'Juxiangyuan' Seedless Orange: A New Mutant with Male and Female Sterility

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Abstract: Seedless is a highly valued commercial characteristic in the citrus industry, both for fresh consumption and for processed markets. In this study, the ‘Succari Sweet Orange’ (WT) and its seedless mutant ‘Juxiangyuan’ (MT), which originated from a bud mutation, were selected to study the formation of a citrus seedless phenotype. Microscopic analysis of MT’s floral organs, including anther and ovary cross-sections, provides insights into its seedless phenotype compared to the original seeded cultivar. Additionally, pollen features, viability, and in vitro germination were examined to determine the cause of seedlessness. MT exhibited significant developmental deformities in both male and female gametes, with pollen grain analysis indicating a high rate of deformity (41.48%), low viability (9.59%), and minimal in vitro germination (9.56%). Hybridization experiments were conducted to assess male and female sterility and pollen incompatibility. Both WT and MT exhibited parthenocarpic development. Notably, MT fruit produced with an average of 3.51 seeds pollinated to WT, despite severe pollen abortion of MT. MT, however, produced seedless fruit through self-breeding or cross-breeding with other varieties, demonstrating stable female sterility. Despite reduced pollen quantity and viability in the seedless mutant ‘Juxiangyuan’, its seedlessness primarily stems from female sterility. This study contributed to a deeper understanding of seedless formation in ‘Juxiangyuan’ and provided valuable information relevant to its commercial cultivation.

Keywords: citrus; female sterility; male sterility; pollen abortion; seedless

1. Introduction

Citrus stands out as a leading fruit crop globally, with efforts concentrated on improving fruit quality and tapping into expanding processed markets [1]. However, a significant challenge in this pursuit is the substantial seed content prevalent in citrus fruits, hindering consumer acceptance [2]. Conversely, seedless citrus fruits hold sway over both local and international markets, especially in the juice industry [3], making the development of seedless cultivars a crucial goal in citrus breeding.

Bud sports, resulting from genetic variations in somatic cells, lead to qualitative and quantitative phenotypic alterations in plants and are observed in many vegetatively propagated plants, including citrus [4]. In citrus, improved trait selection has been facilitated by bud sports (chimeras) selection, spontaneous mutation, and induced mutation breeding strategies [5]. Seedless cultivars in fruit crops are often obtained through bud sports [6]. For instance, the Seedless ‘Ougan’ mandarin originated from a bud sport mutation discovered in Zhejiang Province in 1996, maintaining all horticultural traits of the wild-type ‘Ougan’ mandarin [7]. Similarly, HUANONG NO 1 is an excellent cultivar derived from a bud sport
variation of Ponkan mandarin found in the National Citrus Breeding Center of Huazhong Agricultural University, Wuhan, China [8]. In Korea, more than 30 citrus varieties have been developed from bud sport mutants [9].

Numerous commercially significant citrus cultivars have emerged worldwide from conventional breeding programs, bud sport selections, or chance seedling variations [10]. Notable among the seedless cultivars are Satsuma mandarin (C. unshiu M.) [11,12], navel orange (C. sinensis O.) [13], and Clementine mandarin (C. reticulata B.) [14]. The induction of seedlessness in citrus mandarin cultivars can be attributed to various factors [15], including male sterility, female sterility, incompatibility, parthenocarpy, and early embryo abortion [16].

Male sterility in plants primarily results from anther abnormalities, leading to pollen abortion, a significant factor in seedless fruit formation [17,18]. Pollen abortion typically arises from developmental anomalies in microspores or tapetum, impacting pollen fertility [19]. For example, the seedless ‘Ougan’ mandarin (C. reticulata L.) arose from male sterility due to abnormal microspore development [20,21].

Morphological female sterility in citrus is primarily observed in lemon, citron (C. medica L.), and lime, where flowers may become sterile due to abnormal development or pistil abortion [22]. On the other hand, embryo sac sterility stands out as a significant factor in female sterility [23]. A seedless Ponkan (Citrus reticulata Blanco cv. Lipeng No. 2) resulted from a mutation causing embryo sac sterility compared to normal seedy Ponkan [24]. Self-incompatibility also plays a role, preventing self-fertilization through multi-allele S-RNase in the pistil [25]. For instance, ‘Shatian’ pummelo (C. grandis L.) exhibits self-incompatibility [26].

