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Self-fertility and preferential cross-fertilization in mango (*Mangifera indica*)

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Abstract

Mango (*Mangifera indica* L.) is a tropical fruit tree originated from Southeastern Asia, which is cultivated worldwide in regions with tropical and subtropical climates. Mango cultivation area has significantly extended in the last decades often to regions where environmental conditions are not the most favourable for optimal mango flower development and fruit set and, consequently, several reproductive problems had been described in this species. Some of them could be related to self-incompatibility, but so far information is not clear on the possible self-incompatibility system operating in this crop. In this work we study pollen tube growth, following self and cross pollination, in three mango cultivars ('Osteen', 'Kent' and 'Kensington'). Paternity was also determined in the offspring of two of these cultivars - 'Osteen' and 'Kent' - and in a solid block of an additional cultivar, 'Keitt'. Sequential examination of pollen tube

growth showed that pollen tubes grew to the base of the style in both cross- and self-pollinated pistils of the different cultivars examined. Paternity analysis of ‘Osteen’ and ‘Keitt’ offspring in monovarietal orchards showed the production of fruits resulting from self-fertilization in both cultivars. The results from the multivarietal orchard corroborate this fact for ‘Osteen’ and ‘Kent’, but showed a preference for outcrossing in ‘Osteen’. Pollen tube behaviour and paternity analysis show self-fertility in this species but the higher proportion of fruits resulting from outcrossing suggests a preference for cross-fertilization.

Keywords

Self-incompatibility, pollen tube, paternity analysis, mango, *Mangifera indica*

1. Introduction

Mango (*Mangifera indica* L.) belongs to the Anacardiaceae, a family that contains about 70 genera and 850 species (Bompard and Schnell, 1997) including other species of agronomic interest such as pistachio (*Pistacia vera* L.), cashew (*Anacardium occidentale* L.), ambarella (*Spondias dulcis* Forst.), or yellow and purple mombins (*Spondias mombin* L. and *Spondias purpurea* L., respectively). The genus *Mangifera* includes about 70 species, mostly restricted to tropical Asia, of which at least 12 are of local commercial importance.

Mango is an andromonoecious evergreen tropical tree that has been cultivated for millennia in Southern Asia. Cultivation spread outside its centre of origin and domestication throughout many tropical and subtropical regions of the world along

trading routes where selections of the cultivars best adapted to particular conditions was made. Currently, mango is one of the five most important fruit species worldwide (together with bananas, oranges, grapes and apples) with a total world production that has reached about 40 million tons in 2013 (FAOSTAT, 2016). A few countries (India, China, Thailand, Indonesia and Mexico) account for over 70% of world production, being India, with about 18 millions tons, the main mango producing country.

Mango inflorescences are composed thirsoids that present a high number of flowers with a variable proportion of both hermaphrodite and male flowers. Mango trees have low fruit set, usually lower than 0.1% under open pollination, due to a massive drop of flowers and developing fruitlets (Singh, 1960). This situation is common to other fruit tree species and different reasons, such as environmental factors (Sturrock, 1966; Huang et al., 2010), alternate bearing (Dambreville et al., 2013), high number of malformed flowers (Sturrock, 1966), empty and degenerated embryo sacs (Shuraki and Sedgley, 1996), immature female reproductive organs (Sturrock, 1966; Martinez-Pallé and Herrero, 1998), or starch deficiencies in flowers (Alcaraz et al., 2010, 2013), have been proposed to explain this low flower to fruit ratio. However, information is still elusive to explain the reasons behind the massive premature flower abscission in mango.

