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Self-incompatibility, parthenocarpy and reduction of seed presence in 'Afourer' mandarin

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ABSTRACT

Seedlessness is one of the main requirements for the *Citrus* fresh market. 'Afourer' mandarin has been registered as self-incompatible, producing seedless fruit in the absence of cross-pollination. Under field conditions, a high number of seeded fruits is produced, which reduces its quality and commercial value. The purpose of this work was to characterize self-incompatibility type of 'Afourer' mandarin, to determine its parthenocarpic capacity, and to reduce the presence of seeds under field conditions. Four experiments were performed, including net-covered trees during flowering period (experiments 1 and 2), single flower emasculation and self-pollination (experiment 3), *in vivo* pollen germination, pollen tube development and ovule viability (experiment 1), and field application of copper sulfate ($CuSO_4$) and gibberellic acid (GA_3) during flowering period (experiment 4); open pollination was the control in all situations. Under open pollination, relatively low seedless fruit percentage was found (7% and 34%), whereas with anti-bee nets this percentage reached 98–99% of the fruits. Relatively high fruit set percentage and fruit number per tree was found in net-covered trees and in bagged or emasculated flowers, indicating that 'Afourer' mandarin presents facultative parthenocarpy. Emasculated or self-pollinated flowers resulted in similar seedless fruit number, indicating an autonomous parthenocarpy. In flowers from open or net-covered trees, similar pollen tube growth was registered until 6 days after anthesis, but, thereafter, growth was interrupted in self-pollination condition, reaching only 40% of the style length, which suggests a gametophytic self-incompatibility system. GA_3 applied during flowering period reduced the percentage of seeded fruits and seed number per fruit; the most efficient treatment was three applications of GA_3 ($50\,mg\,l^{-1}$) combined with $CuSO_4$ ($25\,mg\,l^{-1}$), that increased seedless fruit from 19% to 31% and reduced from 3.7 to 2.3 the number of seeds per fruit.

1. Introduction

Global trade of *Citrus* fruit demands high cosmetic and organoleptic characteristics; in recent years, seedless fruit has been one of the main requirements for this market. The definition of 'seedless fruit' has changed over time. In the 1980s, it was defined as those fruits with less than five seeds. Barry (2004) proposes a stricter classification, considering exclusively one seed per 100 fruits. Marketing requirements are variable, having fewer exigencies when cultivars in high demand for their organoleptic quality are concerned.

'Afourer', originated in Morocco, and also known as 'W. Murcott' or 'Nadorcott', is probably a hybrid of Murcott and an unknown pollinator parent (Nadori, 1998, 2004). It is reported as self-incompatible and produces seedless fruit in the absence of cross-pollination (Bono et al., 2000; Chao, 2005). However, under field conditions, a high percentage of seeded fruits is produced (Agustí et al., 2005). A similar behavior is observed in Uruguayan citriculture, where this cultivar presents high yields and quality fruit, but seed presence reduces its commercial value.

Self-incompatibility is defined as the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination (de Nettancourt, 1977). Self-incompatibility has been classified as gametophytic, when incompatibility phenotype of the pollen is determined by its own (haploid) S genotype, or as sporophytic, which is regulated by the diploid S genotype of the pollen-producing plant (Newbigin et al., 1993). The incompatible reaction could occur along different phases of pollen tube growth through the gynoecium (de Nettancourt, 1977; Newbigin et al., 1993). In general, it has been stated that when it occurs in the stigma, a sporophytic incompatibility is operating, and when pollen tube starts growing through the style, but could not progress more

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than 50% of it, it indicates a gametophytic self-incompatibility (de Nettancourt, 1977; Newbigin et al., 1993). The most widespread form of self-incompatibility in plants is the gametophytic type (Newbigin et al., 1993).

