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# SEEDLESS MECHANISM OF A NEW CITRUS CULTIVAR 'HUAMI WUHEGONGGAN' (*CITRUS SINENSIS* × *C. RETICULATA*)

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## Abstract

In this study, the seedless mechanism of a new citrus cultivar 'Huami Wuhegonggan' (HMWG) (*Citrus sinensis* × *C. reticulata*) was assessed. Results from pollen number per anther, abnormal pollen frequency, pollen viability and germination frequency analyses suggested that HMWG pollen had low fertility. Thus, male sterility contributed partly to the seedlessness of HMWG. Embryonic development of self- and cross-pollinated HMWG was abnormal. After self- and cross-pollination, globular and heart-shaped embryos were observed after 2-3 weeks and heart- and torpedo-shaped embryos were detected at 4 weeks. However, embryos began to degenerate at 5 weeks and debris from degenerated embryos, or an empty cavity, was observed in the ovary at 7 weeks. These results demonstrate that seedlessness of HMWG results from male sterility and embryo abortion.

**Key words:** Citrus, Seedless mechanism, Male sterility, Embryo abortion.

## Introduction

Seedlessness is a desirable economic trait for both the fresh and processed citrus markets since seedless fruits possess no seeds, generally making consumption and processing easy and convenient. At present, most commercially produced citrus fruits such as oranges, mandarins and lemons are seedless (Vardi *et al.*, 2008; Wang *et al.*, 2012). Therefore, development of seedless fruit cultivars has become a major breeding objective for citrus breeders around the world. Seedless citrus fruits can be obtained through parthenocarpy (Talon *et al.*, 1992), male sterility (Hu *et al.*, 2005; 2007; Zhang *et al.*, 2006; Xiao *et al.*, 2007; Yu *et al.*, 2011), embryo abortion (Chen *et al.*, 1998; Chen *et al.*, 2002; Wang *et al.*, 2009; Chai *et al.*, 2011; Yu *et al.*, 2011), abnormal embryo sacs and unfertilized ovules (Yamasaki *et al.*, 2009), embryo sac abortion (Wang *et al.*, 2008; Zhou *et al.*, 2011; Xiao *et al.*, 2007), self-incompatibility (Yamamoto *et al.*, 2006; Distefano *et al.*, 2009; Wang & Lü, 2009; Ye *et al.*, 2009; Ngo *et al.*, 2010; Zhang *et al.*, 2012), polyploidy (Grosser *et al.*, 2010; Grosser & Gmitter, 2011) and triggered pollination during fruit development (Wen & Cai, 2000).

Bud sport selection has been widely used to creat novel cultivars in vegetatively propagated plants. Bud mutations often arise in citrus cultivars that are maintained by vegetative propagation (Zhang & Deng, 2006). Many excellent seedless citrus cultivars such as Satsuma (*Citrus unshiu* Marc.), Washington navel orange (*C. sinensis*), 'Wuzishatangju' mandarin (*C. reticulata* Blanco), 'Xiangshui' lemon [*C. limon* (L.) Burm. F.], Oroblanco (*C. grandis* × *C. paradisi*) and Tahiti lime [*C. latifolia* (Yu. Tanaka) Tanaka] have been obtained through bud sport selection (Zhang & Deng, 2006; Zhang *et al.*, 2012). In general, bud sports arise from a change in the nature of the gene (termed a "point" mutation) produced by chromosomal aberrations or abnormal segregation, leading to the loss, duplication, or rearrangement of genes. However, information on the potential mechanism of genetic alterations that results in seedlessness in citrus is very limited. It is necessary to carry out research on the seedless mechanism of citrus that has originated from bud variation.

Gonggan (*C. sinensis* × *C. reticulata*) is an ancient and excellent native Chinese citrus cultivar that grows in Guangdong province, China. It has been cultivated for over 1000 years (Ji *et al.*, 2011). Gonggan is one of the most popular citrus cultivars in China due to its beautiful fruit shape, golden fruit pericarp, honey taste and aroma, tender fresh texture with few slags, high sugar level and low acidity, as well as the ability to easily separate the capsule from the sarcocarp. However, Gonggan is seedy with an average of 10 seeds per fruit which seriously hinders its economic value and further development. Therefore, breeding a seedless Gonggan is essential to meet the needs of consumers, and increase revenue for the majority of growers. In 2006, a novel seedless Gonggan tree resulting from a bud mutation was found in a Gonggan orchard in Deqing county, Guangdong province, China. The mutation was registered and released as a new variety with the name of Huami Wuhegonggan (HMWG) (Qin *et al.*, 2014). In this study, the seedless mechanism of this new cultivar was investigated through fertility of the male gamete and embryo sac, embryonic development, compatibility of self-pollinated HMWG and cross-pollinated HMWG (HMWG × Gonggan, HMWG × Wuzishatangju). The results of this study can help to reveal the seedless mechanism of HMWG and assist in the establishment of seedlessness in other citrus cultivars originating from bud mutations.

## Material and Methods

**Plant materials:** Five cultivars, namely Huami Wuhegonggan (*C. sinensis* × *C. reticulata*), Gonggan (*C. sinensis* × *C. reticulata*) Wuzishatangju (*C. reticulata*

Blanco), Kiyomi (hybrid cultivar of *C. unshiu* Marc. cv. Unbergii Nakai × *C. Sinesis* cv. Toroveta) and Chuntianju (*C. reticulata* Blanco), were used as plant materials. These plants were cultivated in an orchard of South China Agricultural University.

