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# Flowering and Seed Development Characteristic of Citrus Derived from Somatic Hybridization of Mandarin Satsuma (*Citrus unshiu* Marc.) and Siam Madu (*Citrus nobilis* Lour.)

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**ABSTRACT**

The somatic hybridization between Mandarin Satsuma (*Citrus unshiu* Marc.) and Siam Madu (*Citrus nobilis* Lour.) is expected to produce progenies having sweet seedless fruit. The research was aimed to study flowering biology, fruit and seed development to identify parthenocarpic lines derived from somatic hybridization. The research was carried out at Pacet Experimental Station of ICABIOGRAD, Cianjur, West Java (1150 m asl), during August 2019-July 2020. The research materials were 15 citrus lines derived from somatic hybridization between Mandarin Satsuma and Siam Madu, of which 5 plants (± 2 years old) per line were prepared. Observation was carried out on flowering phenology, flower morphology, fruit development, pollen viability, and stigma receptivity. Seedless fruits from un-pollinated, self-pollinated, and cross-pollinated flowers of each line were investigated. The results showed that flower morphology of the 15 citrus lines varied in the number of petals, flower diameter, pistil length relative to the stamen, and number of stamen. The development from bud emergence to fruit ripening also varied among and within lines, ranging from 212 to 316 days. Among the lines, FS 25 showed a stronger character towards stimulative parthenocarpy. FS 84 and FS 89 were potential sources of pollen (pollenizer) to produce seedless fruits.

## INTRODUCTION

Citrus cv Siam Madu (*Citrus nobilis* Lour.) is the most widely consumed citrus fruit in Indonesia due to its sweetness, but it contains many seeds (15-21 seeds/fruit) and has less attractive colour, thus making it less competitive to imported oranges. On the other hand Mandarin Satsuma (*Citrus unshiu* Marc.) is a seedless cultivar with bright color (Agustí et al., 2014). An attempt to generate seedless cultivars with sweet taste was performed through somatic hybridization between *Citrus nobilis* Lour. and *Citrus unshiu* Marc. (Husni et al., 2010) to overcome interspecific constraints.

Citrus seedlessness is mostly due to parthenocarpy, in which the fruit is developed in the absence of fertilization (Ali et al., 2013; Dexter-Boone et al., 2019). Obligatory parthenocarpy takes place when seedless fruits are always produced regardless of the occurrence of pollination and/or fertilization. Mandarin Satsuma (*Citrus unshiu* Marc.) is an obligatory parthenocarpic cultivar that shows a high degree of seedlessness with a high fruit set (Agustí et al., 2014; Dexter-Boone et al., 2019). Facultative or stimulative parthenocarpy occurs when seedless fruits are produced only when pollination takes place (Mesejo et al., 2013;

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Rezaldi et al., 2019). The advantage of having a parthenocarpic cultivar is due to a more stable fruit production even when pollination is lacking (Liu et al., 2018).

Somatic hybridization of Mandarin Satsuma (*Citrus unshiu* Marc.) and Siam Madu (*Citrus nobilis* Lour.) (both 2n=18) was aimed to obtain seedless clones with sweet taste (Husni et al., 2010). The somatic hybridization resulted in 84 lines. Most of them have 2n=36 chromosome number, except few clones were found to have only 2n = 35 chromosomes. Martasari et al. (2014) reported that among the 84 lines, 61 lines were identical to Siam Madu, meaning that fusion between the two species did not take place; 7 lines were cybrid, the protoplast resemble combination of the two parents; and 16 lines were the hybrid resulted from fusion of the the two parents. The 16 hybrid lines were studied by Annisa et al. (2018) and reported that some of the hybrids were either diploid or tetraploid, nevertheless, leaf morphology resembles a combination of the two parents. Observation on seedlessness and other fruit qualities need to be carried out, and 15 hybrid lines were selected for further investigation. Therefore, the purpose of this research was to investigate flowering biology, fruit and seed development characteristics of the selected lines.

## MATERIALS AND METHODS

The research was carried out at Pacet Experimental Station (1150 m above sea level), Cianjur, West Java (6° 45.111' S and 107° 2.786' E), during August 2019 – July 2020. The materials were ± 2 years old plant of 15 hybrid lines (code FS: fussion) with 3 plants each line derived from somatic hybridization between Mandarin Satsuma and Siam Madu. The accessions were the collection of ICABIOGRAD (Annisa et al., 2018; Martasari et al., 2014). The lines were propagated by grafting into JC rootstocks in June 2017. Flowering biology was descriptively observed comprising flower morphology, flower and fruit development, pollen viability, and stigma receptivity.

### Flower Morphology

Observations were made on 3 inflorescences per plant for all plant samples, covering flower diameter at full bloom; number of petals and stamen per flower; stamen and pistil length. Petal color was determined using RHS Color Chart and the quantitative data were analyzed using F and Tukey Test ($\alpha$ = 5%).

### Flowering Phenology

### Flower and Fruit Development

Flower development was observed on 3 inflorescences per plant. Observations were based on Biologische Bundesanstalt Bundessortenamt and Chemical Industry (BBCH) scale (BBCH, 2001; Reykande et al., 2013). Observation on flower development until fruit ripening was classified into 4 stages (BBCH scale), i.e. stage 5: from flower buds' appearance until bulging buds when petals forming a hollow ball; stage 6: from flower open to petals are aborted; stage 7: from the ovary enlargement to fruits are of final size; and stage 8: from fruits color break until ripe for consumption.

