for pollen germination *in vitro* was of 25 °C, while the most favorable
14 temperature to accelerate *in vivo* pollen tube growth depended on the particular male-female
15 interaction and ranged between 15 and 25 °C. Furthermore, temperature appears to have an effect on
16 self-incompatibility reaction by affecting the place where pollen tubes are arrested. Overall, our
17 results show that pollen performance is not only an inherent characteristic of the pollen genotype,
18 but is largely dependent on the particular male-female combination and on genotype-temperature
19 interactions.

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21 **Keywords:** Citron, Mandarin, Pollen tube growth rate, Pummelo, Self-incompatibility, Temperature
22 stress

23

1. Introduction

Pollen viability and performance play an important role in the sexual reproductive success of plants, finally materialized by fertilization of the ovules and the resultant formation of seeds.

Extensive research work characterizing pollen performance in different plant species revealed that pollen tube growth and its likelihood to fertilize ovule depend not only on its own genotype (Snow and Spira, 1991) but also on the genotype of the pistil (Stephenson and Bertin, 1983; Willson and Burley, 1983; Herrero and Hormaza, 1996; Hormaza and Herrero, 1996, 1999; Mulcahy et al., 1996) and on the prevailing environmental conditions during flowering and pollination (Young and Stanton, 1990; Stephenson et al., 1992; Johannsson and Stephenson, 1998; Hedhly et al., 2009; Hedhly, 2011).

Characterizing pollen performance is especially relevant for some economically important genus like *Citrus*, in which failure of the sexual reproductive process resulting in parthenocarpic fruit development and seedlessness is a prized character. The importance of *Citrus* for agriculture in a world context is shown by its worldwide distribution and its large-scale production. Grown commercially in more than 100 countries around the world in tropical and sub-tropical climates (approximately 40° N and S of the equator), citrus production (including oranges, grapefruit, tangerines and mandarins, lemons and limes) has experienced continuous growth in the last decades of the twentieth century reaching a total annual production of more than 120 million tons (FAOSTAT, 2010). Despite the economic importance of *Citrus* species little research work has been done to characterize their reproductive biology. To this it could partly contribute the lack of an established boundary between species, subspecies and interspecific hybrids, and also the paucity of knowledge of their self-incompatibility reaction and parthenocarpic behaviour. Although recent work (Distefano et al., 2011) shows that parthenocarpy in mandarin is related to an uncoupling of the onset of fruiting with the reproductive process.

On the one hand, the taxonomy of the genus *Citrus* is not exactly established. Recently, studies suggested that only three *Citrus* types, namely citron (*Citrus medica* L.), mandarin (*Citrus*

1 *reticulata* Blanco), and pummelo (*Citrus maxima* [Burm.] Merrill) constituted true or valid species,
2 and that derived from inter- and intraspecific crosses between these three species are the other
3 important *Citrus* species such as orange, lemon, lime and grapefruit (Scora 1975, 1988; Barrett and
4 Rhodes, 1976). The concept of the three true valid species while the other species were derived
5 from hybridization between them is further supported from various recent studies using biochemical
6 and molecular markers (Herrero et al., 1996; Fang and Roose, 1997; Federici et al., 1998; Nicolosi
7 et al., 2000; Moore, 2001; Barkley et al., 2006; Deng et al., 2007).

8 On the other hand, little is known about the self-incompatibility and parthenocarpy
9 behaviors of *Citrus* species, which are very important traits for fruit production and fruit quality
10 since they result in seedless fruit. In particular, seedlessness is currently a very important feature in
11 the evaluation of commercial citrus fruits for fresh consumption.

12 Also *Citrus* breeding strategies have been indeed hampered by factors associated with
13 reproductive biology (sterility, incompatibility, nucellar embryony, juvenility). The control of self-
14 incompatibility is thought to be gametophytic, based only on the observation of the pollen tubes
15 growth, which usually arrest after they have covered some distance through the gynoecium (Soost,
16 1965). While crosses between mandarin cultivars show a clear arrest of pollen tubes in the style
17 (Distefano et al., 2009), the incompatibility reaction has also been reported to occur along different
18 phases of pollen tube growth through the gynoecium in different *Citrus* species (Soost, 1965;
19 Nettancourt, 1977, 1997; Sedgley and Griffin, 1989), but more research is needed to fully
20 characterize it as is the case for other plant families with a gametophytic self-incompatibility system
21 such as Solanaceae (de Nettancourt 1977), Rosaceae (Sassa et al., 1993; Tao et al., 1997),
22 Scrophulariaceae (Xue et al., 1996), Papaveraceae (Franklin-Tong et al., 1993, 1995).

23 In the present study, pollen-pistil interaction has been characterized in the three *Citrus*
24 ancestral species under different temperature treatments. We first carried out a pollen germination
25 *in vitro* experiment under five temperature regimes (10, 15, 20, 25, 30 °C), reflecting the spectrum
26 (from cool to hot) of temperature conditions that can be found during citrus blooming season.