**Meiosis of microsporocyte:** Normal flower buds were randomly collected from five trees of HMWG and Gonggan, then fixed in Carnoy's fluid (anhydrous alcohol/glacial acetic acid/chloroform=5:3:2) (v:v:v) for 24 h. The fixed flower buds were rinsed through a tertiary alcohol series (95%→90%→85%→80%→75%→70%) and stored in 70% alcohol at 4°C for further use. Meiosis of sample microsporocytes was observed following the procedure of Yang & Cheng (2005).

**Assay of pollen viability and germination percentage:** Pollen viability and germination percentage were assayed according to Ye et al. (2009). Three replicates were used, each with four Petri dishes consisting of approximately 800 pollen grains per dish.

**Observation of pollen morphology:** Flower buds were randomly collected from HMWG and Gonggan trees and stored in a drying apparatus at room temperature until pollen spread naturally. Pollen grains were collected and dry-stored at -20°C for further use. Pollen number per anther, abnormal pollen frequency, pollen morphology, polar and equatorial views of pollen grains, ornamentation of pollen exines and pollen grain morphology were observed and recorded under an optical microscope (OLYMPUS BH-2) or a scanning electron microscope (SEM) (FEI-XL30, 15KV).

**Artificial hybridization in the field:** Male fertility of HMWG was determined using Kiyomi, Gonggan and Chuntianju as the female parent, and HMWG as the male parent. There were four replicates, each with five trees consisting of 50 pollinated flowers per tree. Fruit setting frequency was invested before fruit ripening and seed number per fruit was recorded after fruit ripening.

**Pollination and fertilization of HMWG:** Pistils, including the stigma, style and ovary, were collected at 2, 4, 6, 8, 16, 24, 48, 72, 96, 120 and 168 h from five trees of self-pollinated HMWG, cross-pollinated HMWG × Gonggan, and HMWG × Wuzishatangju, respectively. Sections were made following the procedures of Shi & Hou (2004) and Tao *et al.* (2004). Pollination and fertilization of HMWG was observed with a fluorescent microscope (OLYMPUS $BH_2$-RFCA) and photos were obtained by a digital microphotograph system (OLYMPUS DP70).

**Mature embryo sac of HMWG:** Pistils from HMWG flower buds were collected and fixed in Carnoy's fluid (anhydrous alcohol/glacial acetic acid=3:1) (v:v) for 24 h. The fixed pistils were rinsed through a tertiary alcohol series (95% → 90% → 85% → 80% → 75% → 70%) and stored in 70% alcohol at 4°C for further analysis. Sections were made following the method of Xue *et al.* (1995). Mature embryo sacs of HMWG were observed using an optical microscope (OLYMPUS BH-2) and photo records were made by using a digital microphotograph system (OLYMPUS DP70).

**Embryonic development of self-pollinated and cross-pollinated HMWG:** Young fruits were collected at 2, 3, 4, 5, and 7 weeks after self-pollination (WASP) of HMWG×HMWG, and 2, 3, 4, 5, and 7 weeks after cross-pollination (WACP) of HMWG × Gonggan and HMWG× Wuzishatangju. Materials were prepared following the procedures of Ye *et al.* (2009). Slides were observed using an optical microscope (OLYMPUS BH-2) and photos were obtained by a digital microphotograph system (OLYMPUS DP70).

**Statistical analysis:** Statistical analyses were performed using DPS 7.05 software. Differences between compared sets were considered significant using the LSD test at p≤0.05.

## Results

**Pollen viability and germination percentage:** Significant difference in pollen number per anther, pollen viability, pollen germination and abnormal pollen frequencies were detected between HMWG and Gonggan (Table 1, Fig. 1). Pollen number per anther, pollen viability and pollen germination frequency of HMWG were 3250, 44.5% and 7.6%, respectively, which were significantly lower than the same parameters (5200, 85.6% and 33.0%) of Gonggan. Abnormal pollen frequency of HMWG (65.5%) was significantly higher than that of Gonggan (13.7%) (Table 1). Higher abnormal pollen frequency, lower pollen number per anther, pollen viability and germination frequency could give rise to a reduction in pollen fertility, which contributed partly to the seedlessness of HMWG.

**Pollen morphology:** As shown in Fig. 1 and Table 2, no significant differences in pollen morphology and size were observed between HMWG and Gonggan. For both cultivars, the pollen grains showed a tetra-colporate spherical shape in polar view (Fig. 2 B1, B2) and an aperture and colporation in equatorial view (Fig. 2 C1, C2). Ornamentation of the pollen exine in HMWG and Gonggan showed a smooth pollen exine (Fig. 2 D1, D2). However, many abnormal pollen grains with a rough surface were observed in HMWG. Compared to normal pollen grains, those abnormal pollen grains presented various shapes with fewer apertures and larger colporations (Fig. 2 E-H).

**Table 1. Pollen viability and pollen germination frequency of HMWG and Gonggan.**

| Cultivars | Pollen number per anther | Abnormal pollen frequency (%) | Pollen viability (%) | Pollen germination frequency (%) |
|---|---|---|---|---|
| HMWG | 3250 b | 65.5 a | 44.5 b | 7.6 b |
| Gonggan (CK) | 5200 a | 13.7 b | 85.6 a | 33.0 a |

Different letters within the same column indicate significant differences (LSD test; p≤0.05).

