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Production of Triploid Germplasm by Inducing 2n Pollen in Longan

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Abstract: Longan (*Dimocarpus longan* Lour.) is of great economic significance in South China for its unique taste and nutritional properties. However, longan breeding is mainly based on seedling selection, which generally results in small fruits, low flesh recovery, and few seedless germplasm. Triploid breeding is a central way to improve these problems. In this study, microspore chromosomes were doubled by colchicine and high-temperature treatment to create triploids in longans. The relationship between the development process of male gametophyte of longans and the morphological changes of male flower buds was established. Cytological observation showed that when the male flower buds were in stage I (when the diameter of the flower bud is 1.4–2.0 mm), most of the microspores were at the pachytene to diakinesis stage of meiosis, and the chromosome doubling induction effect was the best at this stage. The results showed that the 2n pollen rate induced by a high temperature of about 38 °C was higher than that induced by colchicine treatment. The highest 2n pollen rate was 5.7% and 5.5% based on the microscopic measurement method and the abnormal separation in tetrad stage estimation method, respectively. Four triploids were successfully obtained from artificial pollination with 2n pollen, with a triploid induction rate of 0.6%. This study will promote ploidy breeding in longan.

Keywords: polyploidy breeding; microspore; meiotic; colchicine; high temperature

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1. Introduction

Longan (*Dimocarpus longan* Lour.) belongs to the family of Sapindaceae and is a characteristic subtropical fruit tree [1]. It has a long history of cultivation in China and is widely cultivated in Fujian, Guangdong, Guangxi, Hainan, and Taiwan and locally cultivated in Yunnan and Sichuan at a small scale [2]. With rich primary and secondary metabolites, longan fruit has long been used as both food and traditional Chinese medicine. It is very popular among consumers and has become one of the most important economic fruit trees in South China. However, cultivated longan cultivars is few. The industry in China is currently dominated by only two main cultivars, namely 'Shixia' and 'Chuliang', while the industry in Thailand and Vietnam depends on 'E-dor' [3]. At present, most high-quality varieties produce small fruit with low flesh recovery. A few varieties with aborted seed and high flesh recovery have little commercial cultivation value due to some other shortcomings beyond small fruit size, such as low and unstable seed abortion rate [4–6].

Polyploid and diploid plants differ in morphology, physiology, and cytology; these genotypic and phenotypic differences are mainly caused by gene dosage effects and allelic

diversity [7]. Polyploid fruit trees are huge, with strong stress resistance and seedlessness or smaller seed [8–12]. Triploids have two basic characteristics: 'macrogenicity' caused by the enlargement of somatic cells and 'sterility' caused by the disorder of meiosis, which can simultaneously improve fruit size and increase the flesh recovery of longans [13,14]. However, a polyploid germplasm of longan has not been found up until now. Therefore, it is of great significance to carry out longan polyploidy breeding.

Polyploidy breeding includes asexual polyploidization and sexual polyploidization. Asexual polyploidization is the doubling of somatic chromosomes via artificial induction by physical or chemical methods. However, this means always produces chimeras, which makes it difficult to isolate and identify polyploids and thus prolongs the breeding period [15]. Sexual polyploidization has been achieved by cross breeding between unmeiotic gametes or polyploid parents, of which 2n gametes are more widely used [16]. Sexual polyploidization of 2n gametes has more biological advantages than asexual polyploidization, such as obtaining higher incidence, higher genetic heterozygosity, and creating heterozygotic polyploidy populations with high genetic diversity through gene infiltration from affinity diploid plants [17].

The 2n gametes of plants are mostly produced by abnormalities of meiosis [18], which involves nuclear recombination (first division restitution (FDR) and second division reorganization (SDR)), abnormal meiotic spindle, and abnormal cytokinesis. The frequency of 2n gametes produced by plants in nature is very low. Most plants produce less than 2% of 2n pollen [2]. Therefore, it is necessary to increase the frequency of 2n male gametes by artificial induction and improve the efficiency of polyploidy breeding. The induction of 2n male gametes is usually achieved by physically or chemically interfering with the meiosis of microsporocytes.