Second, to characterize the influence of temperature in the putative site where the pollen tube growth is arrested in self-incompatible crosses we characterized pollen tube behaviour in self-pollinated clementine. Third, to check for inter-species compatibilities and characterize the influence of the pistilar genotype and of temperature regimes on pollen tube growth, we carried out a diallel experiment (self- and cross-pollination) among the three ancestral species under four temperature regimes.

2. Materials and methods

2.1. Plant materials

In the present work four *Citrus* genotypes were used. Three genotypes belong to the three *Citrus* ancestral type species: the semi self-compatible ‘Dancy’ mandarin, ‘Vozza Vozza’ citron regarded as self-compatible, and the self-incompatible ‘Sha Tian Yu’ pummelo. The semi self-compatibility in ‘Dancy’ mandarin is not a barrier to perform this pollination experiment, since a half of pollen tubes grow normally in any pollination. The fourth and self-incompatible cultivar ‘Comune’ clementine (*C. reticulata* Blanco) was used as a known reference for self-incompatibility test (Distefano et al., 2009). Kim et al. (2011) reported the S genotype of several *Citrus* genotypes; however information about the genotypes used in this study is not evident. Trees, older than 10 years, of these four *Citrus* varieties were grown at the “Primosole” Experimental Farm of Catania University adopting standard cultural practices.

2.2. *In vitro* pollen germination

To obtain fresh pollen, 40 flowers were collected one day before anthesis from three trees of each genotype (clementine, mandarin, citron, pummelo). The anthers were removed and left to dehisce for 24 h at room temperature at about 25 °C. Fresh pollen was immediately used for testing *in vitro* pollen germination at four temperature regimes (10, 15, 20, 25, 30 °C) in Petri dishes on a solidified germination medium consisting of 100 g l⁻¹ sucrose, 0.1 g l⁻¹ H₃BO₃, 0.3 g l⁻¹ Ca(NO₃)₂,

0.1 g l⁻¹ KNO₃, and 10 g l⁻¹ agarose (Mesejo et al., 2005). Pollen germination was arrested after 24 h by freezing at -20 °C. This procedure was adopted as it revealed its efficacy in preserving pollen morphology for microscopic observation (Hedhly et al., 2005a). Pollen was scored as germinated when the length of the pollen tube exceeded the diameter of its pollen grain. For each treatment, germination was recorded in two Petri dishes by counting three complete fields, until reaching at least 100 pollen grains in each field, in each Petri dish (6 replicates).

2.3. *In vivo pollen germination and pollen tube growth*

To evaluate the effect of the genotype and of temperature regime on pollen behavior *in vivo*, a diallele experiment between the three ancestral species under field conditions and four temperature regimes was carried out. In the field experiment, flowers of clementine, mandarin, citron and pummelo were emasculated at balloon stage, hand-pollinated (10 flowers per treatment), bagged in cotton tissue, and sampled after 10 days. For the controlled chambers experiment, 120 flowers at balloon stage were collected randomly from three trees for each genotype, emasculated, and immediately placed in trays with soaked florist foam. Ten flowers per treatment were either self-pollinated or cross-pollinated in all possible combinations, except for clementine that was only self-pollinated. After pollination, trays were immediately placed in the controlled temperature chambers at 15 °C, 20 °C, 25 °C and 30 °C. To compare pollen performance across experiments, all *in vitro*, *in vivo*, and *in planta* pollinations were performed with the same batch of pollen. Self- and cross-pollinated flowers of the four species were collected three days after pollination, fixed in a FAA (formalin, glacial acetic acid, 70% ethanol, 1: 1: 18 v/v; Johansen, 1940), and maintained at 4 °C until microscopic observation.

2.4. *Microscopic observations*

Pollen grain germination and pollen tube growth were monitored on squash preparations. Pistils fixed in FAA were washed three times in water, one hour each, and left in 5% sodium sulfite

solution overnight (Jefferies and Belcher, 1974). Pistils were, then, softened in 5% sodium sulfite solution in a microwave for 45 seconds. Before squashing the preparations, 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 K₃PO₄ (Linskens and Esser, 1957), the preparations were observed under a fluorescence microscope (Leica DM 2500 of Leica Microsystems GmbH using I3 filter excitation 450-490 nm, Wetzlar, Germany). Pollen tube growth was recorded as the length of the longest pollen tube in the stigma-style, measured in 10 pistils/genotype. The number of pollen tubes reaching the base of the style was also recorded.

2.5. Statistical analyses

For statistical analysis, to normalize data and homogenize variances, all count data were square-root-transformed before carrying out the analyses of variance. In the case of pollen germination *in vitro*, the removal of 10 °C observations allowed a better normalization of data. In the case of pollen tube numbers at the base of style under growth chamber conditions, the analysis was carried out only among the crosses in which some pollen tubes were reported. Statistical analyses were done using R 2.13.1 (The R Foundation for Statistical Computing, 2011; <http://www.R-project.org>).