In self-incompatible *Citrus* cultivars, pollen tubes are short and spiraling, with irregular callose deposition (Kahn and De Mason, 1986), while in compatible combinations, pollen tubes have thin walls, grow straight and form regular-sized callose plugs, which reach the base of the style (Distefano et al., 2009). A gametophytic self-incompatibility seems to be operating in most *Citrus* hybrids (Distefano et al., 2009). However, different degree of incompatibility has been reported for Clementine cultivars (Ton and Krezdorn, 1966), suggesting that in *Citrus*, self-incompatibility reactions between style and pollen tubes, could be classified by the degree of incompatibility of each portion of the style (Ngo et al., 2001; Yamamoto and Tominaga, 2002). The incompatibility reaction acquisition also seems to be related to pistil maturation, being operative no longer before anthesis (Distefano et al., 2009; Ngo et al., 2001).

One of the main aspects in the production of seedless fruit in self-incompatible varieties, but with viable gametes, is avoiding cross-pollination with compatible cultivars. In *Citrus*, pollination with other cultivars is mainly entomophilous, being bees the main pollinators (Moffett and Rodney, 1971). The distance that pollen can be transported by bees was determined by Chao et al. (2005) in California, using molecular markers, reaching 500 and 960 m for pollen of 'Clementina de Nules' and 'Afourer' mandarins, respectively. This information indicates the difficulties in isolating facultative parthenocarpic cultivars of cross-pollination, in commercial production conditions. Temperature is an important factor conditioning *Citrus* pollination, fecundation and seed set. It has been proved that optimum temperature for pollen germination *in vitro* is 25 °C, while the most favorable temperature to accelerate *in vivo* pollen tube growth, depended on the particular male–female interaction, ranging between 15 and 25 °C (Distefano et al., 2012). Hand pollination of 'Afourer' with pollen of Clementines and hybrids as 'Fortune', 'Nova' and 'Ortanique' shows high capacity for seed formation in Spain (Bono et al., 2000).

Different attempts to reduce *Citrus* fruit seeds has been performed in the last years; gibberellic acid (GA_3) applied at anthesis impairs fertilization by either enhancing ovule abortion or reducing pollen tube growth in 'Clemenules' flowers under cross-pollination conditions (Mesejo et al., 2008). In addition, copper sulfate ($CuSO_4$) applied at full bloom, to 'Afourer' trees under cross-pollination, increases the percentage of seedless fruits and reduces seed number per fruit (Mesejo et al., 2006).

Self-incompatible *Citrus* cultivars can present high parthenocarpic ability, as Satsuma mandarin, or low parthenocarpic capacity, as Clementines, associated mainly to the lower active gibberellins levels in the ovaries during the first stage of fruit growth (Talón et al., 1990). Supporting this, exogenous GA_3 applied during blossom, increase fruit set of seedless Clementine mandarins, though is non-effective in Satsuma mandarin (Talón et al., 1992). Even more, in *Citrus*, when gibberellins synthesis is disrupted by applying paclobutrazol, fruit set diminished (Ben-Cheikh et al., 1997; Rivas et al., 2010).

There are no reports on the self-incompatibility and parthenocarpic type of 'Afourer' mandarin, and scarce information is available about the ability of other cultivars to pollinate it under open pollination, or the agronomical practices to control seed formation. Considering this background, the objectives of our work were (1) to study self-incompatibility of 'Afourer' mandarin and determine its parthenocarpic type and capacity and (2) to reduce the presence of seeds with spring sprays of gibberellic acid and copper sulfate.

2. Materials and methods

2.1. Parthenocarpy and self-incompatibility characterization

2.1.1. Characterization of parthenocarpy

Experiment 1. In an orchard located in Paysandú, Uruguay (31° SL), a plot of 'Afourer' mandarin grafted on 'Trifoliate orange' [*Poncirus trifoliata* (L.) Raf.] with fertigation was used. Twenty 2-year-old homogeneous trees were selected; half of them were covered by anti-bees net fixed on wooden structures to prevent cross-pollination, from the start of flowering to the end of petal fall. The other 10 trees were used as controls (open pollination). At harvest, all fruits per tree were counted and weighed. Presence of seeds was evaluated in 20 randomly selected fruits per tree. Air temperature and relative humidity was registered with sensors situated in the canopy of control and net-covered trees.