One of the reasons could be the existence of a self-incompatibility mechanism. Since Darwin (Darwin, 1876), considerable knowledge has been accumulated on self-incompatibility mechanisms in flowering plants (Darwin, 1876; Charlesworth and Willis, 2009; De Nettancourt, 2013; Gibbs, 2014). Incompatibility systems create barriers to avoid self-fertilization, promoting gene exchange and preventing genetic erosion (Takayama and Isogai, 2005; Charlesworth and Willis, 2009). The possible existence of self-incompatibility in mango was proposed based on results particularly

from Indian cultivars, as 'Dushehari' or 'Dasheri', 'Chausa', 'Bombay Green' and 'Langra' (Singh et al., 1962; Mukherjee et al., 1968; Sharma and Singh, 1970; Ram et al., 1976). Recently, it has also been suggested in 'Malika' and 'Amrapali' which are progeny of 'Dushehari' (Dutta et al., 2013), and 'Ataulfo' in Mexico (Gehrke-Vélez et al., 2012). However, results based on fruit set are difficult to interpret due to the low fruit set, even following hand-pollination, in this species. An evaluation of the pollen tube behaviour has been reported in a number of cases (Maklad et al., 2011; Dutta et al., 2013) showing different time requirements for self and cross pollen tubes to reach the base of style. Even some studies report the presence of mango seeds without endosperm after self-pollination, although no differences in pollen tube growth were found between self- and cross-pollinated flowers (Mukherjee et al., 1968). Seed degeneration and fruitlet drop during the month following initial fruit set has also been reported (Sharma and Singh, 1970; Singh and Sharma, 1972), with higher fruit drop after self-pollination (Dag et al., 1997). However, so far a clear-cut procedure to evaluate self-incompatibility in mango is still missing.

The availability of molecular tools that allow the paternity analysis of the embryos produced can be highly useful to advance in the understanding of possible self-incompatibility mechanisms in mango. The first studies with molecular markers in mango to evaluate outcrossing rate were performed with isozymes (Dag et al., 1997; Dag et al., 1998; Dag et al., 2009), pointing out a high rate of outcrossing in fruits from plants placed near pollinizer trees. Later, studies continued with microsatellite and AFLP markers showing similar results (Schnell et al., 2006; Santos and Neto, 2011).

With the objective of advancing in the knowledge of self-incompatibility mechanisms in mango, in this work we studied pollen tube kinetics and dynamics along

the style after controlled hand pollinations, and used microsatellite markers to analyse seed paternity after open pollination.

2. Material and methods

2.1. Pollination and pollen tube growth

Plant material and pollinations

Three mango cultivars ('Osteen', 'Kent' and 'Kensington') maintained at the IHSM La Mayora, Málaga, Spain, mango germplasm collection at latitude 36º45'N, longitude 4º4' W and altitude 35 m above sea level were used as pollen donor and recipient genotypes. Most mango cultivars produce monoembryonic seeds while some produce polyembryonic seeds, in which only one of the embryos is of gametic origin and the rest are somatic (Aron et al., 1998). 'Osteen' and 'Kent' are monoembryonic cultivars whereas 'Kensington' is polyembryonic. A diallel cross was performed in which each cultivar was hand-pollinated with pollen from the other genotypes including self-pollination. Flowers of the female recipients were collected in the morning, from 08:30 to 10:30 hours, before anther dehiscence. Then, these flowers were emasculated and placed on wet paper inside a Petri dish, in the lab, each dish containing 20 flowers. For each cross, six dishes were prepared to allow for six fixing times, with a total of 18 Petri dishes per female recipient genotype. Flowers from the pollen donors, after collection in the field, were also preserved in the lab until anther dehiscence. Female recipient flowers were pollinated in the lab, from 14:00 to 15:00 hours, by rubbing the dehisced anther of a flower of the pollen donor on the stigma of the female recipient flower. After pollination, flowers were fixed every four hours, at 4, 8, 12, 16, 20 and 24 hours in formalin: acetic acid: ethanol (FAA) at 1:1:18 (v/v/v) (Johansen, 1940).