The ‘Succari Orange’ (Citrus sinensis O. cv. Succari) (WT), introduced in China in 1988 from Egypt, is renowned for its acidless quality, but it bears approximately 10 seeds in each fruit [27]. In response, its seedless mutation of ‘Juxiangyuan’ (MT) was selected in Hunan province, boasting a significant reduction in seed numbers (0–2 seeds) [28]. MT exhibits sweet, juicy, and seedless flesh, addressing the absence of seedless and acidless orange varieties on the market. This study aimed to delve into the mechanisms of seedlessness in MT, encompassing a comprehensive analysis of fruit quality and flower characteristics, an exploration of the fertility of the male and female gametes, and an examination of seed development in self-pollinated and cross-pollinated instances of MT (MT × WT, MT × ‘Dahong’ sweet orange). The results showed that both male and female sterility exist in ‘Juxiangyuan’, while female sterility is notably the primary cause of its seedlessness. The outcomes of this research will hopefully provide valuable insights into the mechanisms of seedlessness.

2. Materials and Methods

2.1. Plant Materials

Flower buds at various developmental stages, including pre-anthesis, were harvested in April in 2022, aligning with the customary flowering period in Hunan province, China (average annual temperature 17.6 °C, average annual sunshine hours 1625.2 h). The samples were meticulously obtained from both the WT and MT, originating from a grove in Yongxing, Hunan province. Fruit samples designated for quality analyses and seed presence determination were procured during the latter part of November in the same year.

2.2. Fruit Quality Measurements

A total of 15 fruits from both the WT and MT were selectively harvested and subjected to a comprehensive analysis encompassing fruit weight, peel color, titratable acidity (TA), soluble solids (TSSs), and seed count [29].

2.3. Pollen Morphology Observation and Male Fertility Analysis

Before anthesis, ten flower buds were sampled from two cultivars, and all pollen samples were freshly harvested. Pollen micromorphology was examined using a JSM-
6380LV scanning electron microscope (SEM; Jeol, Tokyo, Japan). Pollen viability and germinability were assessed through staining with 1% acetic acid magenta (w/v), which was adjusted as appropriate according to Chai [30]. In total, 200 pollen grains were investigated with an optical microscope (Zeiss Axio Imagine 2.0, Carl Zeiss AG, Jena, Germany) for each experiment. Viable pollen grains were identified as well-stained grains, while unstained or feebly stained and empty grains were classified as unviable.

Freshly collected pollen grains were distributed on slides with a culture medium and kept in germination chambers at 25 °C (±2 °C) for 24 h. Germination was analyzed in 1000 grains from both the MT and WT. Pollen grains with a pollen tube larger than the grain diameter were considered germinated [31]. Analyses were conducted under a bright field light microscope, and images were recorded using photomicrographs or digital image capturing.

Fresh pollen samples were immediately fixed in a mixture of FAA (formalin–acetic acid–alcohol). Subsequently, the samples underwent dehydration using a graded ethanol series (50%, 70%, and 95%). After dehydration, the samples were subjected to critical point drying, mounted on copper stubs, and sputter-coated with gold. The prepared samples were examined under a scanning electron microscope (SEM). The SEM allowed for high-resolution images of the pollen samples to be obtained. Representative images were captured to illustrate the morphological features of the pollen [32].

Pre-anthesis flowers of the WT and MT were fixed in 1% glutaraldehyde and 4% formaldehyde in 0.1 M of phosphate buffer at pH 7.2. After anthesis dissection, the excised anthers were dehydrated in ethanol series, embedded in technovit 7100 (KULZER) resin, and grouped and polymerized in molds (approximately 20 anthesis per group). Three-micrometer-thick sections were excised with a Leitz microtome equipped with a glass knife and mounted onto glass slides. Cross-sections of the anthesis were stained with 0.05% (w/v) Toluidine Blue O. The slides were observed under a bright field light microscope equipped with a digital camera.

2.4. Female Organs Observation

Fresh pre-anthesis flowers of both WT and MT were collected. Petals and filaments were removed, and the ovary was transected in the middle, exposing the embryo sac, where 50 of them were dissected to count the number of ventricles and ovules per ventricle under a depth-of-field extended microscope (Zeiss, Smartzoom5, Carl Zeiss AG, Jena, Germany) using a digital camera.

2.5. Artificial Hybridization

To further assess the male and female fertility of the MT, artificial hybridization experiments were conducted in the field. The male fertility of the MT was evaluated using the WT as the female parent, and WT and ‘Dahong’ sweet orange were employed as male parents, as well. Similarly, the female fertility of the MT was assessed, with WT, MT, and ‘Dahong’ sweet orange acting as the male parents. Each experiment comprised 4 replicates, with 5 trees occurring in each replicate, totaling more than 500 pollinated flowers per tree. The fruit setting rate was meticulously recorded before fruit ripening, and the seed number per fruit was quantified after fruit ripening [33].