Experiment 2. In Libertad, Uruguay (35° SL), a plot of 5-year-old 'Afourer' mandarin grafted on 'Trifoliate orange' with fertigation was used. Ten trees were covered with anti-bees net from the beginning of flowering until the end of petal fall and 10 uncovered trees were used as controls (open pollination). Two branches with an average of 120 nodes per branch were tagged to evaluate flowering intensity and fruit set. Seeds per fruit were quantified in 200 fruits per tree. Air temperature and relative humidity was registered in the canopy of control and net-covered trees.

Experiment 3. In Paysandú, in a plot of seven-year-old 'Afourer' trees grafted on 'Trifoliate orange', 15 trees were selected and 30 leafy single flowered shoots per tree were labeled, totaling 450 shoots. Three treatments with 150 flowers each were applied: (a) open pollination (control), (b) bagged flowers and (c) flowers emasculated and bagged. Treatments were performed at the end of the flowering period, selecting shoots at 59 state of the *Citrus* BBCH phenological scale (Agustí et al., 1997). Bags were taken off 15 days after that. At maturation all fruits were counted, and fruit set percentage and seeds per fruit were quantified. To assess pollen viability, *in vitro* pollen germination was evaluated in 10 flowers collected at anthesis. Flowers were kept in silica gel during 24 h to promote anther opening, then anthers were removed and kept at 4 °C in a humid chamber during 2 h to achieve pollen pre-hydration. BK germination medium was used (Brewbaker and Kwack, 1963). In a laminar flow chamber, pollen was extracted from anthers with a thin brush, and put in a slide over a solid medium (BK solidified with 1% Phytagel), and then covered with liquid BK medium. Samples were kept in darkness at 25 °C and 70–80% RH during 48 h. Following incubation, samples were fixed with FAA solution (5% formaldehyde, 5% acetic acid, 90% ethanol at 70%) . With an optical microscope (OLYMPUS ECE-Bi), 100 pollen grains (germinated or not) were counted in eight fields of each slide. Pollen was considered germinated when pollen tube length was larger than grain size (Stanley and Linskens, 1974).

2.1.2. Characterization of self-incompatibility

To determine self-incompatibility type *in vivo*, flowers sampled from net-covered and control trees from experiment 1 were used. Pollen germination, pollen tube development and ovule viability were observed by fluorescence microscopy, using an OLYMPUS AH3-RFCA microscope with ultraviolet filter. One hundred single leafy flower shoots, at state 59 of the *Citrus* BBCH phenological scale (Agustí et al., 1997), were tagged before covering the trees with the net. Ten flowers per treatment (covered and uncovered trees) were collected at pre-anthesis and 0, 3, 6 and 9 days after anthesis. Flowers were fixed in FAA and kept at 4 °C until analysis. Flowers were prepared for microscopic observation according to Mesejo et al. (2006). Pollen germination was measured counting 300 germinated grains in four randomly selected visual fields per flower. Pollen tube growth was determined as the percentage of the tube length in the style recorded by the longest pollen tube in each flower. Ovule viability was determined as the percentage of ovules without callose deposition in the chalazal zone, considering a mean of 15 ovules per flower.

2.2. Chemical control of seeds

Experiment 4. In Paysandú, 48 seven-year-old 'Afourer' trees grafted on 'Trifoliate orange' rootstock under fertigation conditions were selected. Eight treatments, including gibberellic acid (GA_3) and copper sulfate ($CuSO_4$), were applied (Table 1).

In GA_3 sprays, water was previously acidified to pH 4.5 and, in all cases, a non-ionic adherent was added. Throughout the period of fruit growth, equatorial diameter of 30 fruits per tree was measured. At harvest, fruit number and yield per tree were quantified. A randomized sample of 100 fruits per plant was used to estimate final fruit size and seed number.