121

122 *Microscopic observations*

123 To monitor pollen tube growth rate in the stigma and style, the fixed pistils were
124 washed in distilled water, and left in 5% sodium sulphite for 24 hours. Samples were
125 autoclaved for five minutes at 1 Kg/cm^2 in sodium sulphite (Jefferies and Belcher,
126 1974), and then the individual styles were squashed onto glass slides with 0.005% (w/v)
127 aniline blue in 0.15N K_3PO_4 (O'Brien and McCully, 1981). Microscope slides were
128 preserved at 100% relative humidity at 4ºC overnight. Pollen tubes were visualized with
129 a Leica DM LB2 epifluorescent microscope (Leica, Wetzlar, Germany) with a 340-380
130 excitation filter and LP 425 barrier filter after squashing the coverslip. Number of pollen
131 tubes in the stigma and in the upper, middle and basal sections of the style was counted.
132 Pollen tube kinetics at the style level was evaluated as the percentage of the style length
133 traversed by the longest pollen tube.

134

135 **2.2. Outcrossing rate**

136 *Plant material*

137 Two experiments were performed, one in a multivarietal mango orchard with pollen
138 available from a wide range of cultivars, and the other in an orchard with two adjacent
139 monovarietal blocks. For the first experiment, fruits for paternity analysis were obtained
140 in a collection of mango varieties at the IHSM La Mayora, Málaga, Spain. The planting
141 pattern is 3m x 4m, showing the same distance between trees throughout the plot. Fruits
142 were collected, from September to November, from three trees of 'Osteen' and 'Kent'

143 that were surrounded by different mango varieties. After seed extraction, the tissue of
144 the embryonic axis was used for DNA extraction.

145 For the second experiment, fruits for paternity tests were obtained from a
146 commercial mango orchard in Benamocarra (Málaga, Spain). Two opposite plots, one
147 with only 'Osteen' and the other with only 'Keitt' trees, were used; an additional row of
148 'Kent' was also present at the border of the 'Osteen' plot (Fig. 4). The distance between
149 rows of trees was 3.5 m. In each plot fruit samples were taken from trees at different
150 distances from the opposite plot. Ten fruits from two trees were collected in each row,
151 and the seeds were removed for DNA extraction as described above.

152

153 *DNA extraction and SSR analysis*

154 DNA from the embryos was extracted using the method reported by Hormaza (2002).
155 Initial amplification was performed with 16 SSR loci, previously developed for mango
156 by Viruel et al., (2005), that allowed assigning paternity. PCR amplifications were
157 performed in 15µl volumes containing 15 ng of genomic DNA, 16 mM $(NH_4)_2SO_4$, 67
158 mM Tris-HCl pH 8.8, 0.01% Tween20, 2 mM $MgCl_2$, 0.1 mM each primer, 0.625 mM
159 each dNTP and 0.5 units of Biotaq™ DNA polymerase (Bioline, London, UK).
160 Forward primers were labeled with florescent dyes on the 5' end (Proligo, France).
161 Reactions were carried out on an I-cycler thermocycler (Bio-Rad Laboratories,
162 Hercules, CA, USA) using the following temperatures: a step of one minute at 94ºC
163 followed by 35 cycles of 30 s at 94ºC, 30 s at 50ºC of annealing temperature and an
164 extension step of five minutes at 72ºC.

165 PCR amplified fragments were analysed by capillary electrophoresis in a CEB™
166 8000 capillary DNA analysis system (Beckman Coulter, Fullerton, CA, USA). The

results allowed the selection of 7 primers (LMMA6, LMMA7, LMMA8, LMMA9, LMMA10, LMMA11 and LMMA15) that were enough to discriminate the different genotypes available as pollen donors.

For the evaluation of outcrossing rate in the monovarietal block experiment, the protocol was performed as described above for the multivarietal orchard but, in this case, only three loci, LMMA9, LMMA10 and LMMA11, were used since they allow unequivocal differentiation between the two varieties, 'Osteen' and 'Keitt'.