2.6. Statistical Analysis

Data on pollen viability, in vitro germination, morphological characteristics, and ovule development were subjected to one-way analysis of variance (ANOVA) using SPSS software (Version 6.4, SAS Institute, Cary, NC, USA). Tukey’s honestly significant difference (HSD) post hoc test was employed to identify statistically significant differences (p < 0.05) between the WT and MT groups for each analyzed parameter.
3. Results
3.1. Fruit Characteristics of MT Compared to WT

Distinct characteristics were observed in the newly cultivated mutant variety compared to the control cultivar. Notably, an upright growth habit was demonstrated by MT, forming a naturally rounded head-shaped canopy with denser and more upright branches characterized by tuft shoots indicative of vigorous growth (Figure 1A,B). MT exhibited larger fruit size in both vertical and horizontal diameters accompanied by notable increases in single fruit weight (Figures 1C,D and 2B,C). Remarkably, the average seed number per fruit in MT was dramatically reduced to only 1.1 seeds, while WT exhibited an average of 17.9 seeds per fruit (Figures 1 and 2). Other fruit qualities, including soluble solids and titratable acids content, showed almost no differences (Figure 2D–F).

![Figure 1. Comparison of fruit size and seed number between WT and MT. The tree of WT (A) and MT (B); the fruit of WT (C) and MT (D). Bar = 2 cm.](image)

![Figure 2. Fruit characteristics of WT and MT. Seed number per mature fruit (A); vertical and horizontal diameters of fruit (B); fruit average weight (C); fruit TSS contents (D); fruit TA contents (E); fruit edible rate (F). ** shape represents \( p < 0.01 \).](image)
3.2. Floral Morphology and Quality of Two Cultivars

The flowers of both WT and MT exhibited intact structures, with similar sizes and morphologies of stamens and petals (Figure 3). Before pollination, ovary and stamen cross-sections of WT and MT were examined. However, observations revealed pollen grain abortion, with a nearly 6.8-fold reduction in the number of full pollen grains in MT compared to WT (Figure 4A,B,E). Within the ovary, notable disparities were noted in the number, insertion, size, or even the complete absence of ovules in MT compared to WT (Figure 4C,D,F). These microscopic morphological characteristics suggested indications of sterility in both the androecium and gynoecium of MT.

Figure 3. Flower characteristics of (A) WT and (B) MT (bar = 1 cm); comparison of the number of petals (C); stamen number (D); petal length and width; pistil length and width (E).

**Figure 2.** Fruit characteristics of WT and MT. Seed number per mature fruit (A); vertical and horizontal diameters of fruit (B); fruit average weight (C); fruit TSS contents (D); fruit TA contents (E); fruit edible rate (F). **shape represents** p < 0.01.
3.3. Pollen Morphology and Viability

SEM revealed that pollen grains from MT exhibited distortion and shrinkage, contrasting with the spherical and full morphology observed in most mature pollen grains from WT. Notably, the aberration rate was significantly higher in MT (41.48%) compared to WT (3.4%) (Figure 5A,B; Table 1). For normal pollen grains, MT displayed smaller sizes, with a pollen equatorial axis length nearly 1.3 times smaller than WT. Despite these size differences, both cultivars exhibited similar smooth ornamentation of pollen exines and a tetra-colporate spherical shape in polar view, the shape of pollen grains rectangular-obtuse in equatorial outlines, with ornamentation microreticulate-perforate (Figure 5C,D; Table 1).

Figure 4. Floral dissection observation. Anther cross-section of WT (A); anther cross-section of MT (B) (bar = 200 µm); ovary cross-section of WT (C); ovary cross-section of MT (D) (bar = 1 mm); comparison of intact pollen grains per pollen sac of anther in WT and MT (E); comparison of ovules numbers in cross-sections of WT and MT (F). **, Significant at $p$-value < 0.01.
Figure 5. Pollen morphology and viability of WT and MT. Pollen grains by SEM (×500) of WT (A) and MT (B); equatorial view of pollen grains in WT (C) and MT (D); pollen viability of WT (E) and MT (F) assayed by staining (bar = 50 µm); in vitro pollen germination of WT (G) and MT (H) on artificial medium (bar = 100 µm).
Table 1. Comparison of morphology and viability of pollen grains between WT and MT. * Significant at \( p \)-value < 0.05; ** Significant at \( p \)-value < 0.01.