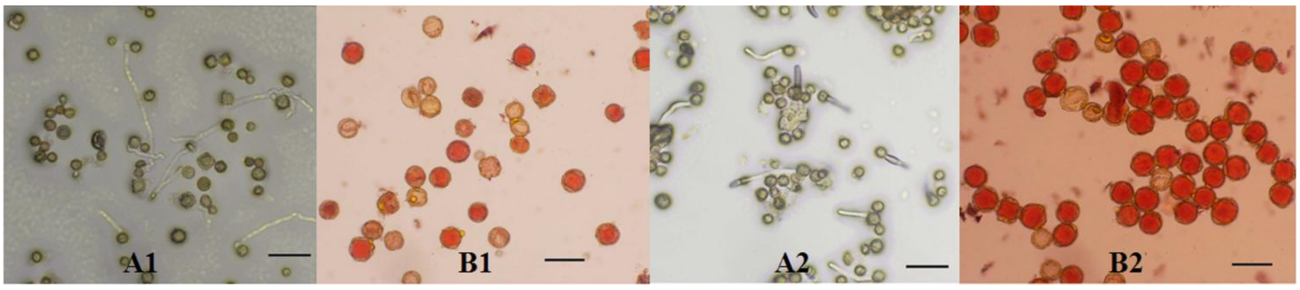

Fig. 1. Pollen viability and germination frequency of HMWG and Gonggan. A1 and A2, pollen germination frequency of HMWG (A1) and Gonggan (A2); B1 and B2, pollen viability of HMWG (B1) and Gonggan (B2). Bars = 40 µm.

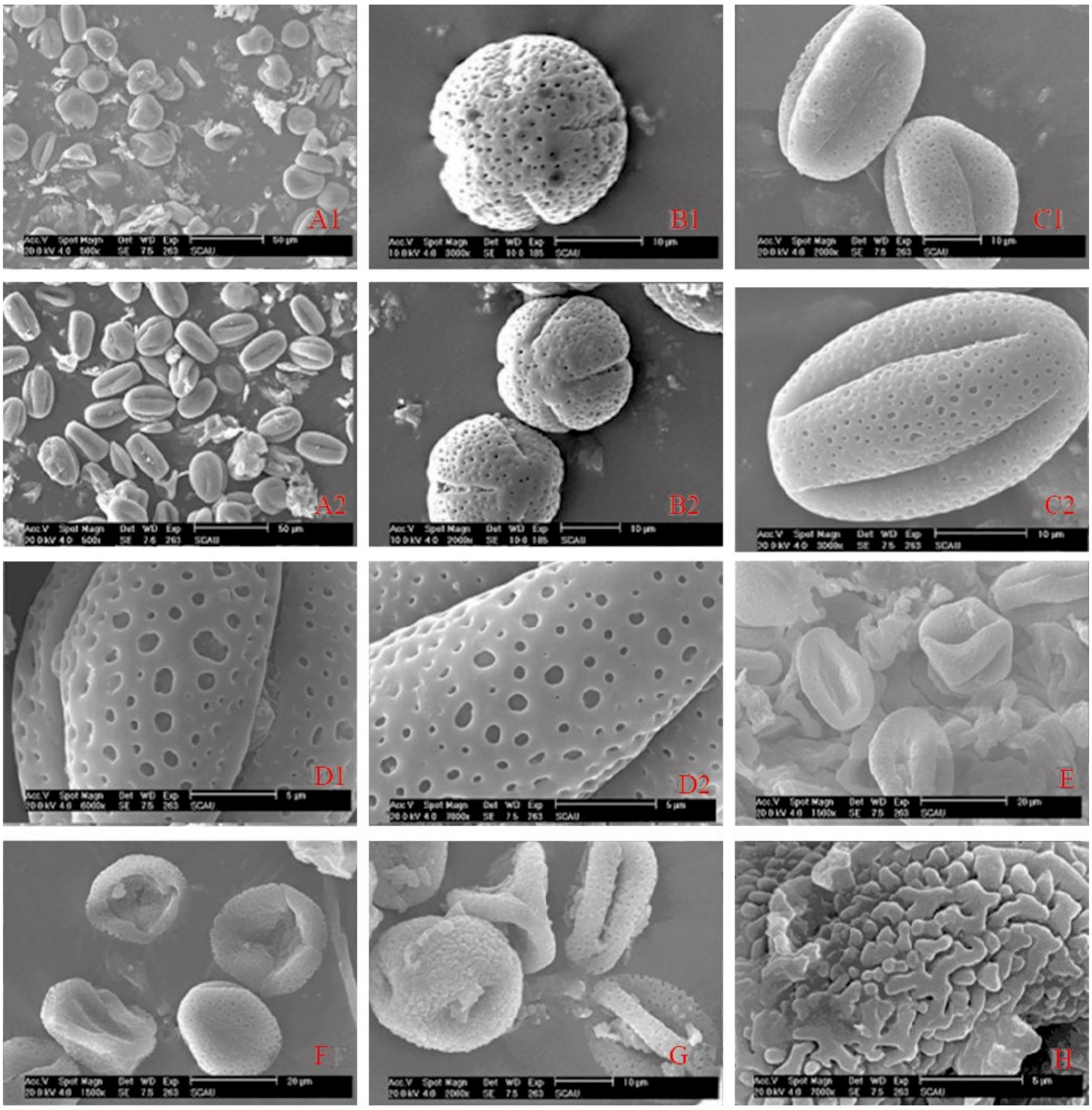

Fig. 2. Pollen morphology of HMWG and Gonggan. A1 and A2, pollen grains of HMWG (A1) and Gonggan (A2) (×500); B1 and B2, polar view of a pollen grain from HMWG (B1) (×3000) and Gonggan (B2) (×2000) showing tetra-colpate-lobed circular; C1 and C2, the equatorial view of a pollen grain from HMWG (C1) (×2000) and Gonggan (C2) (×3000) showing aperture and colporate; D1 and D2, ornamentation of the exine in HMWG (D1) and Gonggan (D2) pollen showing a smooth surface (×7000); E-G, abnormal pollen grains of HMWG; H, abnormal pollen grains of HMWG with a rough surface. Bars in A=50 µm, B, C and G=10 µm, D and H=5 µm, E and F=20 µm.