Temperature stress may change the localization and distribution of the cytoskeleton to produce 2n gametes [19]. During the diplotene to diakinesis stage of the meiotic process of pollen mother cells, the frequency of 2n pollen was found to be the highest (58.03%) when male buds of aspen were treated with a high temperature (38 °C or 41 °C) for 6 h [20]. The greatest amount of diploid pollen produced by the treatment of 42 °C for 4 h was up to 22.04% of the total yield in persimmon [21]. Roses grown in the greenhouse obtained a significant increase in 2n pollen ratio of up to 4.6% by applying a high temperature (close to 32 °C) during the day, when plants bloom in recurrent cycles [22]. Microtubule inhibitors such as colchicine can inhibit meiosis, resulting in the production of 2n pollen. Studies showed that a high 2n pollen induction rate of up to 62.10% could be achieved by injections of 0.6% colchicine four times with 2 h intervals in *Populus* × *popularis* [23]. The highest rate of 2n pollen production was 28.71% in *Eucalyptus*, when the flower bud size was from 3.5 to 4.0 mm under a treatment with 0.5% colchicine solution for 6 h [24]. The incidence rate of 2n pollen in 'Wuhefeng' jujube substantially increased by up to 17.36% by injection with colchicine 0.3% [25].

The flower bud differentiation period of longans was from January to April, while the inflorescence development and flower organ differentiation were from March to April. The flowering period was in April [26]. The female and male flowers were in the same flower cluster and same tree. The flower cluster belonged to conical cyme with much side inflorescence and a large number of flowers. Flowers mainly included female and male flowers. Flowers in a single cluster opened alternately in the order of male and female, i.e., "male-female-male" or "female-male-female" [27].

In this study, we analyzed the relationship between microspore development and male flower bud formation in longans to guide the induction of 2n pollen. At the same time, according to the cytological observation, the optimal induction period of longan 2n pollen was found. During this period, high temperature and colchicine treatment were separately used to treat longan male flower buds, and the better method for inducing 2n pollen to produce a longan triploid was determined.

2. Materials and Methods

2.1. Plant Material

The material used in this study was from 10-year-old longan trees of the main cultivar 'Shixia' (SX), which were all planted in the Longan Germplasm Resource Garden, College of Horticulture, South China Agricultural University.

2.2. Male Flower Development and the Microspore Meiotic Process

Male flower buds were collected when they were light green in color, with sepals completely closed. The transverse diameter of each male bud was measured with a vernier caliper for grading. The male bud at each stage was photographed. The buds were fixed with Carnot's fixative for 24 h, then transferred to 70% ethanol and stored at 4 °C. Before observation, the preserved flower buds were taken out and soaked in pure water for 5 min to remove the alcohol residue on the surface. The anthers were cut from the flower buds with a dissecting needle and placed in a centrifuge tube containing a 3% enzymatic hydrolysis solution (Macklin). The tube was placed in an incubator set at 37 °C in darkness and allowed to undergo enzymatic hydrolysis for 5 h. After the enzyme solution was removed, 500 μL of distilled water was added to tube and set for 10 min. The water was then sucked out and the sample in the tube was suspended in 500 μL of Carnot fixative and fixed for 30–60 min. The sections of meiotic stages were observed according to the improved method of the wall-removed hypotonic-drying method [28]. Microsporocytes were then prepared by squeezing the anthers with forceps and stained with 5% Giemsa staining solution for 10 min. Representative cells at various stages of meiosis were photographed with an optical microscope OLYMPUS BX-53 under a magnification of 1000×.

2.3. 2n Pollen Induction

The experiment was carried out over 7–17 March 2020 at a natural temperature of 14–27 °C in Guangzhou city. The 2n pollen grains were induced by three methods: cotton wool soaking with colchicine, injection with colchicine, and high-temperature treatment. Forty flower clusters were treated for each method.

The colchicine cotton wool soaking method was performed according to Zhou et al. [29]. Panicles of the same size and developmental state were selected. When most of the male flower buds were 1.4–2.0 mm in diameter, the side inflorescences were wrapped with absorbent cotton to make the colchicine fully contact the bud and soak the sepal. A syringe was used to inject and wet the absorbent cotton with 0.5% or 0.7% colchicine solution containing 1.5% dimethyl sulfoxide (DMSO). The panicle was placed in a black plastic bag to prevent the colchicine from being decomposed by light (Figure 1a,b). Colchicine was supplemented every 12 h. The cotton wool was carefully removed 5 d later, and the colchicine remaining on the surface of the flower buds was washed with distilled water. At the same time, flower branches in the same state were treated with distilled water and served as a control.

