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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