### Pollen Viability

Result from preliminary experiment shows that modified pollen germination medium/PGM (Fariroh et al., 2011) is a more suitable medium for citrus pollen germination testing than the original PGM (Schreiber & Dresselhaus, 2003) or Brewbaker and Kwack/BK (Khan & Perveen, 2014) (data is not shown). Therefore, the modified PGM (PGMm) was used for pollen germination testing of the 15 lines. The pollen was collected at full bloom before anther dehiscence, and germinated in concave object glasses with 6 replications, incubated at 16ºC for 24 and 48 hours (Distefano et al., 2012), then was observed under a light microscope. Germinated pollen were those with pollen tubes longer than the diameter.

### Stigma Receptivity

Stigma receptivity was observed on 3 inflorescences per plant, 3 plants per line. The stigma is receptive when exudate appears on the surface (observation was made using a loop) accompanied by changes in color (RHS color chart). Observation was carried out at three different stages i.e. bulging flower bud, flower starts to open and flowers at full bloom.

### Fruit and Seed Development

To study the seedlessness among the lines, 4-35 flowers per plant were emasculated and bagged (without pollination) and some other (5-29 flowers) were self-pollinated and bagged until 4 days after pollination. The unequal number of samples used in this treatment was due to the limited number of flowers in each plant. Twelve lines were used in this treatment, the other 3 lines (FS 106, FS A5 and FS 70) were excluded due to the insufficient number of flowers. Cross pollination was performed using FS 25 and FS 89 as female parents due to tendency to seedlessness (preliminary observation). Fruit and seed sets as well as fruit development were observed from those without pollination, self-pollination, and cross-pollination. Seedlessness was observed when the fruits reached half of maximum size. The number of empty/abortive seed and full seed were counted. Fruits with ≤ 6 full seeds are categorized as parthenocarpic fruit (Mariana et al., 2018).

## RESULTS AND DISCUSSION

### Flower Morphology

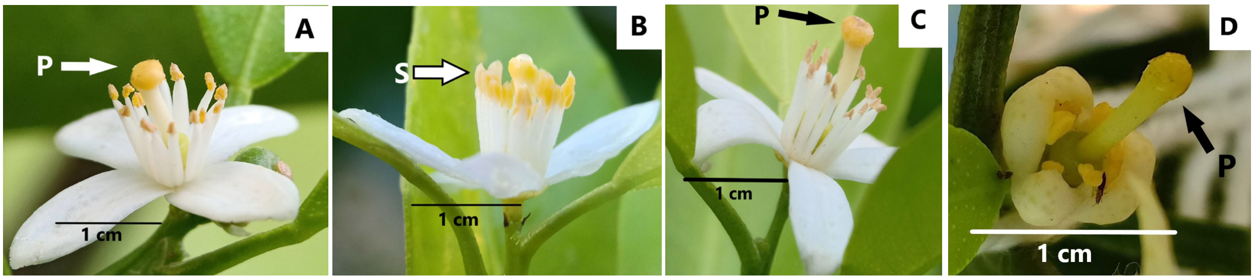
The 15 citrus lines have normal complete flowers, but the flower dimension varies among the lines. The length of petals varied among lines and ranged between 10-14 mm at full bloom. Similar phenomena were also observed on diameter of flower (ranged 18-30 mm), length of stamen (ranged 7-9 mm) and length of pistil (ranged 7-12 mm). Number of petals (ranged 4-5) and stamen (ranged 14-18) per flower were not significantly different among lines (Table 1). In general, most lines have longer pistil than the stamen, except FS A6 that has shorter pistil than the stamen, whereas FS A12 and FS A13 have pistil and stamen at the same length. The variations of flower characters did not seem to be consistent within a line. The similarity to that of parent stocks, Mandarin Satsuma (Dorji & Yapwattanaphun, 2011) and Siam Madu (variety description) was that the pistil is longer than the stamen so that it protrudes among the stamens and makes it more exposed for cross pollination.

The pistil length of line FS 106 is only significantly different from line FS A6, but not from other lines. IPGRI (1999) classified the citrus flower based on the distance of the stamens to the pistil into 3 groups i.e. pistil length is shorter, equal to, or longer than the stamen. Although in general the pistil is longer than the stamen, however, all the three types of flower morphology were found in all lines (Fig. 1A, 1B, 1C). Rarely, abnormal flowers (Fig. 1D) was also found in all lines (<5%), in which the style and stigma protrudes much longer than the stamen at flower bud stage.