To determine treatment effect on pollen germination, two *in vitro* assays were carried out according to the methodology described in experiment 3 (Section 2.1.1). *Assay A*: before tree spray, 15 flowers per tree were sampled at anthesis (state 61 of the *Citrus* BBCH phenological scale, Agustí et al., 1997) and processed for pollen extraction, which were placed into BK germination medium (control treatment), or into a BK with GA_3 (50 mg l^{-1}), $CuSO_4$ (125 mg l^{-1}) or GA_3 (50 mg l^{-1}) + $CuSO_4$ (125 mg l^{-1}) added to the medium. *Assay B*: one day after the third application, 15 flowers from control, GA_3 (50 mg l^{-1}), $CuSO_4$, or GA_3 + $CuSO_4$ treated trees were collected and their pollen was placed in a BK germination medium for evaluation. To evaluate *in vivo* field treatment effect on pollen germination, pollen tube development and ovule viability, 5 flowers per tree were sampled at 0, 3, 6 and 9 days after anthesis and observed by fluorescence microscopy as in experiment 1 (Section 2.1.2).

2.3. Statistical analyses

A randomized single plot design, with 6–10 replications and a tree as experimental unit was used in experiments 1, 2 and 4. For microscopic determinations (experiments 1, 3 and 4), flowers were the experimental unit. Continuous variables were analyzed by general linear models; proportions were previously transformed to arcsine, and mean differences were compared by Tukey's test.

3. Results and discussion

3.1. Parthenocarpy and self-incompatibility characterization

3.1.1. Characterization of parthenocarpy

Under open pollination, relatively low seedless fruit percentage was found in experiments 1 and 2 (Tables 2 and 3). Differences between them could be explained by pollen donor cultivars situated near the experimental plot, environmental conditions and/or bee efficiency in each location. In experiment 1, 'Ortanique' and 'Satsuma' mandarins were the surrounding cultivars, whereas in experiment 2, 'Fino' lemon, 'Nova' and 'Montenegrina' mandarins were the nearest cultivars. In both locations, the average mean temperature from anthesis to petal fall was 16–17 °C, varying between 12 and 24 °C, thus no restrictive temperature for *in vivo* pollen tube growth (Distefano et al., 2012). However, several pollen behavior, depending on pollen and pistil genotypes at different temperatures, have been recently proved (Distefano et al., 2009, 2012), which can be associated to seed set capacity, and could explain our results. An average of 4–5 seeds per fruit was found in experiments 1 and 2, respectively (Tables 2 and 3).

In net-covered trees (cross-pollination prevented), 99% and 98% of the fruits were seedless in experiments 1 and 2, respectively (Tables 2 and 3). No significant differences in temperature or relative humidity inside or outside net coverage were registered (data

Table 1
Treatments applied for chemical control of seeds in 'Afourer' mandarin (experiment 4).

Treatments	25–35% open flowers	35–50% open flowers	50–60% open flowers
Control	–	–	–
$CuSO_4$ (125 mg l^{-1}) (2)[z]	+	+	–
$CuSO_4$ (125 mg l^{-1}) (3)	+	+	+
GA_3 (10 mg l^{-1}) (2)	+	+	–
GA_3 (10 mg l^{-1}) (3)	+	+	+
GA_3 (50 mg l^{-1}) (2)	+	+	–
GA_3 (50 mg l^{-1}) (3)	+	+	+
$CuSO_4$ (125 mg l^{-1}) + GA_3 (50 mg l^{-1}) (3)	+	+	+

[z] (2) Two sprays and (3) three sprays.

Table 2
Percentage of seedless fruits, fruit number and yield per tree, fruit weight and number of seeds per fruit, in open pollination and net-covered trees of 'Afourer' mandarin. Data correspond to the average of 10 trees per treatment (experiment 1).

Treatment	Seedless fruits (%)	Fruit number tree^{-1}	Yield (kg tree^{-1})	Fruit weight (g)	Seeds per fruit[y]
Open pollination	34 b[z]	117 a	10 a	85 a	4 a
Net-covered trees	99 a	115 a	10 a	87 a	0.04 b

[z] Different letters in columns indicate significant differences (Tukey, $p \leq 0.05$).
[y] Data correspond to total seeds divided by all evaluated fruits.

Table 3
Fruit set, seedless fruit percentage and seed number per fruit, in open pollination and net-covered trees of 'Afourer' mandarin. Data correspond to the average of 10 trees per treatment (experiment 2).