**Table 2. Comparison of pollen equatorial and polar length between HMWG and Gonggan.**

| Cultivars | Equatorial length (µm) | Polar length (µm) | Equatorial length/polar length |
|---|---|---|---|
| HMWG | 27.2 a | 28.1 a | 0.97 a |
| Gonggan (CK) | 26.9 a | 28.1 a | 0.95 a |

Different letters within the same column indicate significant differences (LSD test; $p \leq 0.05$).

**Table 3. Fruit setting frequency and average seed number per fruit in different cross combinations.**

| Cross combinations | No. of pollinated flowers | Fruit setting frequency (%) | Seed No. per fruit |
|---|---|---|---|
| HMWG×HMWG | 225 | 3.4 c | 1.1 d |
| Kiyomi×HMWG | 113 | 11.5 b | 11.5 a |
| Chuntianju×HMWG | 242 | 33.5 a | 7.2 b |
| Gonggan×HMWG | 178 | 4.5 c | 5.6 c |

Different letters within the same column indicate significant differences (LSD test; $P \leq 0.05$).

**Meiosis of microsporocyte:** As shown in Fig. 3 A-M, all microsporocytes from Gonggan and approximately 85% of microsporocytes from HMWG could form a normal dyad after the first meiosis and a tetrad after the second meiosis. At the end of the second pollen mitosis, four equal microspores were observed in each pollen grain (Fig. 3K-M). However, a few chromosome bridges, asynchronous meiosis and other types of variations such as triads and pentads were observed during meiosis of some HMWG microsporocytes (Fig. 3N-Q).

**Pollination and fertilization of HMWG in the field:** Fruit setting frequency and average seed number per fruit were normal using Kiyomi, Gonggan and Chuntianju as the female parent, and HMWG as the male parent (Table 3). These results indicate that the male gamete of HMWG was fertile.

**Pollination and fertilization of HMWG:** Three pollination combinations, namely HMWG×HMWG, HMWG × Gonggan and HMWG × Wuzishatangju, were carried out to study self- and cross-compatibility of HMWG. After dyeing with staining agents, pollen grains became golden-yellow, the pollen tube became fluorescent green, and a bright spot of sperm and vegetative nuclei was detected at the apex of the pollen tube (Fig. 4). For self-pollination of HMWG × HMWG and cross-pollination of HMWG × Gonggan, pollen grains germinated on the stigma at 2-4 h after pollination (Fig. 4 A1, A2 and B1, B2). For cross-pollinated HMWG × Wuzishatangju, no pollen grains germinated on the stigma at 2 h after pollination (Fig. 4 A3). Pollen grains began to germinate on the stigma 4 h after pollination (Fig. 4 B3). Pollen tubes elongated constantly on the stigma at 6-16 h (Fig. 4 C1-C3, D1-D3 and E1-E3) and entered into the stigma at 24 h after self- and cross-pollination (Fig. 4 F1, F2 and F3). These results indicate that the pollen of self- and cross-pollinated HMWG germinated normally on the stigma.

Forty-eight hours after self- and cross-pollination, pollen tubes entered into the style and grew downward through the style, and vascular bundles were observed around the pistillar chord (Fig. 4 G1, G2, and G3). Seventy-two hours after self- and cross-pollination, pollen tubes reached the bottom of the style (Fig. 4 H1, H2 and H3). These results indicate that the pollen tubes of self- and cross-pollinated HMWG could grow normally in the style.

The pollen tubes entered into the ovary and continued to grow along the spermophore from 96 h to 120 h after self- and cross-pollination (Fig. 4I1-I3 and J1-J3). Pollen tubes entered the ovule through the micropyle to complete fertilization at 168 h (Fig. 4 K1, K2 and K3). These results show that seedlessness in HMWG is not self-incompatible.

**Embryo sac and embryonic development:** Seven cells and eight nuclei were separated from the ovule of HMWG (Fig. 5B). The mature embryo sac had one egg cell, two synergids, three antipodal cells and a large central cell containing two polar nuclei (Fig. 5B). These results indicate that embryo sac fertility of HMWG was normal. Globular embryos were observed at 2-3 WASP (Fig. 5 A1-4) and heart-shaped embryos were observed at 4 WASP (Fig. A5-7). Torpedo-shaped embryos were observed while nucellar and integument cells of globular and heart-shaped embryos began to degenerate and the nucleus disappeared by 5 WASP (Fig. A8-10). Nucellar and integument cells shrank and an empty cavity, or debris from degenerated embryos, was observed in the ovary at 7 WASP (Fig. A11-12).

At 2 WACP of HMWG × Gonggan and HMWG× Wuzishatangju, globular embryos were observed (Fig. 5 B1-B2 and Fig. C1-C2). Globular embryos developed into heart-shaped embryos at 3 WACP (Fig. 5 B3-B4 and C3-C4) and heart-shaped embryos developed into torpedo-shaped embryos at 4 WACP (Fig. 5 B5-B7 and C5-C7). Nucellar and integument cells of torpedo- and heart-shaped embryos began to degenerate and the cavity of the embryo sac began to increase at 5 WACP (Fig. 5 B8-B9 and C8-C9). Debris from degenerated embryos or the empty cavity was observed in the ovary at 7 WACP (Fig. 5B10-B11 and C10-C11). These results suggest that embryo abortion occurred in the process of embryonic development of HMWG. Therefore, embryo abortion was the main cause of seedlessness of HMWG.