The second method of colchicine treatment involving injection was conducted according to Liu et al. [30]. Small holes with a diameter of 1.0 mm were drilled deep into the xylem in flowering shoots about 20 cm below the main panicle. A ring cut was carried out about 10 cm below the holes to prevent downward transport of colchicine. Then, 0.5% or 0.7% colchicine solution was transfused through these holes with injection needles connected to a bottle containing 6.0 mL of colchicine solution. The transfusion device was fixed with tape (Figure 1c,d). The flow rate was adjusted to one drop every 1 min, and colchicine was supplemented every 12 h. The device was removed 10 d later. The control was with the same treatment, but distilled water was injected into the flowering shoot instead of colchicine solution.

Figure 1. Display of three methods for 2n pollen induction. (**a**,**b**) Cotton soaking with colchicine. (**c**,**d**) Injection with colchicine using an infusion apparatus (showed by the arrows). (**e**,**f**) High-temperature treatment with trees covered by plastic shed and heated with unit heater.

In the high-temperature treatment, the whole tree was covered with transparent plastic film when most of the flower buds were at about 1.4 mm (Figure 1e,f). Two unit heaters (AIRMATE, WP20-X3, 20–2000W) were used to adjust the temperature in the plastic shed to 38 ± 2 °C, and a digital thermometer was used to record the temperature change in real time. The film and unit heaters were removed 10 d later. The trees outside the plastic shed was used as a control group.

2.4. Estimation of the Frequency of 2n Pollen

To examine the effect of different 2n pollen induction methods, 2n pollen obtained by different induction methods were identified based on the microscopic measurement and the abnormal division ratio at the tetrad stage of the longan microspore mother cell. As for the microscopic measurement method, a drop of pollen suspension was placed on a glass slide, a drop of acetic acid magenta was added, and then it was covered with a cover glass. More than 500 pollen grains were measured under the microscope (Olympus CX51) at a magnification of 200×, and the experiment was repeated three times. Large pollen which was 1.28 times the size of of 1n pollen in diameter was regarded as 2n pollen.

$$\text{2n pollen ratio (\%)} = \text{number of total large pollen/number of total pollen} \times 100\%$$

The method for estimating the proportion of abnormal divisions in the tetrad stage of longan microspore mother cells was based on the study of Xue et al. [31]. The occurrence of 2n pollen originates from the formation of dyads (Dy) and triads (Tr). Theoretically, one tetrad (Te) produces four 1n pollen, one dyad produces two 2n pollen, and one triad produces one 2n pollen and two 1n pollen. The number of dyads, triads, and tetrads were counted separately. The number of observed dyads, triads, and tetrads during meiosis enabled an evaluation of the theoretical frequency of 2n pollen. The frequency of 2n pollen (F) was estimated as follows:

$$F = \frac{2Dy + Tr}{2Dy + 3Tr + 4Te} \times 100\% \quad (1)$$

where Dy = number of dyads, Tr = number of triads, and Te = number of tetrads.

2.5. Artificial Pollination Using 2n Pollen

About 1500 female flowers were artificially pollinated by 2n pollen when they were in full bloom (Figure 2). To avoid interference from other pollen, male flowers in panicles to be pollinated were removed and placed in non-woven bags in advance. On the day of pollination, a 2n pollen suspension with 1.0% sucrose solution was gently brushed onto the stigma of all the female flowers in the panicle, which were then placed in non-woven bags. Pollination was performed once a day around 9:00 am until all the female flowers were pollinated.

Figure 2. Artificial pollination with 2n pollen using a brush.

2.6. Ploidy Analysis by Chromosome Counting and Flow Cytometry

After the fruit of the treated groups and untreated group were mature, the seeds were collected and sown immediately in a mixed substrate composed of peat soil, sand, and coconut bran for the germination culture. Fruit set rate (%) = number of collected seeds/number of artificially pollinated female flowers × 100%. When the seeds germinated and the roots grew to about 1 cm, 0.3 mm of root tips were taken for chromosome observation. The method of chromosome counting was according to Zhang et al. [32].