Treatment	Flowers per 100 nodes	Fruit set (%)	Seedless fruits (%)	Seeds per fruit[y]
Open pollination	60 a[z]	15.2 a	7 b	5 a
Net-covered trees	76 a	6.4 b	98 a	0.07 b

[z] Different letters in columns indicate significant differences (Tukey, $p \leq 0.05$).
[y] Data correspond to total seeds divided by all evaluated fruits.

not shown). The use of anti-bees net during flowering period in experiment 1 did not modify the productive behavior of 'Afourer', reaching the same yield, fruit number, and fruit weight as with open-pollination treatment (Table 2). In experiment 2, fruit set in uncovered trees was higher than in net-covered ones; however, more than 6% of fruit set was found in both situations (Table 3). These results indicate that 'Afourer' mandarin presents facultative parthenocarpy. Wide variability concerning parthenocarpic ability is reported among *Citrus* cultivars. 'Satsuma' mandarin presents high fruit set in the absence of cross-pollination (Talón et al., 1990), whereas self-incompatible hybrids like 'Nova' (Papadakis et al., 2009; Rivas et al., 2004) or 'Ortanique' (Borges et al., 2009), and most of Clementines (Talón et al., 1990) have low parthenocarpic capacity. In Clementines, the low ability to set parthenocarpic fruits is associated to low levels of active GAs in the ovaries from anthesis until early stages of cell enlargement (Talón et al., 1990, 1992). Accordingly, exogenous GA_3 increases fruit set of seedless Clementine mandarins, yet is non-effective in Satsuma mandarin (Talón et al., 1992).

In experiment 3, in flowers with cross-pollination prevented (bagged flowers, and emasculated and bagged flowers), all fruits were seedless (Table 4). *In vitro* 'Afourer' pollen germination reached 25%, therefore, the absence of seeds could not be explained by pollen quality, considering that in other *Citrus* cultivars, less than 15% of pollen germination was enough to set seeds (Bermejo et al., 2011). In both treatments, fruit set was higher than 40%, without significant differences between them (Table 4). These results allow us to postulate that 'Afourer' parthenocarpy is autonomous, as it does not depend on the stimulus of pollination to set fruits.

In control flowers (open pollination), fruit set reached 72%; high percentage of seedless fruit and low seed number per fruit was found (Table 4). This result, compared to the low percentage of seedless fruits under open pollination in experiments 1 and 2, could be explained by the little foreign pollen available at the end of the flowering period, when experiment 3 was installed, and by reduced bee activity. The high percentage of fruit set in all treatments may be attributed to the high fruit set capacity of leafy single flower *Citrus* shoots (da Cunha Barros and Gravina, 2006; Rivas et al., 2007) that were used in our experiment. Additionally, as treatments were performed at the end of the flowering period, temperature was more favorable for fruit set (Lovatt et al., 1984). Both factors could also explain the difference with the low fruit set (1%) obtained by Chao et al. (2005) after 'Afourer' flower emasculation.

3.1.2. Characterization of self-incompatibility

Before anthesis, at state 59 of the *Citrus* BBCH phenological scale (Agustí et al., 1997), no pollen was shed in the stigma, which allow us to state that no pollination occurs before anthesis in 'Afourer' mandarin (Fig. 1A). This result indicates that the temporal compatibility, which appears to occur before anthesis in several self-incompatible *Citrus* cultivars, has been avoided (Distefano et al., 2009; Ngo et al., 2001). Net-covered trees, with bees excluded, did not prevent 'Afourer' pollen to reach the stigma. However, there was sensible lower pollen amount than in flowers of control trees (open pollination) (Fig. 1B and C). Pollen tube growth along styles was similar in both conditions until 6 days after anthesis, confirming that 'Afourer' pollen is not only viable, but has also the ability to germinate and start growing in its own flowers (Table 5). Thereafter, pollen tube growth in styles of flowers from net-covered trees was interrupted, reaching only the 40% of the style length. This suggests that the incompatibility reaction occurs in the higher portion of the style, as it has been reported for 'Fortune' and 'Nova' hybrids (Distefano et al., 2009). Our result allow us to propose that in 'Afourer' mandarin, a gametophytic self-incompatibility is operating, given that in this type, pollen tubes cannot progress more than 50% of the style length (de Nettancourt, 1977; Newbigin et al., 1993). Supporting this affirmation, high correspondence between predicted *S* genotypes and pollen tube behavior has been proved in several *Citrus* cultivars (Ngo et al., 2010).