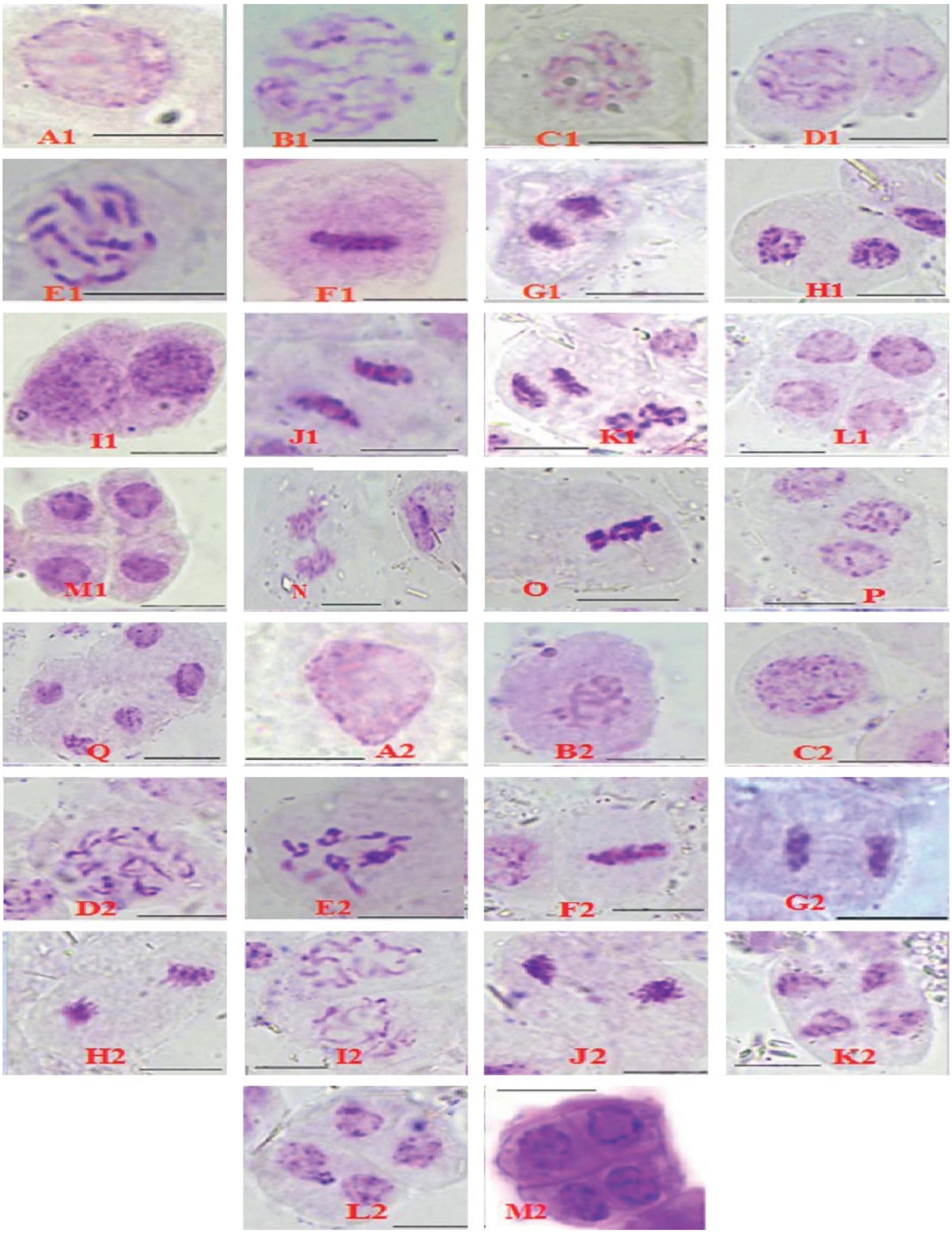

Fig. 3. Meiosis of microsporocytes of HMWG (A1-Q1) and Gonggan (A2-L2). A1, A2, microsporocyte at prophase I-leptotene stage; B1, B2, microsporocyte at prophase I-zygotene stage; C1, C2, microsporocyte at prophase I-pachytene stage; D1, D2, microsporocyte at prophase I-diplotene stage; E1, E2, microsporocyte at prophase I-diakinesis stage; F1, F2, microsporocyte at metaphase I; G1, G2, microsporocyte at anaphase I; H1, H2, microsporocyte at telophase I; I1, I2, microsporocyte at prophase II; J1, J2, microsporocyte at metaphase II; K1, K2, microsporocyte at anaphase II; L1, L2, microsporocyte at telophase II; M1, M2, microsporocyte at tetrad stage; N, chromosome bridges in meiotic prophase I; O, asynchronous meiosis in meiotic prophase II; P, triad in meiotic tetrad stage; Q, pentad in meiotic tetrad stage. Bars = 10 µm.