When the seeds germinated into seedlings, 0.5 cm^2 of young leaves were taken for detecting the ploidy level by FACSMelody flow cytometry (BD Biosciences, San Jose, CA, USA). The diploid 'Shixia' longan (2n = 2x = 30) was used as the external standard. The detection method of flow cytometry was as described by Lai et al. [33].

2.7. Statistical Analysis

The data are presented as means ± SD, and significance of differences between experimental data was evaluated by Duncan's multiple comparison tests using Statistical Product and Service Solutions software (version 16, SPSS Inc., Chicago, IL, USA).

3. Results and Analysis

3.1. Relating the Meiotic Process of Microspore Mother Cells to the Morphological Changes of Male Flower Buds

The principle of 2n pollen induction is to use physical or chemical means to cause meiotic disorder during meiosis of microspore mother cells so that the formed gametes contain doubled chromosomes. Studies have shown that the appropriate induction period is generally from the pachytene to the diakinesis stage in prophase I of meiosis [34]. To improve the induction efficiency, the correlation between the meiotic process of longan microspore mother cells and the morphological changes of male flower buds was studied to accurately determine the appropriate induction period based on flower bud morphology. The results are shown in Table 1 and Supplemental Table S1.

Table 1. Meiotic process at different developmental stages of male flower buds of 'Shixia' longan.

Meiotic Phases		Different Developmental Stages of Male Flower Buds				
		I	II	III	IV	V
Prophase I	Leptotene	28.7% [a] (173 [b])	1.6% (8)			
	Zygotene	11.9% (72)	1.0% (5)			
	Pachytene	13.6% (82)	2.6% (13)			
	Diplotene	27.7% (167)	2.6% (13)			
	Diakinesis	14.1% (85)	9.0% (45)			
Metaphase I		3.7% (22)	14.4% (72)			
Anaphase I		0.3% (2)	8.2% (41)			
Telophase I			6.2% (31)			
Prophase II			8.2% (41)			
Metaphase II			3.6% (18)			
Anaphase II			6.6% (33)			
Telophase II			5.8% (29)			
Tetrad			12.6% (63)	4.5% (9)		
Microspore cells			17.6% (88)	95.5% (191)	100.0% (100)	——

[a] The percentage of microspore mother cells at the corresponding meiotic phases. [b] The statistical number of microspore mother cells at the corresponding meiotic stage.

When the male flower buds were in stage I, the stamens and pistils were clearly visible (Figure 3I), and the microspore mother cells began to undergo meiosis. The microspore mother cells were mainly in the pachytene, diplotene, or diakinesis stage, accounting for 13.6%, 27.7%, and 14.1%, respectively, accounting for 55.4% in total (Figure 4d–f). At stage II, the heights of the stamens and pistils were the same (Figure 3II), and microspore mother cells at all meiotic stages—prophase I (16.8%), metaphase I (14.4%), anaphase I (8.2%), telophase I (6.2%), prophase II (8.2%), metaphase II (3.6%), anaphase II (6.6%), telophase II (5.8%), tetrad stage (12.6%), and microspore period (17.6%)—could be observed (Figure 4). When the male flower buds entered stage III, the stamens were higher than the pistils (Figure 3III), and the microspore mother cells were mainly in the microspore stage (95.5%), with very few in the tetrad stage (4.5%) (Figure 4n–p). From stage IV to V (Figure 3IV,V), the pistils gradually atrophied until the male flowers were fully open, and meiosis ended.

Figure 3. Morphological characteristics of male flower buds at different developmental stages of 'Shixia'. (**I**) The male flower buds were light green, with a diameter of 1.4–2.0 mm, sepals completely closed, and stamens and pistils clearly visible. (**II**) The male flower buds were brown-green, with a diameter of 2.1–3.0 mm, sepals closed, and stamens and pistils equal in height. (**III**) The male flower buds were yellow-green, with a diameter of 3.1–3.8 mm, sepals slightly open, and stamens higher than (atrophic) pistils. (**IV**) The male flower buds are light yellow, with a diameter of 3.9–4.4 mm and sepals open to reveal the stamens. (**V**) The male flowers were yellow and blooming, with a diameter of over 4.4 mm and sepals fully spreading. Scale bar = 1 mm.