<table>
<thead>
<tr>
<th></th>
<th>Pollen Equatorial Axis Length (( \mu )m)</th>
<th>Pollen Polar Axis Length (( \mu )m)</th>
<th>Pollen Aberration Rate (%)</th>
<th>Pollen Viability (%)</th>
<th>Pollen Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>36.2 ± 0.18</td>
<td>19.4 ± 0.38</td>
<td>3.57 ± 0.12</td>
<td>89.8 ± 1.32</td>
<td>87.49 ± 0.58</td>
</tr>
<tr>
<td>MT</td>
<td>28.41 ± 0.66 *</td>
<td>16.17 ± 0.63</td>
<td>41.48 ± 0.46 **</td>
<td>9.59 ± 1.4 **</td>
<td>9.56 ± 0.02 **</td>
</tr>
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Regarding pollen viability, 89.8% of pollen grains from the WT were dynamic, whereas only 9.59% of pollen grains were stainable in the MT. (Figure 6E,F; Table 1). Furthermore, pollen germination revealed that 87.49% of WT grains and 9.56% of MT grains were viable on the artificial medium (Figure 5G,H; Table 1). These results suggested that low fertility in its pollen might contribute to the seedless trait of MT.

Figure 6. Fruit characteristics of emasculation treatment without pollination of WT (A) and MT (B); WT × WT (C); MT × MT (D); WT × MT (E); MT × WT (F); WT × ‘Dahong’ sweet orange (G); MT × ‘Dahong’ sweet orange (H). Bar = 2 cm.
3.4. Effects of Self- and Cross-Pollination on Seed Number in Fruits

An emasculation treatment without pollination was employed to investigate the parthenocarpic ability of WT and MT. Fruit production without pollen was observed in both WT and MT, confirming the parthenocarpic nature of both cultivars (Table 2; Figure 6A,E). Furthermore, WT and MT underwent selfing and hybridization, respectively. To eliminate the possibility of self-incompatibility causing seedlessness between WT and MT, cross-pollination was conducted with another sweet orange cultivar, namely ‘Dahong’ sweet orange, known for its high seed count. The results reveal that WT produced approximately 12 seeds per fruit when self-crossed, while seeds were significantly reduced to about 3 seeds per fruit when crossed with WT or ‘Dahong’ sweet orange. In contrast, MT remained seedless regardless of self-pollination or cross-pollination (Table 2; Figure 6). This indicated that, although pollen quantity and viability were reduced in MT, female sterility was notably the primary cause of its seedlessness.

Table 2. Pollination experiment of different combinations between WT and the seedless mutant.

<table>
<thead>
<tr>
<th>Female Parent</th>
<th>Male Parent</th>
<th>Average Seed Number</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>-</td>
<td>0 c</td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>11.47 ± 0.84 a</td>
</tr>
<tr>
<td>WT</td>
<td>MT</td>
<td>3.51 ± 1.78 b</td>
</tr>
<tr>
<td>WT</td>
<td>‘Dahong’ sweet orange</td>
<td>2.4 ± 0.12 b</td>
</tr>
<tr>
<td>MT</td>
<td>-</td>
<td>0 c</td>
</tr>
<tr>
<td>MT</td>
<td>WT</td>
<td>1 ± 0.70 b</td>
</tr>
<tr>
<td>MT</td>
<td>MT</td>
<td>1 ± 0.53 b</td>
</tr>
<tr>
<td>MT</td>
<td>‘Dahong’ sweet orange</td>
<td>0.67 ± 0.67 c</td>
</tr>
</tbody>
</table>

Note: ‘-’—no pollen was available; WT—‘Succari Orange’; MT—seedless mutant ‘Juxiangyuan’ sweet orange. For a given factor different letters within a column represent significant differences.