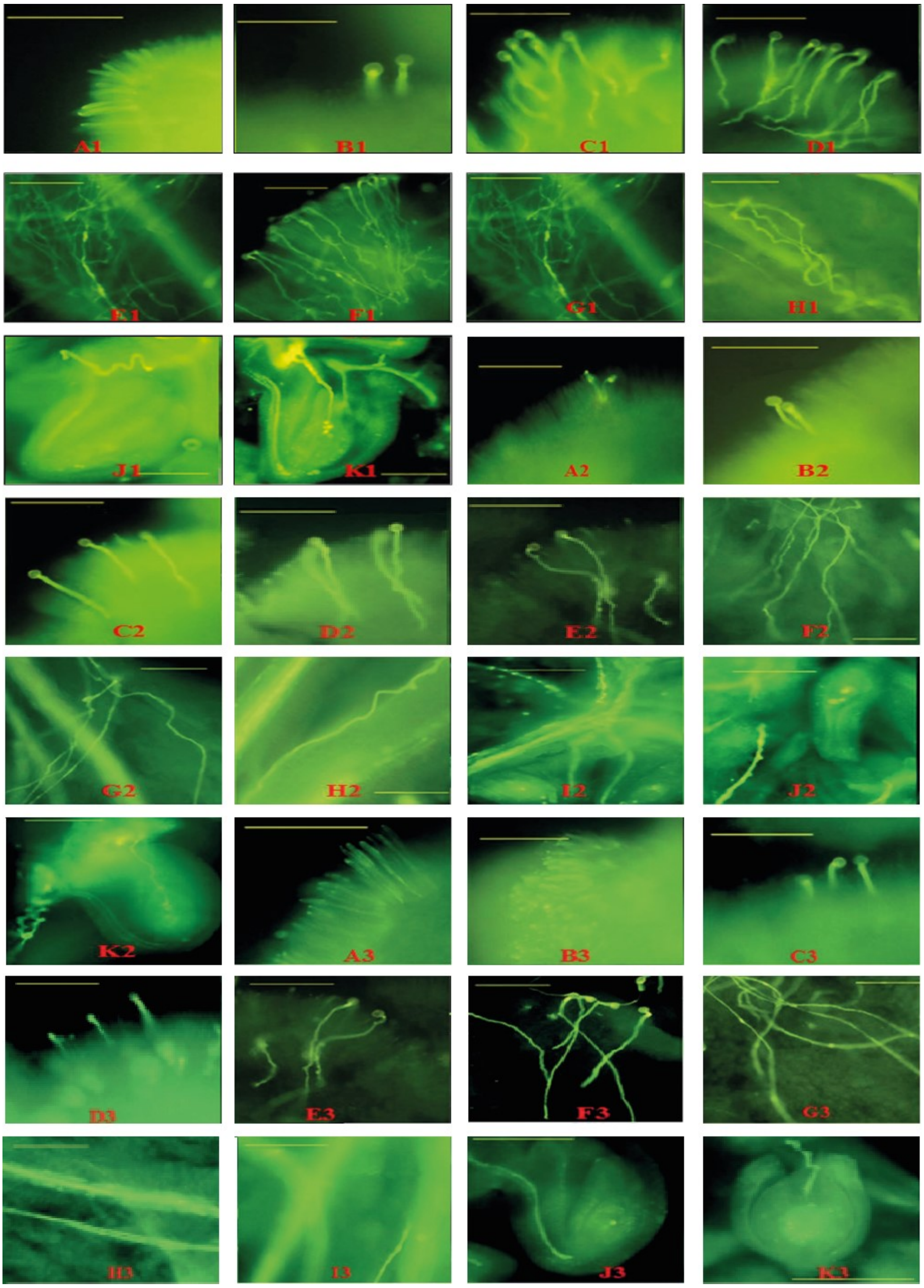

Fig. 4. State of pollen germination at different time intervals after HMWG×HMWG (A1-K1), HMWG × Gonggan (A2-K2) and HMWG × Wuzihatangju (A3-K3) pollination. A, 2 h; B, 4 h; C, 6 h; D, 8 h; E, 16 h; F, 24 h; G, 48 h; H, 72 h; I, 96 h; J, 120 h; K, 168 h. Bars = 200 µm.