3. Results

3.1. Pollen germination *in vitro*

The effect of incubation temperature on the *in vitro* pollen germination of the four *Citrus* varieties was expressed as percentage of pollen grains germinated during 24 hours (Fig. 1). Apart from significant main effects, the analysis of variance revealed a highly significant genotype-temperature interaction (Table 1). No pollen germination was observed for any of the *Citrus* genotypes at the lowest temperature of 10 °C. Pollen grain germination was less than 10% at 15 °C except for clementine in which still no germination was observed. Bigger and more significant

differences were observed at 20 °C when pollen grain germination ranged from 3% for clementine to 22% for citron and pummelo. Mandarin, citron and clementine showed a maximum germination percentage at 25 °C ranging from 28 to 45%. Also pummelo pollen grains showed the highest germination percentage (>90%) at 25 °C. Examining the global trend of pollen germination in response to increasing temperature, a progressive increase in pollen grain germination rate from 15 °C to 25 °C can be observed for all genotypes, followed by a sudden decrease when temperature reached 30 °C except for clementine.

3.2. Interspecific crosses in field conditions

Ten days after self and cross-pollination under field temperature conditions, pollen tubes reached the base of the style in most combinations except for self-pollinated pummelo (black bars in Fig. 2). The length of distance travelled by pollen tubes from the stigma to the ovary varied between species from 5.7 mm (0.9 mm SD) in mandarin, 5.9 mm (1.1 mm SD) in clementine, 12.1 mm (1.3 mm SD) in pummelo, to 14.1 mm (1.7 mm SD) in citron. Although pollen tubes in the self-pollinated citron only reached 60% of the length of the style, a few pollen tubes were observed at the base of the style in a few flowers. In all other combinations pollen tubes reached the base of the style in all flowers and varying number of tubes were recorded ranging from 2 to 40. Thus a highly significant difference between the crosses carried out was found for pollen tube growth and for the number of pollen tubes reaching the ovary (Table 1). Interestingly, the number of pollen tubes at the base of style was not the same in reciprocal crosses, and varied depending on the particular male-female or female-male combination. As a female receptor, pummelo and citron had significantly more pollen tubes at the base of their styles compared to mandarin. However, as a male donor, none of the species was **more** high performing in more than one combination. As a particular male-female combination, pummelo x mandarin and citron x pummelo resulted in the highest number of pollen tubes (>40) reaching the base of the style.

1 3.3. *Interspecific crosses at different temperature regimes*

2 Subjecting flowers to varying temperatures between 15 and 30 °C in controlled growth
3 chambers differentially affected pollen tube growth in the style, and the number of pollen tubes
4 reaching the base of style. The analysis of variance revealed not only a highly significant effect of
5 the crosses made (genotype and combination) and of temperatures assayed, but also of their
6 interaction (Table 1). Thus, pollen performance depended on the particular male-female or female-
7 male combination. Overall, 20 °C, and to a lesser degree 25 °C, promoted pollen tube elongation; 15
8 °C and to a lesser extent 30 °C were inhibitory in most cases. The promoting effect of 20°C can best
9 be seen by the number of pollen tubes reaching the base of style of mandarin, and in the
10 combination citron x pummelo 3 days after pollination (Fig. 3).

11 Overall the longest pollen tubes were observed in citron styles and, at a less degree, in cross-
12 pollinated pummelo styles. Nevertheless, due to the different stigma + style length of the considered
13 species, pollen tubes covered the whole style length in mandarin after three days. Pollen tube
14 growth rate was also very effective in citron x pummelo combination in which, despite the very
15 long style length, a number of pollen tubes reached the base of the style after three days at different
16 temperatures.

17 Furthermore, temperature appears to have a significant effect on the place where pollen
18 tubes were arrested in self-incompatible pollinations (clementine and pummelo). For clementine,
19 while at 15 and 25 °C pollen tubes were arrested before reaching one third of the style length, they
20 traveled significantly longer distance at 20 °C (about two third of the length of style), and less
21 distance -although not significantly different from 15 °C and 25 °C- at 30 °C (25% of the length of
22 style).

23 Comparing mandarin and citron as female recipients, the effect of temperature appeared to
24 be independent of the pollen donors in the first, but varied with the pollen donor in the second. As a
25 pollen donor, no single species was consistently more high-performing in more than one
26 combination. Under our controlled temperature conditions (maintaining flowers for 3 days after

pollination) only the three combinations in which mandarin was used as a female recipient, and the combination citron x pummelo showed some pollen tubes at the base of the style.