**Table 1.** Variation of flower morphology of citrus lines derived from somatic hybridization

| No | Lines | PL (mm) | FD (mm) | SL (mm) | PiL (mm) | ∑P | ∑S |
|---|---|---|---|---|---|---|---|
| 1 | FS 106 | 13.4 ab | 23.5 cd | 8.5 ab | 11.5 a | 4.7 a | 16.1 a |
| 2 | FS A5 | 10.1 d | 19.7 ef | 7.3 b | 8.7 ab | 4.3 a | 14.6 a |
| 3 | FS A6 | 11.9 bc | 23.1 cd | 7.2 b | 6.8 b | 4.7 a | 16.3 a |
| 4 | FS A12 | 12.6 abc | 24.5 bcd | 8.1 ab | 8.8 ab | 4.7 a | 14.1 a |
| 5 | FS A13 | 13.4 ab | 22.8 cde | 8.3 ab | 8.9 ab | 4.7 a | 17.6 a |
| 6 | FS 25 | 11.0 cd | 18.1 f | 7.7 ab | 9.3 ab | 4.3 a | 14.8 a |
| 7 | FS 26 | 12.0 bc | 29.5 a | 7.8 ab | 9.2 ab | 4.7 a | 16.3 a |
| 8 | FS 27 | 13.7 ab | 24.7 bcd | 8.3 ab | 10.1 ab | 5 a | 17.2 a |
| 9 | FS 29 | 12.9 ab | 27.8 ab | 9.0 a | 9.9 ab | 4.7 a | 16.5 a |
| 10 | FS 70 | 12.7 abc | 25.1 bc | 7.3 b | 9.9 ab | 4.7a | 16.1 a |
| 11 | FS 71 | 13.5 ab | 25.1 bcd | 7.9 ab | 9.9 ab | 4.7 a | 15.5 a |
| 12 | FS 84 | 12.6 abc | 24.6 bcd | 7.6 ab | 10.5 a | 5 a | 16.1 a |
| 13 | FS 89 | 13.4 ab | 25.5 bc | 8.4 ab | 11.8 a | 5 a | 14.8 a |
| 14 | FS 91 | 12.4 abc | 21.9 de | 9.2 a | 10.2 a | 4.7 a | 14.7 a |
| 15 | FS G118 | 13.9 a | 27.4 ab | 8.1 ab | 9.4 ab | 4.3 a | 15.7 a |
| F value | | ** | ** | * | * | ns | ns |
| Stdev (%) | | 0.6 | 1.1 | 0.5 | 1.1 | 0.6 | 1.3 |

Remarks: PL = petal length; FD = flowers diameter; SL = stamens length; PiL = pistil length; ∑P = number of petal per flower; ∑S = number of stamen per flower; numbers followed by the same letters within the same column are not significantly different based on Tukey test α = 5%




Remarks: s : stamen; p: pistil

**Fig. 1.** Distance of the stamen to the pistil: A. pistil length is shorter than stamen, B. pistil length is equal to stamen, C. pistil length is longer than stamen, D. abnormal flower

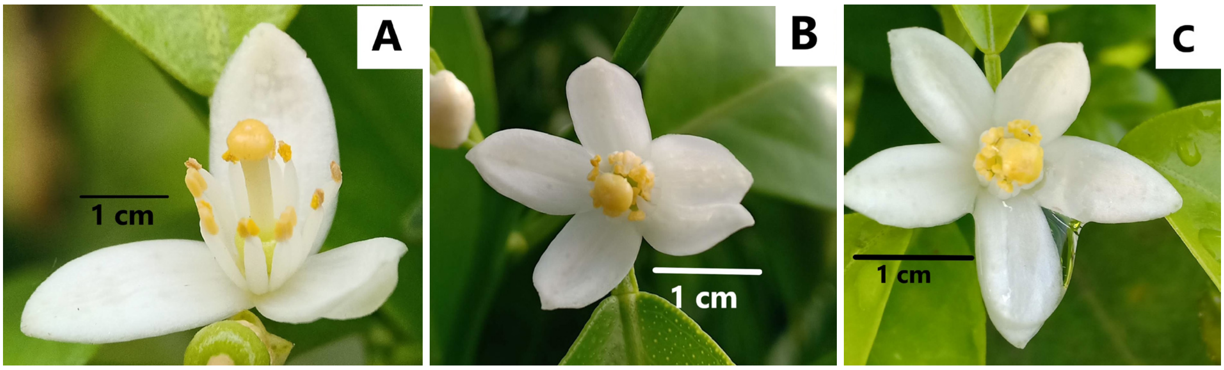



**Fig. 2.** Citrus flower with different numbers of petals: A. 3 strands, B. 4 strands, C. 5 strands

The number of petals was not significantly different among the lines (Table 1), mostly were the same as their parents Siam Madu (5 strands) (Sinaga et al., 1999) and Mandarin Satsuma (4-5 strands) (Dorji & Yapwattanaphun, 2011). In some lines, i.e. FS A12, FS 25, and FS G118, however, the number of petals was 3 (Fig. 2). The number of stamens was not significantly different among the lines. Based on IPGRI (1999) the numbers of stamen classified into 3 groups i.e. < 4 stamens per petal (FS 106, FS A5, FS 25, FS 26, FS 29, FS 70, FS 89, FS 91), 4 stamens per petal (FS A6, FS A13, FS 27, FS 71, FS 84, FS G118) and >4 stamens per petal (FS A12).

Based on the RHS colour chart the petal colour falls into the white colour category (IPGRI, 1999) and is similar with the parental character (Dorji & Yapwattanaphun, 2011; Sinaga et al., 1999). The stigmatic surface has different shade of yellow, from vivid yellow A/YG 12 (106, FS 26), vivid yellow B/YG 9 (FS 27; FS 71), brilliant yellow A/YG A (FS G118, FS 70), brilliant yellow B/YG 13 (FS A6, FS A13, FS 25, FS 89 and FS 91), to brilliant yellow C/YG 13 (FS A5, FS A12, FS 29 and FS 84). Anther colour falls into the yellow-green group (IPGRI, 1999), although they come in different shades such as brilliant yellow C (YG 9), vivid yellow B (YG 9); and light yellow B (YG 10). In general, petal, stigma and anther colours of these lines were similar to their parents, Mandarin Satsuma and Siam Madu (Dorji & Yapwattanaphun, 2011) (Dorji & Yapwattanaphun, 2011; Sinaga et al., 1999).