Paternity analysis

Results from the capillary DNA analysis system were analysed using the Cervus 3.0 software (Marshall et al., 1998; Kalinowski et al., 2007), that allows assignment of paternity to the most likely parents with a known level of statistical confidence by using a likelihood-based approach generating a statistic, ΔLOD, that is defined as the difference in positive log likelihood ratios (LOD) between the top two candidate parents (Hauser et al., 2011). Two confidence levels were used, 80% and 95%.

3. Results

3.1. Pollen germination and pollen tube grow *in vivo*

In spite that the flowers were hand pollinated some flowers did not show pollen tubes in the style in all the crosses. The proportion of pistils with pollen tubes was variable depending on the cultivars; it was higher for 'Kensington' (82%), whereas for 'Osteen' and 'Kent' the values were 56% and 40% respectively, regardless of the pollen donor

189 genotype. Few pollen tubes were observed in the small mango stigmas, with differences
190 in the average number of pollen tubes between cultivars, which were higher for
191 'Kensington', 8.91 ± 0.89, compared to 2.64 ± 0.61 in 'Osteen' or 2.05 ± 0.58 in 'Kent'.
192 Pollen grains rapidly germinated and grew in the style and no rejection of pollen tubes
193 in self-pollinating samples in the three genotypes was observed (Fig. 1).

194 Evaluation of pollen tube kinetics showed that germination proceeded rapidly
195 and, in all crosses, pollen tubes could be observed at the base of the style in some
196 flowers 4 hours after pollination (Fig. 2A, B, C), although the number of pollen tubes
197 did not stabilize at the base of the style until 16 hours after pollination. Pollen tubes
198 reached the base of the style in most of the flowers analysed (Fig. 2D, D, F) with an
199 average of 0.5-1.5 tubes, following both cross and self pollinations, (Fig. 2G, H, I). The
200 best pollen receptor genotype was 'Kensington' with 76% flowers with pollen tubes at
201 the style base, followed by 'Osteen' with 45%, and, finally, 'Kent' with 28 % for all
202 crosses.

203

204 **3.2. Outcrossing rate in a multivarietal mango orchard**

205 Unequivocal paternity, with a 95% confidence level, could be assigned to 91 of the 95
206 'Osteen' fruits analysed. Fruits that did not reach the confidence level were not taken
207 into account. The results from the fruits analysed showed that only 6% of the 'Osteen'
208 fruits resulted from self-fertilization. The paternity analysis showed that in 55% of the
209 cases the pollen donor was 'Kensington' and in 15% 'Keitt'. Other genotypes, also
210 present in the orchard, were assigned as parents at a lower rate (Fig. 3A).

211 The situation was different for 'Kent', in which the paternity analyses showed
212 that half of the fruits (48%) resulted from self-fertilization. In this case, for 62 of the 71

fruits analysed, paternity could be assigned with a confidence level of 80% (45 fruits with 95% confidence level, and 15 fruits with 80% confidence level); fruits with a confidence level lower than 80% were not taken into account. For the fruits resulting from cross-pollination, the main pollen donor genotypes were 'Kensington' (18%), 'Keitt' (11%), 'Manzanillo Núñez' (8%) and 'Irwin' (6%). The rest of the genotypes showed a percentage lower than 5% (Fig. 3B).

3.3. Outcrossing rate in monovarietal mango orchards

In this case, most of the fruits resulted from self-fertilization in both 'Osteen' (79%) and 'Keitt' (76%). Most of the outcrossed 'Osteen' fruits had 'Keitt' as pollen donor and less than 7% 'Kent' due to the presence of a row of this cultivar bordering the 'Osteen' plot (Fig. 4). For 'Keitt', most of the outcrossed fruits had 'Osteen' as pollen donor (Fig. 4). In general, the rate of outcrossing decreased with increasing distance from the pollen donor both for 'Osteen' (0.26, 0m; 0.25, 17m; 0.20, 35m; 0.10, 52m; 0.21, 66m) and 'Keitt' (0.35, 25m; 0.35, 60m; 0.15, 95m; 0.10, 130m).