4. Discussion

4.1. Male-female interaction and pollen performance

To analyze the influence of the genotype on pollen performance we carried out first an *in vitro* pollen germination assay to assess pollen viability under different temperature regimes. All the analyzed *Citrus* species showed more or less the same pollen grain germination trend as temperature increased from 10 °C to 30 °C, but different pollen grain germination rate at the same temperature. Furthermore, the ranking of the different genotypes studied was not constant and varied with temperature, which resulted in highly significant genotype-temperature interaction. Overall, the pollen had a sufficient viability, and at optimum temperature pollen germination ranged from >90% for pummelo to 28% for clementine. Variation in *in vitro* pollen germination between different genotypes within the same species and at the interspecific level has been reported in other species reflecting either differences in the genetic background or the origin and adaptation of the pollen-producing parent (reviewed in Hedhly, 2011).

Pollen performance *in vivo* (expressed as pollen tube length in the style and number of pollen tubes reaching the base of style) varied, as well, depending on the genotype of the pollen donor and of the female receptor.

Under field temperature conditions, the ten days after pollination allowed pollen tubes to reach the base of style of all flowers and in all combinations except for self-pollinated flowers of pummelo and citron. Thus, results obtained herein showed that, under field temperature conditions, and based on the number of pollen tubes at the base of style, there is no particular pollen donor or female recipient that is consistently high-performing, but the outcome is rather dependent on the particular male-female and/or female-male combination. Under both field temperature and growth chamber conditions, those combinations that promoted more pollen tube growth in the style and

1 higher number of pollen tubes at the base of style, can be considered compatible pollinations. Thus,
2 based on these results, ‘Sha Tian Yu’ pummelo can be considered self-incompatible, while the
3 small number of pollen tubes reaching the base of style of self-pollinated ‘Vozza Vozza’ citron
4 point out either to be a self-compatible slow progamic phase, or to be a late acting self-
5 incompatibility; however, more experiments are needed to have a definitive answer.

6 Examining the differential behaviour in pollen performance, although some female
7 recipients were somehow deciding factor in more than one combination irrespective of the male
8 genotype, overall, no single pollen donor was consistently **more** high performing, and pollen
9 performance was definitively influenced by the particular male-female or female-male interaction.
10 Differences in pollen performance depending on the pollen and the pistil genotypes have been
11 previously reported in other species (e.g. *Prunus* spp.: Hedhly et al., 2005a; *Citrus* spp.: Distefano
12 et al., 2009). Although in some species the male genotype was the deciding factor in pollen
13 performance and the ranking was consistent across different female genotypes (Charlesworth et al.,
14 1987; Snow and Spira, 1991; Marshall, 1998; Mitchell and Marshall, 1998), results obtained in this
15 and other works (Stephenson and Bertin, 1983; Vaser et al., 1987; Cruzan, 1990; Willson, 1994;
16 Herrero and Hormaza, 1996; Kerwin and Smith-Huerta, 2000; Marshall and Diggle, 2001; Hedhly
17 et al., 2005a) suggest that the outcome of the sexual phase is rather dependent on the specific male-
18 female genotype interaction. Also the different rate of compatibility for pollen tube behavior in
19 interspecific cross condition, are consistent with those obtained in previous works on citrus (Ton
20 and Krezdorn, 1966; Ngo, 2001; Yamamoto, 2006; Distefano et al., 2009).

22 4.2. Temperature variation effects on pollen performance

23 One of the most important environmental factors that could affect pollen performance is the
24 temperature regime during the progamic phase spanning from pollination to fertilization. It has been
25 shown that temperature affects pollen germination (Elgersma et al., 1989; Shivanna et al., 1991),
26 and pollen tube kinetics in the style (Lewis, 1942; Jefferies et al., 1982; Elgersma et al., 1989;

Hedhly et al., 2005b). Studies in sweet cherry (*Prunus avium* L.) showed that temperature affects not only pollen tube growth rate, but also pollen tube dynamics and, as a consequence, influences the amount of the pollen tubes that succeed in reaching the ovary (Hedhly et al., 2005a).

Our results of pollen germination under different *in vitro* incubation temperatures, showed a progressive increase in germination rate from 15 °C to 25 °C, with a sharp decrease when temperature reached 30 °C. All three true ancestral species plus clementine showed their maximum germination percentage at 25 °C. This is in line with results reported for different sweet orange cultivars (Ramos et al., 2008). Thus, the absence of germination at 10 °C, and the optimum germination temperature of 25 °C reflect the subtropical origins of all *Citrus* species examined. Similar relatively high optimum temperatures were reported for other subtropical and tropical species such as *Pistacia* spp. (Acar and Kakani, 2010), olive (Koubouris et al., 2009), and mango (Sukhvibul et al., 2000), while relatively lower optimum temperatures in the range of 15-20 °C are usually reported for temperate fruit trees like apricot (Egea et al., 1992), sweet cherry (Pirlak, 2002), and sour (Cerovic and Ruzic, 1992).