**Flowering Phenology**

**Flower and Fruit Development**

Most of the lines started to flower in July (mid dry season), followed by fruit development up to October (beginning of rainy season). April (end of rainy season) through July is dominated by fruit development and ripening. Meanwhile flower initiation also occurred simultaneously almost all year round, with the peak flowering in July-September and very infrequent during April – July (Table 2). The hybrid lines seemed to inherit the character of Siam Madu which flowers all year round. However, massive flowering occurred during drier months of July till October when the rainfall was medium to low. The development of flower buds from emergence until bulging and ready to bloom (Fig. 3A and 3B) varied among the lines as well as within lines. The shortest (6 days) occured in some flowers of FS 89 and the longest (32 days) in some flowers of FS A13 (Table 3). The development from the bulging flower bud until flower blooming followed by stamen and petal abortion (Fig. 3C) (stage 6) took 2-3 days at the shortest and 3-7 days at the longest (Table 3). The shorter duration means that the flowers of the same lines bloomed more simultaneously than the longer ones. Fitchett et al. (2014) reported that peak flowering dates of five citrus types (orange, tangerine, sweet lemon, sour lemon and sour orange) in Iran are similar over 51-year period, but significantly different between three geographically and climatically distinct cities suitable for citrus cultivation. Over the years citrus flowering date changes. Flowering date advances and delays varies among cities and is affected by the environmental condition. Therefore, these hybrid lines may have different flowering phenology when planted in geographically and climatically different areas.

Ovule enlargement following fertilization (Fig. 3D) until fruit reaches its normal size (Fig. 3E) (stage 7) is the longest developmental stage, and varied among lines, the fastest was 169 days (FS G118) and the slowest was 213 days (FS 25). Variation in duration of the development within the line was lowest in FS 106 (196-208: 12 days) and highest in FS G118 (169-257: 88 days). Fruit loss was common in this stage, which was considered to be due to extreme environmental changes, as sometimes accompanied by fruit cracking (Abobatta, 2019; Ashari et al., 2015; Fitchett, 2013).

Ripening starts when fruit colour changes from green to yellowish green until completely yellow (Fig. 3F) and the fruits were ready to harvest. The period of ripening varied among lines, ranging 11 - 91 days, with the shortest being FS 71 and the longest being FS A13 (Table 3). Dorji & Yapwattanaphun (2011) reported that high rainfall and low light intensity delay citrus fruit ripening and colour change. In addition, the low air temperature will prolong fruit ripening (Abobatta, 2019).