Under open pollination, pollen tube reached the base of the style at 9 days after anthesis, when ovules still remained viable, as no callose layer deposition near the chalazal end occurred (Rodrigo and Herrero, 1998) (Fig. 1D). This information, together with seed presence in fruits, confirms that fertilization normally occurred. No defective pollen tube guidance was observed in 'Afourer' under open pollination, differing from the behavior reported for Satsuma 'Owari' mandarin and 'Valencia' orange (Mesejo et al., 2007), and from the effect of preanthesis GA_3 application induced in 'Clemenules', as an indirect process of the embryo sac degeneration (Mesejo et al., 2008).

3.2. Chemical control of seeds

In experiment 4, the percentage of fruit set and final fruit number were not significantly affected by GA_3 or $CuSO_4$ treatments, reaching at harvest more than 1000 fruits and 100 kg per tree (data not shown). This behavior confirms the high productive capacity of 'Afourer' under open-pollination conditions (Agustí et al., 2005). However, GA_3 treatments (50 mg l^{-1}), when combined with $CuSO_4$ (125 mg l^{-1}), resulted in the lowest fruit weight, reaching significant differences with respect to controls. This reduction in fruit size in the combined treatment may be associated with higher percentage of initial fruit set, registered between 30 and 60 days post-flowering, which resulted in lower fruit diameter from 75 days post-flowering (data not shown).

Seedless fruit percentage in control trees reached 19%, indicating high pollination and fertilization with pollen from cultivars located near the experimental plot. Potential pollinator cultivars in the orchard were 'Valencia' orange (130 m of distance), and 'Clementina de Nules' mandarin (175 m of distance). Combined application of GA_3 (50 mg l^{-1}) and $CuSO_4$ increased seedless fruit percentage to 31% (Table 6). The $CuSO_4$ sprays failed to decrease the percentage of seeded fruit, and seed number per fruit, not confirming, in our conditions, the results of Mesejo et al. (2006) in the same cultivar under Spanish conditions. The response to GA_3 was related to the concentration used and the number of applications—the higher the product concentration and application number, the fewer the seeds per fruit (Table 6).

In vitro pollen germination was reduced by the products added to the medium, being completely inhibited by the combined application of GA_3 (50 mg l^{-1}) and $CuSO_4$ (125 mg l^{-1}) (Table 7, assay A). In flowers sampled after field applications, *in vitro* pollen

Table 4
Fruit set, seedless fruit percentage and seed number per fruit in 'Afourer' mandarin from open pollination, bagged, and emasculated and bagged flowers. Data correspond to the average of 150 single leafy flower shoots per treatment (experiment 3).

Treatment	Fruit set (%)	Seedlees fruits (%)	Seeds per fruit[y]
Open pollination	72 a[z]	73 b	0.47 a
Bagged flowers	50 b	100 a	0.00
Emasculated and bagged flowers	43 b	100 a	0.00

[z] Different letters in columns indicate significant differences (Tukey, $p \leq 0.05$).
[y] Data correspond to total seeds divided by all evaluated fruits.