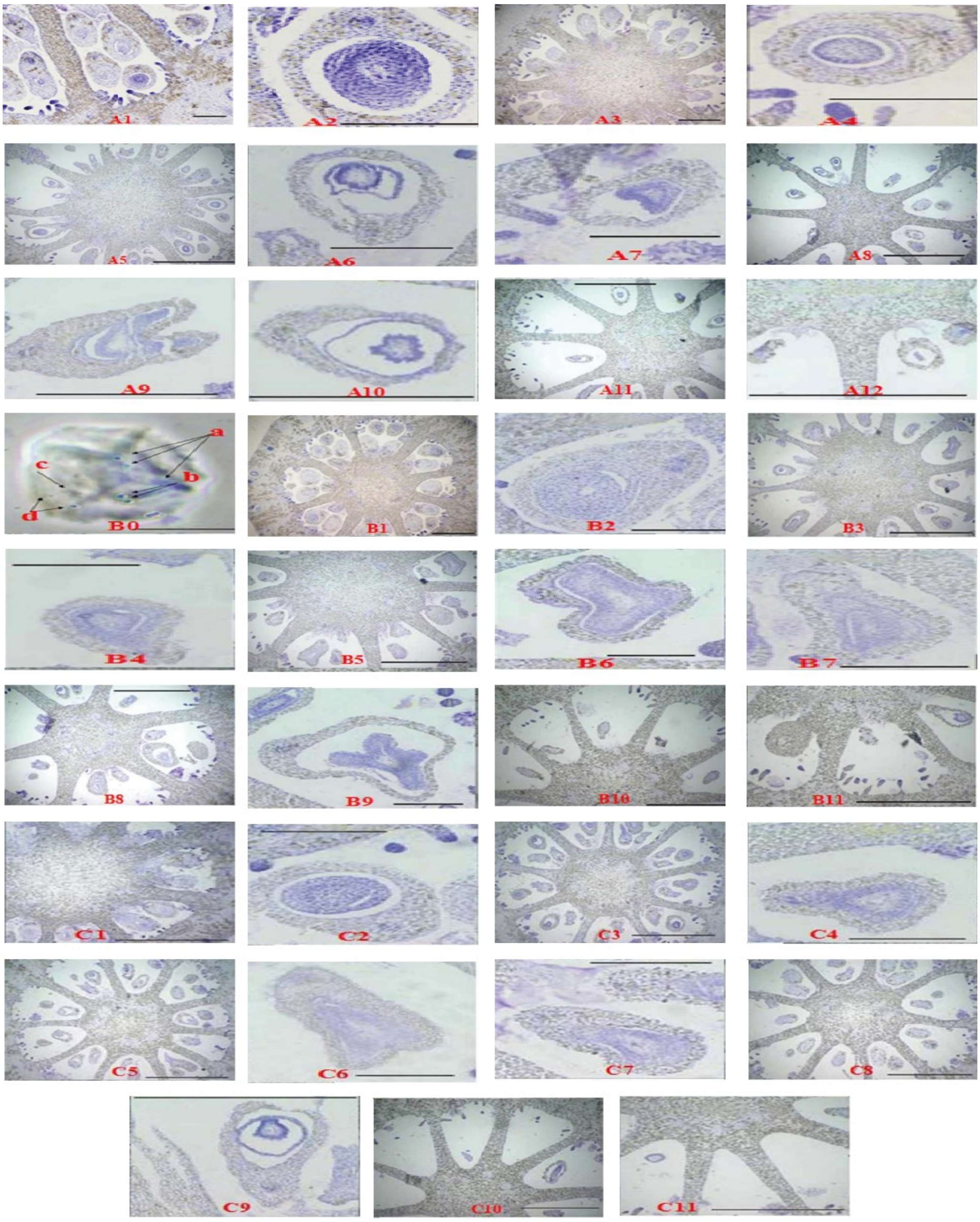

Fig. 5. Embryonic development of self-pollination of HMWG (A), cross-pollination of HMWG × Gonggan (B) and HMWG × Wuzishatangju (C) at different stages. A1-2, B1-2 and C1-2, two weeks (A1, ovary in longitudinal section; B1 and C1, ovary in transverse section; A2, B2 and C2, globular embryo); A3-4, B3-4 and C3-4, three weeks (A3, B3 and C3, ovary in transverse section; A4, globular embryo; B4 and C4, heart-shaped embryo); B5-7 and C5-7, four weeks (A5, B5 and C5, ovary in transverse section; A6, initial development of embryo from globular to heart-shaped stages; A7, B6 and C6, heart-shaped embryo; B7 and C7, torpedo-shaped embryo); B8-9 and C8-9, five WACP (A8, B8 and C8, ovary in transverse section; A9, initial development of embryo from heart- to torpedo-shaped stages; B9, C9 and A10, embryo began to degenerate); B10-11 and C10-11, seven weeks (A11, B10 and C10, ovary in transverse section; A12, B11 and C11, degenerated embryo). B0, mature embryo sac of HMWG (a, three antipodal cells; b, two synergids; c, one egg cell; d, two polar nuclei). Bars in A1, A2, A4, A6, A7, B2, B4, B6, B7, B9, C2, C4, C6 and C7=200 µm; A3, A9, A10, B1 and C9=500 µm; A5, A8, A11, A12, B3, B5, B8, B10, B11, C1, C3, C5, C8, C10 and C11=1 mm; B0=20 µm.

## Discussion

**Polyploidy analysis of HMWG:** Polyploids have more than two sets of chromosomes and are prevalent in plants. Polyploid plants, which are larger and stronger than their diploid counterparts, can produce fruits with fewer or no seeds through semi-sterility (lower pollen viability or abnormal meiosis of microsporocyte) or sterility. Polyploid citrus can be obtained through bud sports, interploid hybridization, somatic cell fusion, embryo rescue, endosperm culture and mutation breeding (Grosser *et al.*, 2010; Grosser & Gmitter, 2011). Currently, triploid, tetraploid, pentaploid, hexaploid, heptaploid, octaploid, decaploid and aneuploid citrus have been found. However, only triploid citrus is an elite trait since it is usually seedless or has fewer seeds (Ruan & Shi, 2011). In this study, branches, leaves, fruit and pollen grains of HMWG were as large or strong as its original cultivar Gonggan. Compared to Gonggan, most microsporocyte meiosis of HMWG was normal and no significant difference in pollen grain size was observed between the two cultivars (Fig. 2-4; Table 2). These results suggest that there was no variation in chromosomal ploidy in HMWG.

**Embryo sac fertility of HMWG:** The embryo sac is an important female organelle for fertilization and consists of a series of developmental processes such as megasporocytes, meiosis, functional megaspore, mono-nucleate embryo sac, two nucleate embryo sac, four-nucleate embryo sac, eight-nucleate embryo sac and mature embryo sac (Sun *et al.*, 2010). Abnormal phenomena can occur at any development stage of the embryo sac and will result in an aborted embryo sac (Sun *et al.*, 2010). Embryo sac abnormality, abortion or sterility often lead to seedless fruits in citrus (Xiao *et al.*, 2007; Wang *et al.*, 2008; Yamasaki *et al.*, 2009; Zhou *et al.*, 2011). Arrested seed development is a characteristic feature of seedless cultivars such as Mukaku Kishu (*Citrus kinokuni* hort. Ex. Tanaka) attributed to abnormal embryo sacs and unfertilized ovules (Yamasaki *et al.*, 2009). Embryo sac abortion is responsible for several citrus cultivars such as Wuhexuegan (*C. sinensis*) (Wang *et al.*, 2008), super late-maturing cultivar JZT (P)-1 (*C. reticulata*) (Zhou *et al.*, 2011), and Lipeng No. 2 ponkan (*C. reticulata*) (Xiao *et al.*, 2007). In the present study, the mature embryo sac from HMWG had one egg cell, two synergids, three antipodal cells and one large central cell containing two polar nuclei. No abnormality or abortion was observed in the mature embryo sac (Fig. 5 B0). After self- and cross-pollination, the HMWG ovule was fertilized and the embryo developed normally. These results suggest that the female gametophyte of HMWG was fertile and therefore female sterility was not the cause for seedlessness of the new cultivar.