4. Discussion

4.1. Pollen germination and pollen tube growth in vivo

Although self-incompatibility had been previously reported for some mango cultivars (Singh et al., 1962; Mukherjee et al., 1968; Sharma and Singh, 1970; Ram et al., 1976; Gehrke-Vélez et al., 2012; Dutta et al., 2013), our results with 'Osteen', 'Kent' and 'Kensington' do not suggest an evident self incompatibility system at the stigma or style level that can significantly affect pollen tube growth. However, this pollen tube

236 behaviour cannot rule out a post-zygotic embryo rejection as a late-acting self
237 incompatibility system (Mukherjee et al., 1968), similarly to what has been suggested
238 for cashew, *Anacardium occidentale*, (Aliyu, 2007; Wunnachit et al., 1992), also a
239 member of the Anacardiaceae.

240 Differences in the number of pollen tubes at the stigma were found between
241 cultivars, which can be due to differences in the adhesion, hydration or germination of
242 the pollen grains. Results in 'Kensington' showed more pollen tubes at the stigmatic
243 region than in 'Osteen' or 'Kent'. This better performance of 'Kensington' as pollen
244 receptor is interesting since this cultivar has polyembryonic seeds, where all the
245 embryos except one are of somatic origin (Maheshwari, 1950). Similar differences in
246 pollen tube behaviour have been recorded in sweet cherry flowers depending on the
247 genotype (Hormaza and Herrero, 1999) or the mating combination (Hedhly et al., 2005,
248 2015).

249

250 **4.2. Selfing and outcrossing in mango**

251 The results show a high outcrossing rate in mango in the line of previous paternity
252 studies using morphological (Singh et al., 1962; Mukherjee et al., 1968; Sharma and
253 Singh, 1970) and molecular traits (Schnell et al., 1994; Dag et al., 1997; Degani et al.,
254 1997b; Dag et al., 1998; Dag et al., 2001; 2009; Santos and Neto, 2011). Those results
255 suggest the presence of negative selection after fertilization of fruits resulting from
256 selfing, a similar situation to that also reported in other subtropical fruit crops such as
257 avocado or lychee (Degani et al., 1995; Degani et al., 1997a; Alcaraz and Hormaza,
258 2011).

As expected, outcrossing rate was higher in the multivarietal than in the monovarietal orchards. In the multivarietal orchard, the high percentage of outcrossing observed in 'Osteen' could be due to a higher synchronization in flowering time with the rest of the genotypes present in the orchard. In contrast, the high proportion of fruits resulting from self pollination in 'Kent' appears related to the fact that, under our environmental conditions, 'Kent' flowering time was significantly later than most of the genotypes in the collection.

In the monovarietal orchards studied, self-fertilization prevailed over cross-fertilization, with a low outcrossing rate for both genotypes, 'Osteen' and 'Keitt'. In those orchards, a decrease in the percentage of fruits derived from outcrossing with increasing distance to the pollinizer was obtained. Similar results have been obtained previously in mango (Degani et al., 1997b; Dag et al., 1998; Dag et al., 2009), and in other subtropical fruit trees such as avocado or lychee (Degani et al., 1995; Degani et al., 1997a; Alcaraz and Hormaza, 2011).

4.3. Preferential cross-fertilization versus cryptic self-incompatibility in mango

Floral incompatibility systems are a common phenomenon in several plant species, resulting in the avoidance of self-fertilization or crossing with genetically close genotypes favouring genetic exchange. The occurrence of self-incompatibility in some mango cultivars has been described previously (Singh et al., 1962; Mukherjee et al., 1968; Sharma and Singh, 1970; Ram et al., 1976; Gehrke-Vélez et al., 2012; Dutta et al., 2013), but no conclusive data about a possible incompatibility system in this species have been put forward. In this study a combination of molecular and microscopic techniques was used. Thus, microsatellite markers were used to assess the paternity of