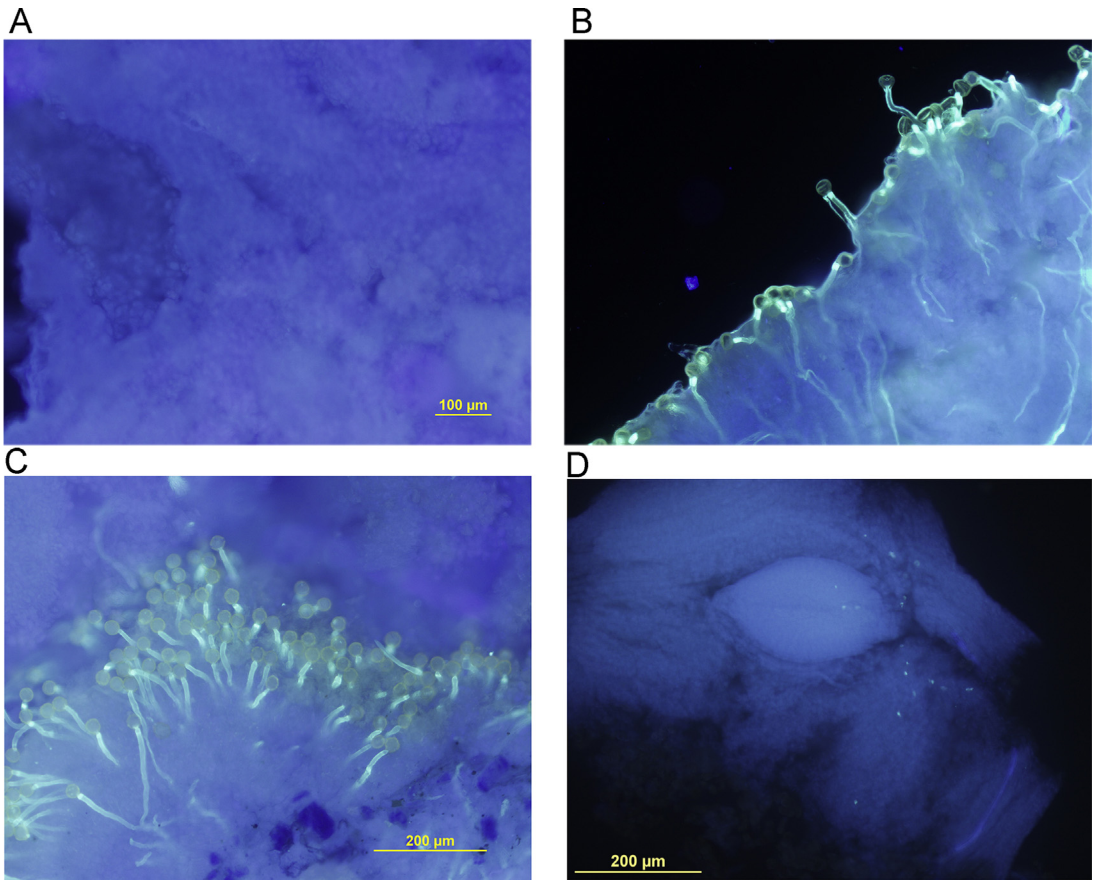

Fig. 1. 'Afourer' flowers sampled from net-covered trees and open-pollination trees. Stigma before anthesis with absence of pollen (A), pollen germinated 3 days after anthesis on the stigma of flowers from net-covered trees (B) pollen germinated 3 days after anthesis on the stigma of flowers collected from open-pollination trees (C), viable ovule at 9 days after anthesis (D).

germination was significantly reduced with respect to control, but germination percentage never diminished below 18% (Table 7, assay B). *In vivo*, no clear reduction of pollen germination in the stigma was found after field treatments (data not shown). However, 3 days after anthesis, the number of pollen tubes growing in the style was reduced by 40% with three field applications of $GA_3 + CuSO_4$. Beside this, 6 days after anthesis, pollen tubes growth could reach 50–75% of the style length in all treatments and no significant ovule degeneration until 9 days after anthesis could be verified as a result of field applications. These results explain the low efficiency in reducing seed number per fruit of the field treatments.