Our results confirmed the self-incompatibility of clementine (Ton and Krezdorn, 1966; Distefano et al., 2009) and pummelo (Ngo, 2001; Yamamoto, 2006) showing pollen tube growth arrest in the first quarter of the style following self-pollination under field temperature conditions. However, characterizing pollen tube growth in these self-incompatible crosses and under different temperature regimes, revealed significant differences in the place where pollen tubes were arrested. An effect of temperature on the self-incompatibility reaction was reported in pear (Lombard et al. 1972). In *Citrus* species, the incompatibility reaction was classified by the degree of self-incompatibility at different levels of the style (Ngo, 2001; Yamamoto, 2006). In some cases, pollen tubes are arrested very soon at the stigma level (Ton and Krezdorn, 1966), and, in others, they are arrested at the base of the style, in the ovary or ovules (Sage et al., 1994; Gòmez et al., 2004). The influence of temperature reported here on the place where pollen tube are arrested, can explain part

of the conflicting reports in the literature, and suggest a superimposed influence of temperature and also of the specific male and female genotypes interaction.

In compatible pollinations, pollen performance in the style (pollen tube growth and the number of pollen tubes reaching the base of style) did not correspond to pollen performance *in vitro*. This discrepancy can be explained by an effect of a suboptimal artificial germination medium, by a different optimum temperature between pollen germination and pollen tube growth as discussed below, or by an influence of the female recipient used (Hedhly et al., 2005a). Results obtained herein show an optimum temperature for pollen germination *in vitro* of 25 °C, while the most favourable temperature to accelerate *in vivo* pollen tube growth depended on the particular male-female interaction and ranged between 15 and 25 °C. This result is in line with previous reports of a different optima for both parameters (McKee and Richards, 1998; Kakani et al., 2002), suggesting that they are independent processes.

Overall, our result showed that increasing temperature increased pollen germination and pollen tube growth, and that both low and high temperature had a negative effect. Results obtained in this work show how pollen performance is not only an inherent characteristic of the pollen genotype, but is largely dependent on male-female and genotype-temperature interactions.

References

- Acar, I., Kakani, V.G., 2010. The effects of temperature on *in vitro* pollen germination and pollen tube growth of *Pistacia* spp. *Sci. Hortic.* 125, 569–572.
- Barkley, N.A., Roose, M.L., Krueger, R.R., Federici, C.T., 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). *Theor. Appl. Genet.* 112, 1519–1531.
- Barrett, H.C., Rhodes, A.M., 1976. A numerical taxonomic study of affinity relationships in cultivated *Citrus* and its close relatives. *Syst. Bot.* 1, 105–136.

1 Cerovic, R., Ruzic, D., 1992. Pollen tube growth in sour cherry (*Prunus cerasus* L.) at different
2 temperatures. J. Hortic. Sci. 67, 333–340.

3 Charlesworth, D., Schemske, D.W., Sork, V.L., 1987. The evolution of plant reproductive
4 characters; sexual vs. natural selection. In: Stearns, S.C. (Ed.), Evolution of Sex and its
5 Consequences. Birkhauser, Basel, pp. 317–355.

6 Cruzan, M.B., 1990. Pollen-pollen and pollen style interactions during pollen tube growth in
7 *Erythronium grandiflorum* (Liliaceae). Am. J. Bot. 77, 116–122.

8 Deng, Z.N., La Malfa, S., Xie, Y.M., Xiong, X.X., Gentile, A., 2007. Identification and evaluation
9 of chloroplast uni- and trinucleotide sequence repeats in citrus. Sci. Hortic. 111, 186–192.

10 Distefano, G., Gentile, A., Herrero, M., 2011. Pollen–pistil interactions and early fruiting in
11 parthenocarpic citrus. Ann. Bot. 108, 499–509.

12 Distefano, G., Las Casas, G., La Malfa, S., Gentile, A., Tribulato, E., 2009. Pollen tube behavior in
13 different mandarin hybrids. J. Am. Soc. Hortic. Sci. 134, 583–588.

14 Egea, J., Burgos, L., Zorua, N., Egea, L., 1992. Influence of temperature on the *in vitro* germination
15 of pollen of apricot (*Prunus armeniaca* L.). J. Hortic. Sci. 67, 247–250.

16 Elgersma, A., Stephenson, A.G., Nijs, A.P.M., 1989. Effects of genotype and temperature on pollen
17 tube growth in perennial ryegrass (*Lolium perenne* L.). Sex. Plant Reprod. 2, 225–230.

18 Fang, D.Q., Roose, M.L., 1997. Identification of closely related citrus cultivars with inter-simple
19 sequence repeat markers. Theor. Appl. Genet. 95, 408–417.

20 FAOSTAT, 2010. <http://faostat.fao.org/site/339/default.aspx>.

21 Federici, C.T., Fang, D.Q., Scora, R.W., Roose, M.L., 1998. Phylogenetic relationships within the
22 genus *Citrus* (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. Theor. Appl.
23 Genet. 96, 812–822.

24 Franklin-Tong, V.E., Ride, J.P., Read, N.D., Trewavas, A.J., Franklin, F.C.H., 1993. The self-
25 incompatibility response in *Papaver rhoeas* is mediated by cytosolic free calcium. Plant J. 4,
26 163–177.