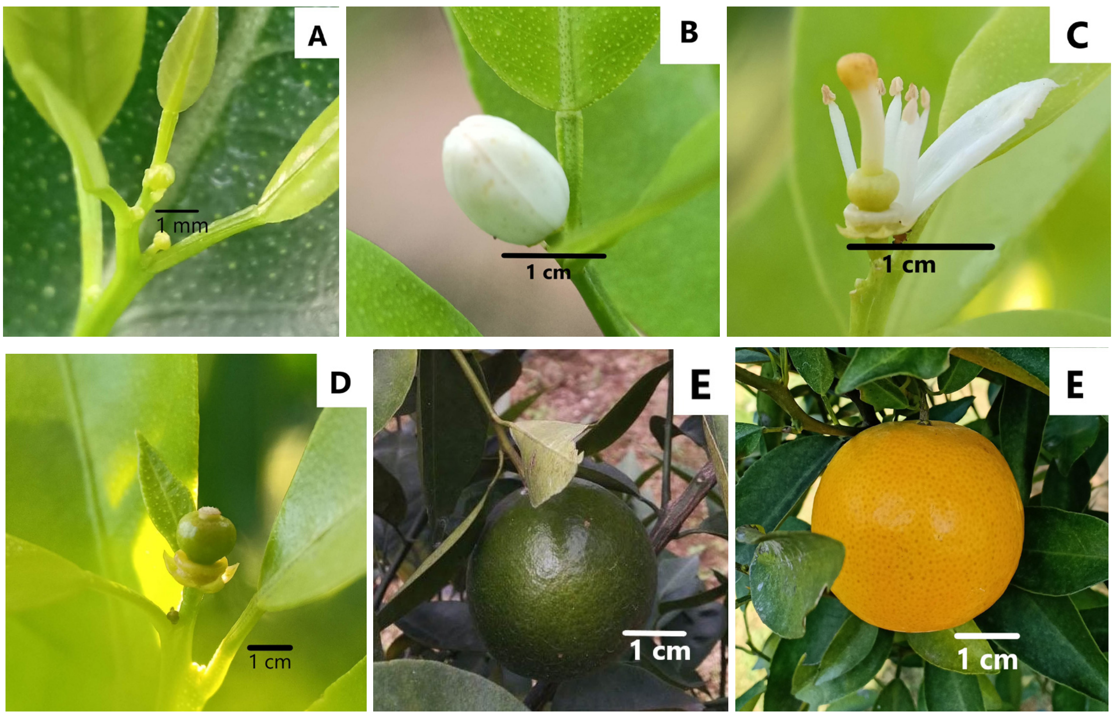
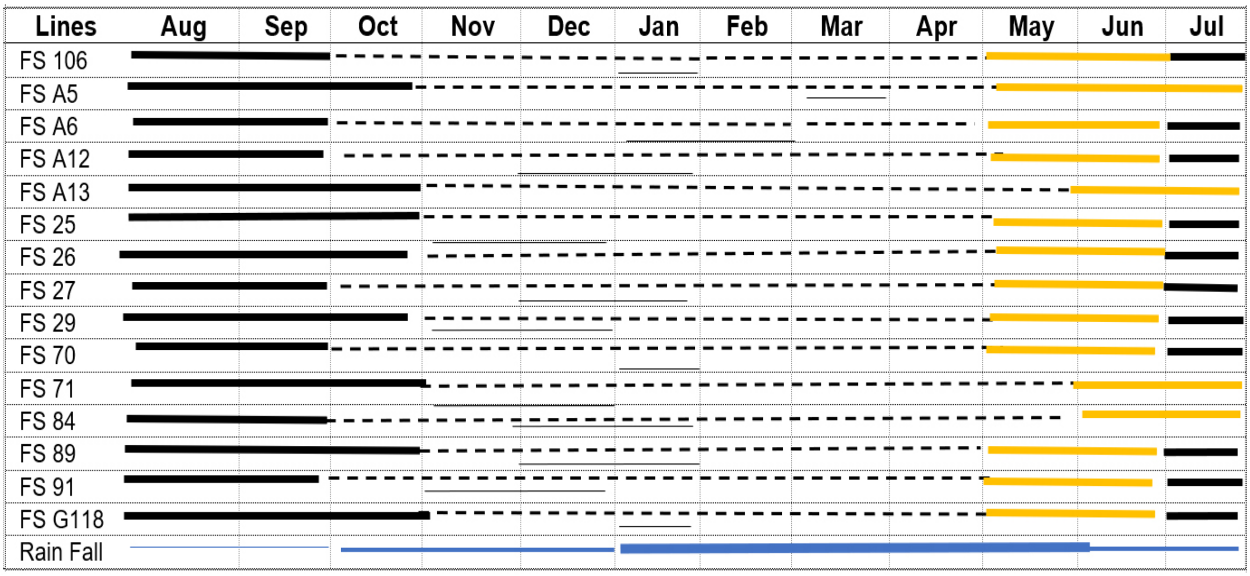



**Fig. 3.** Flower and fruit development: A. Flower bud emergence, B. Flower bud at maximum size, C. The end of flower bloom, D. The beginning of fruit development, E. Fruit at maximum size, F. Fruit ripen and ready for harvest

**Table 2.** Flowering phenology of citrus derived from somatic hybridization and rainfall from Aug 2019 until July 2020




Remarks: ( ) = Peak flowering season; ( ) = flowering season; (- - -) = fruit development; ( ) = Harvest season; ( ) = low rainfall; ( ) = medium rainfall; ( ) = high rainfall; Source rainfall: dataonline.bmkg.go.id

**Table 3.** Flower and fruit development of citrus lines derived from the somatic hybridization

| No | Lines | Duration (days) | | | | Total period of flower and fruit development (days) |
|---|---|---|---|---|---|---|
| | | Stage 5 | Stage 6 | Stage 7 | Stage 8 | |
| 1 | FS 106 | 12-20 | 2-6 | 196-208 | 32-47 | 250-271 (21)[a] |
| 2 | FS A5 | 7-20 | 3-5 | 197-241 | 47-64 | 260-310 (50) |
| 3 | FS A6 | 8-16 | 3-5 | 183-216 | 16-42 | 239-274 (35) |
| 4 | FS A12 | 12-16 | 2-3 | 205-243 | 12-46 | 254-290 (36) |
| 5 | FS A13 | 20-32 | 3-5 | 190-230 | 70-91 | 292-313 (21) |
| 6 | FS 25 | 12-16 | 2-4 | 213-244 | 28-63 | 268-296 (28) |
| 7 | FS 26 | 13-24 | 3-6 | 173-218 | 15-39 | 220-278 (58) |
| 8 | FS 27 | 12-23 | 2-4 | 181-230 | 28-50 | 245-274 (29) |
| 9 | FS 29 | 12-23 | 3-6 | 183-229 | 18-38 | 235-268 (33) |
| 10 | FS 70 | 13-26 | 2-6 | 191-245 | 22-27 | 237-286 (49) |
| 11 | FS 71 | 12-26 | 2-6 | 194-243 | 11-58 | 231-316 (85) |
| 12 | FS 84 | 8-23 | 2-5 | 181-242 | 13-57 | 234-277 (43) |
| 13 | FS 89 | 6-22 | 3-5 | 203-255 | 24-78 | 246-301 (55) |
| 14 | FS 91 | 12-20 | 3-7 | 196-230 | 17-39 | 265-293 (28) |
| 15 | FS G118 | 7-24 | 2-5 | 169-257 | 18-46 | 212-255 (43) |

Remarks: [a]Number in the brackets: the difference between the shortest and longest fruit development within a line. The higher the number, the longer the harvest periode

The total duration of flower and fruit development from flower bud emergence until the fruit ready for harvest varied among the 15 lines, ranging from 212-316 days. The fastest development was detected at FS G118 (212 days) and the longest was at FS A13 (292 days) (Table 3). However, the fruits of FS A13 matured within 21 days (the first and the last matured fruit), while FS G118 revealed 43 days, and the longest one was FS 71 in 85 days. This data indicated that FS A13 would have a shorter harvest period than FS 71. This information is useful for planning the harvest.