Mesejo et al. (2006) reported that pollen of 'Fortune' mandarin was completely inhibited *in vitro* by the addition of $CuSO_4$ (25 mg l^{-1}). However, when it was applied to 'Clemenules' flowers hand pollinated with 'Fortune' pollen, *in vivo* pollen germination was reduced only if $CuSO_4$ was sprayed at pre-anthesis or 2 h before pollination, but no reduction was registered if treatment was performed 24 h after pollination. GA_3 (10 mg l^{-1}) sprayed the days around anthesis to 'Clemenules' single flowers, enhanced ovule abortion, reduced pollen tube growth, and consequently diminished seed set under cross-pollination conditions. Treatment effectiveness depended on physiological flower state, being anthesis, simultaneously with pollination, the most efficient moment (Mesejo et al., 2008).

Our results in 'Afourer' mandarin indicate that one of the main factors to optimize GA_3 sprays applied to whole trees is to cover as many flowers in sensitive phenological stages as possible (61–67, BBCH, Agustí et al., 1997), in agreement with Mesejo et al. (2008) working in 'Clemenules'. For future research

Table 5
Number of germinated pollen, pollen tube growth (as a percentage of pistil length recorded) and percentage of viable ovules, in single leafy flowers of 'Afourer' mandarin sampled from 0 to 9 days after anthesis, under open pollination and net coverage. Data correspond to 10 flowers per treatment and date (experiment 1).

Days after anthesis	Treatment	Number of germinated pollen	Pollen tube growth (% of pistil length)	Viable ovules (%)
0	Open pollination	0	–	100
	Net-covered trees	0	–	100
3	Open pollination	159	19	100
	Net-covered trees	1	20	100
6	Open pollination	604	37	100
	Net-covered trees	70	42	100
9	Open pollination	727	95	100
	Net-covered trees	65	40	100

Table 6
Seedless fruit percentage and seed number per fruit concerning total fruits (TF) and seeded fruits (SF) in 'Afourer' mandarin. Data correspond to the average of 6 trees per treatment (experiment 4).

Treatment	Seedless fruits (%)	Seeds per fruit (TF)	Seeds per fruit (SF)
Control	19 b[z]	3.7 a	4.6 a
$CuSO_4$ 125 mg l^{-1} (2)[y]	20 ab	3.5 ab	4.4 ab
$CuSO_4$ 125 mg l^{-1} (3)	26 ab	3.0 abc	4.0 ab
GA_3 10 mg l^{-1} (2)	21 ab	3.1 abc	3.9 ab
GA_3 10 mg l^{-1} (3)	25 ab	3.1 abc	4.0 ab
GA_3 50 mg l^{-1} (2)	25 ab	3.2 abc	4.2 ab
GA_3 50 mg l^{-1} (3)	30 ab	2.5 bc	3.6 ab
GA_3 50 mg l^{-1} + $CuSO_4$ 125 mg l^{-1} (3)	31 a	2.3 c	3.3 b

[z] Different letters in columns indicate significant differences (Tukey, $p \leq 0.05$).
[y] (2) Two applications: 25–35% open flowers (OF) + 35–45% OF; (3) three applications: 25–35% OF + 35–45% OF + 50–55% OF.

Table 7
Effect of GA_3 (50 mg l^{-1}) or $CuSO_4$ (125 mg l^{-1}) on the *in vitro* pollen germination of 'Afourer' mandarin in two assays: (A) products added to the medium and (B) field applications at anthesis. Data correspond to the average of 6 trees per treatment (experiment 4).

Treatment	Pollen germination (%)	
	Products added to the medium (A)	Field applications (B)
Control	25 a	46 a
GA_3 (50 mg l^{-1})	14 b	20 b
$CuSO_4$ (125 mg l^{-1})	4 c	18 b
GA_3 (50 mg l^{-1}) + $CuSO_4$ (125 mg l^{-1})	0 d	20 b

it would be interesting to start treatment applications earlier than in our experiment, *i.e.* approximately 10% of open flowers (stage 61) and repeating applications every 4–5 days until the end of flowering.

4. Conclusions

'Afourer' mandarin presents a gametophytic self-incompatibility system and an autonomous facultative parthenocarpy. Under the climatic conditions of Uruguay, 'Afourer' produces seedless fruits in isolation from cross-pollination; in open pollination, GA_3 applied during the flowering period reduces the percentage of seeded fruits and seed number per fruit, but does not eliminate them completely.

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