1 Franklin-Tong, V.E., Ride, J.P., Franklin, F.C.H., 1995. Recombinant stigmatic self-incompatibility
2 (S-) protein elicits a Ca^{2+} transient in pollen of *Papaver rhoeas*. Plant J. 8, 299–307.

3 Gómez, N.L., García, E.A., Castillo, A.M., Corona, T., Almaguer, G., 2004. Pollen tube growth in
4 mandarin. Rev. Fit. Mex. 27, 177–182.

5 Hedhly, A., 2011. Sensitivity of flowering plant gametophytes to temperature fluctuations. Environ.
6 Exp. Bot. 74: 9-16.

7 Hedhly, A., Hormaza, J.I., Herrero, M., 2005a. Influence of genotype–temperature interaction on pollen
8 performance. J. Evol. Biol. 18, 1494–1502.

9 Hedhly, A., Hormaza, J.I., Herrero, M., 2005b. The effect of temperature on pollen germination, pollen
10 tube growth and stigmatic receptivity in peach (*Prunus persica* L. Batsch.). Plant Biol. 7, 476–483.

11 Hedhly, A., Hormaza, J.I., Herrero, M., 2009. Global warming and sexual plant reproduction. Trends
12 Plant Sci. 14, 30–36.

13 Herrero, R., Asins, M.J., Carbonell, E.A., Navarro, L., 1996. Genetic diversity in the orange subfamily
14 Aurantioideae. I. Intraspecies and intragenus genetic variability. Theor. Appl. Genet. 92, 599–609.

15 Herrero, M., Hormaza, J.I., 1996. Pistil strategies controlling pollen tube growth. Sex. Plant Reprod. 9,
16 343–347.

17 Hormaza, J.I., Herrero, M., 1996. Dynamics of pollen tube growth under different competition regimes.
18 Sex. Plant Reprod. 9, 153–160.

19 Hormaza, J.I., Herrero, M., 1999. Pollen performance as affected by the pistilar genotype in sweet
20 cherry (*Prunus avium* L.). Protoplasma 208, 129–135.

21 Jefferies, C.J., Belcher, A.R., 1974. A fluorescent brightener used for pollen tube identification *in vivo*.
22 Stain Technol. 49, 199 – 202.

23 Jefferies, C.J., Brain, P., Stott, K.G., Belcher, A.R., 1982. Experimental systems and mathematical
24 model for studying temperature effects on pollen-tube growth and fertilization in plum. Plant Cell
25 Environ. 5, 231–236.

- 1 Johannsson, M.H., Stephenson, A.G., 1998. Effects of temperature during microsporogenesis on pollen
2 performance in *Cucurbita pepo* L. (Cucurbitaceae). Int. J. Plant Sci. 159, 616–626.
- 3 Johansen, D.A., 1940. Plant Microtechniques. McGraw-Hill, New York.
- 4 Kakani, V.G., Prasad, P.V.V., Craufurd, P.Q., Wheeler, T.R., 2002. Response of *in vitro* pollen
5 germination and pollen tube growth of groundnut (*Arachis hypogaea* L.) genotypes to
6 temperature. Plant Cell Environ. 25, 1651–1661.
- 7 Kerwin, M.A., Smith-Huerta, N.L., 2000. Pollen and pistil effects on pollen germination and tube
8 growth in selfing and outcrossing populations of *Clarkia tembloriensis* (Onagraceae) and their
9 hybrids. Int. J. Plant Sci. 161, 895–902.
- 10 Kim, J.H., Mori, T., Wakana, A., Ngo, B.X., Sakai, K., Kajiwarra, K., 2011. Determination of self-
11 incompatible *Citrus* cultivars with S_1 and/or S_2 alleles by pollination with homozygous S_1
12 seedlings (S_1S_1 or S_2S_2) of 'Banpeiyu' pummelo. J. Japan. Soc. Hortic. Sci. 80, 404–413.
- 13 Koubouris, G.C., Metzidakis, I.T., Vasilakakis, M.D., 2009. Impact of temperature on olive (*Olea*
14 *europaea* L.) pollen performance in relation to relative humidity and genotype. Environ. Exp. Bot.
15 67, 209–214.
- 16 Lewis, D., 1942. The physiology of incompatibility in plants. I. Effect of temperature. Proc. R. Soc.
17 London Ser. B. Biol. Sci. 131, 13–26.
- 18 Linskens, H.F., Esser, K., 1957. Über eine spezifische anfärbung der pollenschläuche im griffel und die
19 zahl der kallosepfropfen nach selbstdung und fremddung. Naturwissenschaften 44, 1–2.
- 20 Lombard, P.B., Williams, R.R., Scott, K.G., Jeffries, C.J., 1972. Temperature effects on pollen tube
21 growth in styles of Williams' pear with a note on pollination deficiencies of comice pear.
22 Compte Rendue du Symposium 'Culture du Poirier', 4-8 septembre, 265–279.
- 23 Marshall, D.L., 1998. Pollen donor performance can be consistent across maternal plants in wild
24 radish (*Raphanus sativus*, Brassicaceae): a necessary condition for the action of sexual
25 selection. Am. J. Bot. 85, 1389–1397.