**Pollen Viability**

The experiment during preliminary study on pollen germination media provided information that modified PGM (PGMm) was consistently yielding in a higher pollen germination than other media (PGM and BK), and therefore was considered as a more suitable medium (data not shown). It was also identified that pollen taken at full bloom had the highest germination percentage compared to at the bulging stage or before blooming. In general pollen germination of FS 25 was the lowest and FS A6 and FS 26 were highest among the lines (Table 4). The differences of pollen germination among the lines is thought to be genetically controlled. Thus, the low pollen germination percentage is possibly inherited from Mandarin Satsuma that has a sterile cytoplasmic gene (Fujii et al., 2016; Goto et al., 2016).

**Stigma Receptivity**

The shade of yellow stigma changed over time starting from the flower open and was brightest at full bloom. Exudate was also secreted when the flower started to open and continued to increase until the flower was fully bloomed. The presence of exudate was clearly visible as a shiny layer at the center of the stigma surface. The amount of the exudate was not measured but was supposedly reached maximum at full bloom at which time the stigma was receptive and ready for pollination. The receptive period of stigma that coincided with anther dehiscence during 07.00-09.00 h occurred across the lines. Therefore, self pollination could lead to pollen germination. On the other hand, cross pollination that led to fertilization was also possible.

**Fruit and Seed Development**

Observation on fruit development was carried out on 12 lines, due to insufficient number of available flowers of FS 106, FS A5 and FS 70. The result showed that fruit set in the absence of pollination was low (< 25%). Without pollination FS 25, FS 29, FS 89 and FS G118 failed to set fruit, but some other lines set fruits with full seeds (FS A6, FS A13, FS 26, FS 71 and FS 91) (Table 5). It is possible that those full seeds were somatic (adventitious) embryos that were formed in the absence of fertilization (due to the absence of pollination), a rare phenomenon, but also was found in Valencia oranges (Koltunow, 2012). Fei et al. (2019) reported that the formation of adventitious embryos was not related to the development of endosperm as a result of fertilization. Therefore the unpollinated flower of Valencia orange could develop into seeded fruit.

Most lines set fruits when self-pollinated, except FS 29 that did not set fruit either from un-pollinated or self-pollinated flowers (Table 5) which indicated that FS 29 is a self-incompatible line. The percentage of fruit set and seedless fruits from self-pollinated flowers was higher than that of un-pollinated flowers indicating a tendency towards stimulative parthenocarpy, as also shown by the high number of abortive/empty seeds (stenospermocarpy). Most parthenocarps are resulted from the hybridization of two distant genotypes (Picarella & Mazzucato, 2019), so does in this case (somatic hybridization of *Citrus unshiu* Marc. + *Citrus nobilis* Lour.).

**Table 4.** Pollen germination of citrus lines derived from somatic hybridization on modified PGM

| Incubation | Pollen germination (%) | | | | | | | | | | | | F value | Stdev (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FS 106 | FS A6 | FS A12 | FS A13 | FS 25 | FS 26 | FS 27 | FS 70 | FS 71 | FS 84 | FS 89 | FS G118 | | |
| 24 h | 3.88 bc | 24.24a | 8.97abc | 5.62bc | **0.32c** | 16.75ab | 5.55bc | 10.32abc | 1.35bc | 4.33bc | 7.52bc | 8.46abc | ** | 8.1 |
| 48 h | 9.61ab | 30.48a | 10.73ab | 14.11ab | **1.03b** | 29.88a | 8.98ab | 10.71ab | 4.22b | 8.82ab | 11.37ab | 17.21ab | * | 11.9 |

Remarks: Numbers followed by the same letters within the same row are not significantly different based on Tukey test α = 5%

**Table 5.** Fruit set and seed set from un-pollinated and self-pollinated flowers

| No | Lines | Un-pollinated | | | | Self-pollinated | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | No poll flower | No set fruit (%) | No seedless fruit (%) | Numbers of seed | No poll flower | No fruit set (%) | No seedless fruit (%) | Numbers of seed |
| 1 | FS A 6 | 18 | 1(5.6) [a] | 0(0) [b] | 9 (2)[c] | 17 | 4 (23.5)[a] | 0 (0)[b] | 6-19 (0-7)[c] |
| 2 | FS A 12 | 4 | 1 (25) | 1(100) | 0 (0) | 5 | 5(100) | 3(60) | 0-13 (0-5) |
| 3 | FS A 13 | 17 | 3(17.7) | 0 (0) | 7-20 (1-7) | 16 | 7(43.8) | 3(42.9) | 0-23 (0-6) |
| 4 | FS 25 | 4 | 0 (0) | - | - | 16 | 9(56.3) | 8(88.8) | 0-16 (0-3) |
| 5 | FS 26 | 35 | 6(17.1) | 3(50) | 0-20 (0-14) | 27 | 17(62.9) | 6(35.3) | 0-22 (0-13) |
| 6 | FS 27 | 12 | 1(16.7) | 1(100) | 0 (6) | 10 | 8(80) | 6(75) | 0-30 (0-12) |
| 7 | FS 29 | 4 | 0 (0) | - | - | 15 | 0 (0) | - | - |
| 8 | FS 71 | 18 | 1(5.6) | 0 (0) | 12 (0) | 17 | 1(5.9) | 0 (0) | 11 (4) |
| 9 | FS 84 | 23 | 1 (4.4) | 1(100) | 0 (4) | 18 | 14(77.8) | 7(50) | 0-23 (0–10) |
| 10 | FS 89 | 26 | 0 (0) | - | - | 29 | 10(34.5) | 6(60) | 0-20 (0–9) |
| 11 | FS 91 | 16 | 1(6.3) | 1(100) | 4 (0) | 14 | 7(50) | 4(57.1) | 0-17 (0-11) |
| 12 | FS G 118 | 13 | 0(0) | - | - | 18 | 6(33.3) | 3(50) | 0-22 (0-4) |