4. Discussion

Seeds play a crucial role as a source of phytohormones needed for fruit development, including fruit size regulation [34]. Additionally, fruit size significantly impacts the marketability of citrus fruits [35]. Typically, seedless fruits, resulting from mutations that prevent seed initiation, tend to be smaller than their seeded counterparts. For example, fruit defects with smaller sizes are often exhibited by parthenocarpic mutant seedless tomatoes [36], and seedless chili fruits weigh less compared to seeded ones [37]. This trend was also observed in seedless avocados, which were consistently smaller than their seeded counterparts [38]. Similarly, the seedless mutant of ‘Hongjiangcheng’ sweet orange was smaller than its seedy control [39]. However, in contrast, larger fruit size, including both vertical and horizontal diameters, as well as single fruit weight, were exhibited in this trial by MT in comparison to WT. Plant hormonal status, including levels of auxin and gibberellin, can influence early fruit development in seedless citrus fruits [40,41]. Speculatively, hormonal changes and regulatory processes during the sterility phase of MT may have contributed to its larger fruit size. This unique characteristic, combined with its seedlessness, further enhances MT’s commercial value.

The extent of male (pollen) sterility varies among cultivated citrus varieties, often resulting in seedless or few-seeded fruits when grown without cross-pollination [42]. Similar phenomena were observed in other citrus species like clementine (C. clementina L.), which produced seedless fruit without cross-pollination but exhibited improved fruit characteristics when cross-pollinated with specific citrus varieties [43–45]. In the progeny of Satsuma, the degree of male sterility was primarily determined by the number of pollen grains per anther, a trait inherited by the progeny of ‘Okitsu No. 46’ [17]. In the case of MT, pollen fertility was notably low, consistently resulting in seedless fruits whether through self-pollination or cross-pollination with WT or the ‘Dahong’ sweet orange. Despite severe pollen abortion in MT, fruits developed seeds when a small number of viable pollen grains were artificially hybridized with WT. This suggested that although the male gamete of MT
experience substantial abortion, the presence of even a limited number of viable pollen grains is adequate for pollination and fertilization, indicating that male sterility may not be the primary factor that contributes to the seedlessness of MT.

All seedless fruits belong to the general category of parthenocarpy, encompassing various recognized types [46]. Parthenocarpy can be further categorized into obligatory parthenocarpy, consistently resulting in seedless fruit, and facultative parthenocarpy, which produces seedless fruit only when pollination is prevented [47]. In this study, both WT and MT fruits were obtained without pollination, representing vegetative parthenocarpy. Notably, similar to WT, the majority of commercial citrus cultivars exhibit some level of parthenocarpy in the absence of pollination [48]. Citrus seedlessness typically arises from a combination of factors rather than a single cause. Japanese citrus breeding programs often utilize male sterility in combination with female sterility and parthenocarpy [49]. Female sterility is directly related to seediness [50]. Consequently, MT was always a seedless fruit regardless of self-pollination or cross-pollination. Cross-sectional observation of the ovary during the bud stage revealed that the female abortion of MT was due to the developmental defects of the ovule, which was the key reason for the occurrence of MT sterility.

The external environment, in addition to common sterility types, also exerts a significant impact on flower development and fruit seed formation. Seedlessness can be achieved by combining parthenocarpy with factors that inhibit the normal fertilization process [51]. Certain environmental factors, such as low or high temperatures, chromosomal aberrations, and some chemical treatments, can induce seedlessness [52]. The erratic setting of both seeds and fruits is particularly evident when trees are exposed to sub- or supra-optimal temperatures during the flowering phase [53]. Exposure to different temperatures during flower bud development alters the expression of the self-incompatibility reaction [54]. The in-depth molecular biological mechanism affecting MT seedlessness still requires further exploration. Future investigations should focus on identifying the key abortion stages in MT by conducting histomorphological observations of male and female gametophytes at different bud stages, complemented by omics analyses, such as laser capture microdissection-based RNA-seq, to gain insights into crucial genes and molecular regulatory networks during flower development [55–57]. MT is a rare acid-free and seedless variety known for its advantage in terms of fruit size, which contributes to its commercial value. Genetic verification is essential to unravel the seed abortion mechanism in MT, paving the way for a comprehensive understanding of seedlessness in citrus varieties and promoting the seedless breeding process of citrus.

5. Conclusions

The findings in this study strongly indicate that, although pollen quantity and viability are reduced in the seedless mutant ‘Juxiangyuan’, female sterility is notably the primary cause of its seedlessness. Even with the introduction of fertile pollen, the persistence of seedlessness in MT strongly suggests that ovule failure was the primary factor contributing to this phenomenon. This insight enhances our understanding of the complex mechanisms underlying the reproductive biology of the MT cultivar and emphasizes the critical role of ovule development in the manifestation of its unique seedless phenotype. Further exploration of the specific factors influencing ovule development in MT may provide valuable insights for horticulturists and researchers working on seedless fruit varieties.

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Conflicts of Interest: The authors declare no conflicts of interest.

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