- 1 Marshall, D.L., Diggle, P.K., 2001. Mechanism of differential donor performance in wild radish,
2 *Raphanus sativus* (Brassicaceae). Am. J. Bot. 88, 242–257.
- 3 McKee, J., Richards, A.J., 1998. The effect of temperature on reproduction of five *Primula* species.
4 Ann. Bot. 82, 359–374.
- 5 Mesejo, C., Martínez-Fuentes, A., Reig, C., Rivas, F., Agustí, M., 2005. The inhibitory effect of
6 CuSO₄ on pollen germination and pollen tube growth and its application for the production of
7 seedless fruit. Plant Sci. 170, 37–43.
- 8 Mitchell, R.J., Marshall, D.L., 1998. Nonrandom mating and sexual selection in desert mustard: an
9 experimental approach. Am. J. Bot. 85, 48–55.
- 10 Moore, G., 2001. Oranges and lemons: clues to the taxonomy of *Citrus* from molecular markers.
11 Trends Genet. 17, 536–540.
- 12 Mulcahy, D.L., SariGorla, M., Mulcahy, G.B., 1996. Pollen selection – past, present and future.
13 Sex. Plant Reprod. 9, 353–356.
- 14 Nettancourt, de D., 1977. Incompatibility in Angiosperms. Springer, Berlin Heidelberg, New York.
- 15 Nettancourt, de D., 1997. Incompatibility in Angiosperms. Sex. Plant Reprod. 10, 185–199.
- 16 Ngo, B.X., Wakana, A., Park, S.M., Nada, Y., Fukudome, I., 2001. Pollen tube behaviors in self-
17 incompatible and self-compatible *Citrus* cultivars. J. Fac. Agr. Kyushu U. 45, 443–457.
- 18 Nicolosi, E., Deng, Z.N., Gentile, A., La Malfa, S., Continella, G., Tribulato, E., 2000. Citrus
19 phylogeny and genetic origin of important species as investigated by molecular markers. Theor.
20 Appl. Genet. 100, 1155–1166.
- 21 Pirlak, L., 2002. The effects of temperature on pollen germination and pollen tube growth of apricot
22 and sweet cherry. Gartenbauwissenschaft 67, 61–64.
- 23 Ramos, J.D., Pasqual, M., Salles, L.A., Chagas, E.A., Pio, R., 2008. Stigma receptivity and *in vitro*
24 citrus pollen grains germination protocol adjustment. Interciencia 33, 51–55.
- 25 Sage, T.L., Bertin, R.I., Williams, E.G., 1994. Ovarian and other late-acting self-incompatibility
26 systems. In: Williams, E.G., Knox, R.B., Clarke, A.E. (Eds.), Genetic Control of Self-

1 Incompatibility and Reproductive Development in Flowering Plants. Kluwer, Dordrecht,
2 pp.116–140.

3 Sassa H., Nishio T., Kowiyama Y., Hirano H., Koba T., Ikehishi H., 1993. Self-incompatibility (S)
4 alleles of the Rosaceae encode members of a distinct class of the T2/S ribonuclease superfamily.
5 Mol. Gen. Genom. 250, 547–557.

6 Scora, R.W., 1975. On the history and origin of *Citrus*. Bull. Torr. Bot. Club 102, 369–375.

7 Scora, R.W., 1988. Biochemistry, taxonomy and evolution of modern cultivated citrus. Proc. Sixth Int.
8 Citrus Congress 1, 277–289.

9 Sedgley, M., Griffin, A.R., 1989. Sexual Reproduction of Tree Crops. Academic Press, London.

10 Shivanna, K.R., Linskens, H.F., Cresti, M., 1991. Response of tobacco pollen to high humidity and heat
11 stress: viability and germinability *in vitro* and *in vivo*. Sex. Plant Reprod. 4, 104–109.

12 Snow, A.A., Spira, T., 1991. Pollen vigor and the potential for sexual selection in plants. Nature 352,
13 796–797.

14 Soost, R.K., 1965. Incompatibility alleles in the genus *Citrus*. Proc. Am. Soc. Hortic. Sci. 87, 176–180.

15 Stephenson, A.G., Bertin, R.I., 1983. Male competition, female choice, and sexual selection in plants. In:
16 Real, L. (Ed.), Pollination Biology. Academic Press, New York, pp. 109–149.

17 Stephenson, A.G., Lau, T.C., Quesada, M., Winsor, J.A., 1992. Factors that affect pollen performance.
18 In: Wyatt, R. (Ed.), Ecology and Evolution of Plant Reproduction. Chapman & Hall, New York, pp.
19 119–134.