Remarks: (-): no fruit was set, thus, no seed set; Number in brackets: [a] percentage of number of developing fruits from number of pollinated flowers; [b] number of seedless fruit from number of set fruits; [c] ranged number of abortive seeds per fruit (0 means completely seedless), e.g. 6-19 (0-7): number of full seed in 4 set fruit ranged 6-19, and number of abortive seed per fruit ranged 0-7

The high percentage of seedless fruits from self-pollinatiom of FS 25 (reached 88.8%) strengthened the notion of stimulative parthenocarpy (stenospermocarpy), where pollination and fertilization are still needed for fruit development, but the embryo fails to develop into seeds, and become abortive (Picarella & Mazzucato, 2019; Yam & Hagins, 2011). This phenomenon resembles a late acting self-incompatibility, and supported by the fact that FS 25 had a very low pollen viability (Table 4). Meanwhile FS 26 produced relatively low seedless fruit following self-pollination (Table 5) indicating the higher self-compatibility levels (Liu et al., 2018)however, they can also be pollinated by other varieties of pollen. Here we found two varieties that are different from other peaches: 'Daifei' and 'Liuyefeitao'. 'Daifei' produces less pollen, which needs artificial pollination, honeybee pollination, and the fruit setting depends on other varieties of peach pollen. 'Liuyefeitao' exhibits strictly self-pollination, hence pollen from other species is rejected. To explore the mechanism of this phenomenon, we performed a high-throughput sequencing of the stigma (including style. On the contrary, FS 29 failed to set fruit and seed from un-pollinated as well as self-pollinated flowers. These confirmed that the line was neither parthenocarp nor self-compatible. Among the lines derived from somatic hybridization (*Citrus unshiu* Marc. + *Citrus nobilis* Lour.), FS 25, FS 27, FS 84 and FS 89 were considered as potential stimulative parthenocarps. However only FS 25 and FS 89 were observed further for seedlessness due to lack of flowers on FS 27 and FS 84.

Fruit dimension of un-pollinated flowers was not significantly different among lines, but they were very small regardless of seedlessness (Table 6). Derived from self-pollinated flowers, the fruit dimension of seedless and seeded fruits were not significantly different, except FS 25. The seedless fruits of all these somatic hybridization lines were categorized as small (class C and D) in SNI 3165:2009 (BSN, 2009). The small size of seedless fruit is ascribed to be due to the absence of fruit development hormones (Dexter-Boone et al., 2019; Yam & Hagins, 2011) within the seeds. In this case, however, the seeded fruit from self-pollination were also categorized as small. Meanwhile, the total sugar ranged from 9 - 12 °Brix, indicating that it has the sweet taste of the parent, Siam Madu (Sinaga et al., 1999).

Cross pollination of FS 25 x FS 84 and FS 25 x FS 89 resulted in high fruit set and seedless fruits (Table 7). On the contrary cross-pollination of FS 25 x FS G118 resulted in high fruit set but low percentage of seedlessness (40%). These finding gives a hint that FS 84 and FS 89 can be used as source of pollen (pollenizer). Planting one of these two lines as a source of pollen among FS 25 might increase production of seedless fruits.