**Self-incompatibility of HMWG:** Self-incompatibility (SI) is one of the main causes of seedless fruits in citrus (Yamamoto *et al.*, 2006; Ye *et al.*, 2009; Ngo *et al.*, 2010; Zhang *et al.*, 2012). In angiosperms, SI can be classified into sporophytic SI (SSI) and gametophytic SI (GSI) according to the genetic control of pollen behavior. Citrus belongs to GSI and the growth of pollen tubes was arrested in the styles of ‘Comune’ clementine (*C. clementina* Hort. ex Tan.) (Distefano *et al.*, 2009), shatinyu (*C. grandis* var. shatinyu Hort.) (Xue *et al.*, 1995), and ‘Xiangshui’ lemon [*C. limon* (L.) Burm. F.] (Zhang *et al.*, 2012), in the pistil of ‘Guanxi’ and ‘Duwei’ pomelo (*C. grandis*) (Wang & Lü, 2009) and in the ovaries of ‘Wuzishatangju’ mandarin (*C. reticulata* Blanco) (Ye *et al.*, 2009). In our study, pollen tubes entered the ovule through the micropyle and completed fertilization after self- and cross-pollination of HMWG (Fig. 4). Therefore, SI is not responsible for the seedlessness of HMWG.

**Male gamete fertility of HMWG:** Male sterility is generally referred to as pollen abortion and it is one of the most important causes for seedlessness in citrus fruits. Male sterility is involved in seedlessness of Guoqing No. 1 satsuma orange (*C. unshiu* Marc.) (Hu *et al.*, 2005), Hongjiang Cheng (*C. sinesis*) (Zhang *et al.*, 2006), ‘Ougan’ mandarin (*C. suavissima* Hort. ex Tanaka) (Hu *et al.*, 2007), Lipeng No. 2 ponkan (*C. reticulata*) (Xiao *et al.*, 2007), seedless Gonggan (*C. reticulata* Blanco) (Wang *et al.*, 2009) and Nanfeng tangerine [*C. reticulata* Blanco var. kinokuni (Tanaka) H. H. Hu] (Yu *et al.*, 2011). In this study, the frequency of abnormal pollen in HMWG was significantly higher than that in Gongan pollen while pollen number per anther, pollen viability and pollen germination frequency of HMWG were significantly lower than the same parameters of Gongan. Abnormal phenomena such as chromosome bridges, asynchronous meiosis, triads and pentads were observed during microsporocyte meiosis of HMWG (Table 1 and Fig. 3). Higher abnormal pollen percentage, lower pollen viability and germination frequency, and abnormal microsporocyte meiosis can reduce pollen fertility (Xiao *et al.*, 2007; Wang *et al.*, 2009; Yu *et al.*, 2011). However, fruit setting rate and average seed number per fruit were normal using Kiyomi, Gonggan and Chuntianju as the female parent, and HMWG as the male parent, indicating that the male gametes of HMWG are fertile (Table 3). These results suggest that HMWG pollen had low fertility; thus, male sterility contributed partly to the seedlessness of HMWG. Similar results were also observed in Lipeng No. 2 ponkan (*C. reticulata*) (Xiao *et al.*, 2007), seedless Gonggan (*C. reticulata* Blanco) (Wang *et al.*, 2009), and Nanfeng tangerine [*C. reticulata* Blanco var. kinokuni (Tanaka) H. H. Hu] (Yu *et al.*, 2011).

**Embryonic development of HMWG:** Embryo abortion is a phenomenon in which stamens and pistils develop normally and can complete fertilization while seedless fruits are produced due to ovule or seed degeneration. The majority of embryo abortions occur during the embryonic period. Embryo abortion contributed greatly to many seedless citrus cultivars such as TAYI seedless ponkan (*C. reticulata*) (Chen *et al.*, 1998), Jishou Shatianyou (*C. grandis* Osbeck) (Chen *et al.*, 2002), Zigui shatian pummelo (*C. grandis* Osbeck) (Chai *et al.*, 2011), Nanfeng tangerine [*C. reticulata* Blanco var. kinokuni

(Tanaka) H. H. Hu] (Yu *et al.*, 2011) and seedless Gonggan (*C. reticulata* Blanco) (Wang *et al.*, 2009). In the present study, embryo abortion occurred in the process of embryonic development of HMWG after self- and cross-pollination (Fig. 5). Therefore, embryo abortion was the main cause of seedlessness in HMWG. Our results were consistent with the findings for Lipeng No. 2 ponkan (*C. reticulata*) (Xiao *et al.*, 2007), seedless Gonggan (*C. reticulata* Blanco) (Wang *et al.*, 2009), and Nanfeng tangerine [*C. reticulata* Blanco var. kinokuni (Tanaka) H. H. Hu] (Yu *et al.*, 2011).

## Conclusion

Embryo abortion was the main cause of seedlessness in HMWG. Higher abnormal pollen percentage, lower pollen viability and germination frequency can give rise to a reduction in fertility, which contributed partly to the seedlessness of HMWG.

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