fruits harvested from ‘Osteen’ and ‘Kent’ trees in a multivarietal orchard. The results showed in ‘Kent’ a similar percentage of cross and self-fertilized fruits, suggesting self-compatibility in this cultivar. However, in ‘Osteen’ only a small proportion of the fruits were the result of self-fertilization. Since the high percentage of cross-fertilization observed in this cultivar could conflict with previous observations showing no production problems in monovarietal orchards of this variety, a second experiment was performed in an orchard with just two cultivars in different opposite plots: ‘Osteen’ and ‘Keitt’. A paternity analysis was made from the fruits harvested from trees belonging to both varieties located at different distances from the pollen donor. The results showed a high majority of fruits produced by self-fertilization especially in the trees farther away from the pollen donor, suggesting that self-fertility is possible in these two mango cultivars.

Therefore, the results suggest self-fertility for ‘Kent’, but in ‘Osteen’, although self-fertilization is possible, a preferential trend for cross-fertilization has been recorded. This could be related to the presence of a cryptic self-incompatibility system in this mango variety that would favour cross-fertilization but, in the absence of pollen from other genotypes, would make self-fertilization possible. The term cryptic self-incompatibility was introduced to refer to the cases in which selfing is possible although fertilization with cross-pollen is preferred (Bateman, 1956). Still, no differences could be observed in pollen tube growth in self- and cross-pollinations, with no apparent incompatibility symptoms at the stigma or style levels. In this sense, the term late acting self-incompatibility was introduced by Seavey and Bawa (1986) and thoroughly discussed recently by Gibbs (2014) to explain the lack of selfed fruits after apparently successful self pollen tube growth in the stigma and style. The results obtained in this work suggest that mango could have a combination of the two systems and additional

work in other cultivars is needed to study if this could be a more widespread process in other cases that have been considered to be self-compatible. Similar results of a preferential selection over offspring have been obtained not only in mango (Dag et al., 1998; 2009), but also in avocado or lychee (Degani et al., 1986; Degani et al., 1995; Degani et al., 1997a; Degani et al., 2003; Alcaraz and Hormaza, 2011). Usually those results have been explained in terms of inbreeding depression (Charlesworth and Charlesworth, 1987). In any case, preferential cross-fertilization without excluding self-fertilization is a bet-hedging strategy either for avoiding inbreeding depression or for ensuring reproduction (Holsinger, 1996; Kruszewski and Galloway, 2006; Cachi et al., 2013).

Conflicts of interest

The authors declare no conflicts of interest.

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482 Figure legends

483 **Figure 1 Pollen tube growth in self-pollinized mango flowers.** (A) 'Kent', (B) 'Osteen' and (C)
484 'Kensington'. Aniline blue stain. Bars = 100µm.

485 **Figure 2 Pollen tube growth in the in a diallel pollination of three cultivars.** Expressed as % of style
486 travelled by the longest pollen tube (mean ± SE). (A, B, C), % of flowers with pollen tubes in the base of
487 style, (D, E, F) and number de pollen tubes in the base of style (mean ± SE), (G, H, I). In pistils of
488 'Kensington, KS' (A, D, G), 'Osteen, OS' (B, E, H), and 'Kent, KT' (D, F, I).

489 **Figure 3 Assignment of paternity in a mango orchard with a collection of varieties.** Expressed as the
490 percentage of fruits originating for each parental genotype. (A) 'Osteen' as maternal genotype and (B)
491 'Kent' as maternal genotype.

492 **Figure 4 Diagram of outcrossing rate in a solid mango block of 'Keitt' adjacent to a solid block of**
493 **'Osteen'.** Expressed as the outcrossing rate for each genotype. On the top of the diagram the rows (r)
494 from which samples were taken and the distance between them in each plot (number of trees and distance
495 in meters) are represented. Bordering the 'Osteen' plot there was a single row with 'Kent' trees.
496 Outcrossing rate results are shown at the bottom of the diagram, and the grey line represents
497 decreasing/increasing of outcrossing rate with distance.