**Table 6.** Fruit dimension produced from un-pollinated and self-pollinated flowers

| No | Lines | Un-pollinated | | | Self-pollinated | | |
|---|---|---|---|---|---|---|---|
| | | Fruit Diameter (mm) | Fruit Height (mm) | Fruit Weight (g) | Fruit Diameter (mm) | Fruit Height (mm) | Fruit Weight (g) |
| 1 | A 6 | 45.1a | 38.3a | 44.2a | 48.1abcd | 43.2ab | 59.2abc |
| 2 | A 12 | - | - | - | 45.1abcde | 39.1abcd | 42.3abcd |
| | A $12^s$ | 19.9a | 17.4a | 3.7a | 34.4abcde | 30.5bcde | 22bcd |
| 3 | A 13 | 44.4a | 38.8a | 48a | 48.6abcd | 44.2ab | 62.2ab |
| | $A13^s$ | - | - | - | 39.4abcde | 34.3abcd | 29.7abcd |
| 4 | FS 25 | - | - | - | 58.6a | 50.7a | 102.5a |
| | FS $25^s$ | - | - | - | 35.8de | 31.2abcde | 28.1bcd |
| 5 | FS 26 | 54.0a | 46.6±a | 73.9a | 49.4a | 43.1ab | 60.5ab |
| | FS $26^s$ | 44.1a | 36.2a | 39.1a | 38.5abcde | 33.8abcd | 32.2bcd |
| 6 | FS 27 | - | - | - | 45.1abcde | 38.7abcd | 43.4abcd |
| | FS $27^s$ | 39.7a | 39.6a | 33.8a | 33.1bcde | 31.9abcde | 19.7cd |
| 7 | FS 29 | - | - | - | - | - | - |
| 8 | FS 71 | 36.2a | 32.8a | 24.7a | 35.4abcde | 32.8abcd | 23.3bcd |
| 9 | FS 84 | - | - | - | 49.8a | 43.9±ab | 62.3ab |
| | FS $84^s$ | 36.9a | 31.6±a | 24.2a | 39.5abcde | 34.5abcd | 30.5abc |
| 10 | FS 89 | - | - | - | 48.3abcd | 42.8ab | 56.9abcd |
| | FS $89^s$ | - | - | - | 35.6acde | 31.1abcde | 22.7bcd |
| 11 | FS 91 | - | - | - | 43.4abcde | 40.1abc | 42.2abcd |
| | FS $91^s$ | 31.4a | 27.4a | 13.7a | 28.7e | 26.5de | 12.3d |
| 12 | G 118 | - | - | - | 45.3abcde | 41.5abc | 45.7abcd |
| | G $118^s$ | - | - | - | 32.5abcde | 30.9bcde | 18.5cd |
| F value | | ns | ns | ns | ** | ** | ** |
| Stdev (%) | | 6.9 | 5.7 | 21.7 | 6.9 | 5.9 | 18.2 |

Remarks: $^s$ = seedless fruit; numbers followed by the same letters in the same column indicate not significantly different based on Tukey test α=5%

**Table 7.** Percentage of fruit set and seed set from cross-pollination and fruit quality

| Crosses line | No poll flower | No Set Fruit (%) | No Seedless Fruit (%) | No. seed | Fruit Diameter (mm) | Fruit Height (mm) | Fruit Weight (g) |
|---|---|---|---|---|---|---|---|
| FS 25 x FS 25 | 16 | 9 (56.3)[a] | 9 (88.8)[b] | 0-16 (0-3)[c] | 58.6 a | 50.7a | 102.5a |
| | | | | | [s]35.8 a | 31.2 a | 28.1c |
| FS 25 x FS 84 | 3 | 3 (100) | 3 (100) | 0 (2-3) | [s]45.7 a | 39.1 a | 44.6abc |
| FS 25 x FS 89 | 3 | 3 (100) | 3 (100) | 0-5 (0-3) | [s]43.8 a | 36.2 a | 38.4abc |
| FS 25 x FS G118 | 6 | 5 (83.3) | 5 (40) | 2-12 (0-8) | 55.6 a | 44.6 a | 76.2a |
| | | | | | [s]48.9 a | 39.7 a | 54.2abc |
| FS 89 x FS 89 | 29 | 10 (34.5) | 10 (60 ) | 0-20 (0-9) | 48.3 a | 42.8 a | 57.0 abc |
| | | | | | [s]35.6 a | 31.1 a | 22.7bc |
| FS 89 x FS 25 | 6 | 3 (50) | 3 (66.6) | 0-9 (1-4) | 56.8 a | 47.8 a | 84.5abc |
| | | | | | [s]44.2 a | 37.6 a | 46.3abc |
| F value | | | | | ns | ns | ** |
| stdev | | | | | 8.5 | 6.5 | 18.8 |

Remarks: number in brackets: [a] number of fruit/number of sampled flowers; [b] number of seedless fruit/number of set fruits; [c] ranged number of abortive seeds per fruit (0 means completely seedless), e.g. 0-16 (0-3): number of full seed in 9 set fruits ranged 0-16, and number of abortive seed per fruit ranged 0-3; [s] = seedless fruit; numbers followed by the same letters within the same column are not significantly different based on the Tukey test α=5%

Most citrus orchards need pollenizer to increase fruit set and fruit size. Seedlessness was one of the main purposes of somatic hybridization. The choice of suitable source of pollen (pollenizer) is important to increase fruit set and fruit size without increasing the seediness of the fruits. This finding showed that crossing of FS 25 with FS 84 or FS 89 and crossing of FS 89 with FS 25 increased the size of seedless fruits. Fruit diameter and fruit height between lines are not significantly different, but fruit weight on cross line FS 25 x FS 25 (seedless) significantly different with cross line FS 25 x FS G118 (Table 7). However, the fruit size still needs to be increased to meet the market demand.

## CONCLUSION

Variation in flower morphology of 15 citrus lines derived from somatic hybridization occurred within a line (number of petals) as well as among lines (petal length, flower diameter, pistil length and stamen length). The lines flowered all year round, with heavy flowering occurring from July until October when the rainfall was low to medium intensity. The duration of flower and fruit development from bud emergence until fruit ripening also varied among and within lines. The shortest was FS G118 (212 days), and the longest was FS A13 (292 days). FS 25, among the 15 lines, showed a stronger character towards stimulative parthenocarpy. FS 84 and FS 89 were potential sources of pollen (pollenizer) to produce seedless fruits. Cultivation systems to reach the desirable size of seedless fruits are needed.

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