20 Sukhvibul, N., Whiley, A.W., Vithanage, V., Smith, M.K., Doogan, V.J., Hetherington, S.E., 2000.
21 Effect of temperature on pollen germination and pollen tube growth of four cultivars of mango
22 (*Mangifera indica* L.). J. Hortic. Sci. Biotechnol. 75, 64–68.

23 Tao, R., Yamane, H., Sassa, H., Mori, H., Gradziel, T.M., Dandekar, A.M., 1997. Identification of stylar
24 RNases associated with gametophytic self-incompatibility in almond (*Prunus dulcis*). Plant Cell
25 Physiol. 38, 304–311.

1 Ton, L.D., Krezdorn, A.H., 1966. Growth of pollen tubes in three incompatible varieties of *Citrus*.
2 J. Am. Soc. Hort. Sci. 89, 211–215.

3 Vaser, N.M., Price, M.V., Montalvo, A.M., Gray, R.N., 1987. Female mate choice in a perennial
4 herbaceous wildflower, *Delphinium nelsonii*. *Evol. Trends Plants* 1, 29–33.

5 Xue, Y.B., Carpenter, R., Dickinson, H.G., Coen, E.S., 1996. Origin of allelic diversity in
6 antirrhinum S locus RNases. *Plant Cell* 8, 805–814.

7 Willson, M.F., 1994. Sexual selection in plants: perspective and overview. *Am. Nat.* 144, S13–S39.

8 Willson, M.F., Burley, N., 1983. *Mate Choice in Plants*. Princeton University Press, Princeton.

9 Yamamoto, M., Kubo, T., Tominaga, S., 2006. Self- and cross-incompatibility of various *Citrus*
10 accessions. *J. Japan. Soc. Hort. Sci.* 75, 372–378.

11 Young, H.J., Stanton, M.L., 1990. Influence of environmental quality on pollen competitive ability
12 in wild radish. *Science* 248, 1631–1633.

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Figures and table legends

Fig. 1. *In vitro* pollen grain germination percentage at different incubation temperatures (10 °C, 15 °C, 20 °C, 25 °C, and 30 °C for 24h) for ‘Dancy’ mandarin, ‘Vozza Vozza’ citron, ‘Sha Tian Yu’ pummelo and ‘Comune’ clementine. (means $\pm$ se).

Fig. 2. Pollen tube growth in the styles of cross- and self-pollinated flowers of ‘Dancy’ mandarin (M), ‘Vozza Vozza’ citron (C) and ‘Sha Tian Yu’ pummelo (P), 10 days after pollination under field temperature conditions. Upper: pollen tube length (black bars) in the stigma-style (grey bars); lower: number of pollen tubes at the base of the style. (means $\pm$ se).

Fig. 3. Pollen tube growth in the styles of cross- and self-pollinated flowers of ‘Dancy’ mandarin (M), ‘Vozza Vozza’ citron (C), ‘Sha Tian Yu’ pummelo (P), and ‘Comune’ clementine (Cl) 3 days after pollination under incubation temperatures of 15 °C, 20 °C, 25 °C, 30 °C. Upper: pollen tube length in the stigma-style. Horizontal black lines indicate the stigma-style length (5.7 mm for ‘Dancy’, 12.1 mm for ‘Sha Tian Yu’, 14.1 mm for ‘Vozza Vozza’ citron and 5.9 mm for ‘Comune’ Clementine); lower: number of pollen tubes at the base of the style. (means $\pm$ se).

Table 1

Analyses of variance of temperature effect on pollen germination *in vitro*, pollen tube growth in the style and number of pollen tubes at the base of the style under field conditions and growth chamber constant temperatures.

Table 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
<i>Pollen germination in vitro</i>						
^a Temperature	3	297.5	99.2	601.8	< 2.2e-16	***
Genotype	3	98.6	32.9	199.4	<2.2e-16	***
Temperature:genotype	9	71.5	7.9	48.2	<2.2e-16	***
Residuals	80	13.2	0.2			
<i>Pollen tube growth in the style in the field</i>						
Cross	8	35.8	4.5	121.3	<2.2e-16	***
Residuals	81	3.0	0.04			
<i>Number of pollen tube at the base of the style in the field</i>						
Cross	8	381.5	47.7	275.92	<2.2e-16	***
Residuals	81	14.0	0.2			
<i>Pollen tube growth in the style in growth chambers</i>						
Cross	9	115.9	12.9	490.3	<2.2e-16	***
Temperature	3	35.2	11.7	447.3	<2.2e-16	***
Cross:temperature	27	19.2	0.7	27.1	<2.2e-16	***
Residuals	359	9.4	0.03			
<i>Number of pollen tube at the base of the style in growth chambers</i>						
^b Cross	5	156.6	31.3	29.7	3.1e-14	***
Residuals	53	55.9	1.0			

^a The 10°C temperature observations was removed from the analysis

^b Anova was done only on those 6 crosses with pollen tubes at the base of the style

Fig. 2

Fig. 3