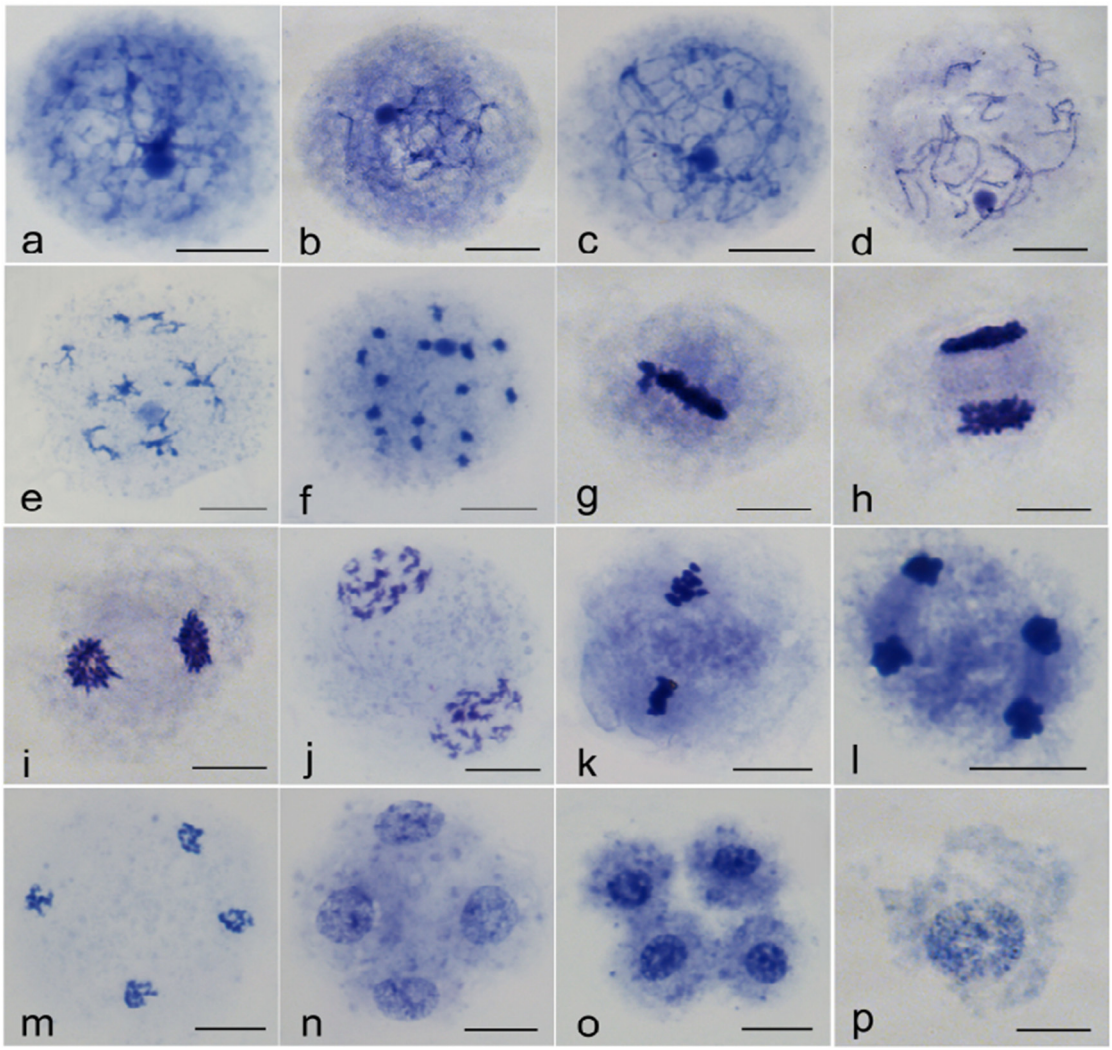

Figure 4. The process of meiosis in 'Shixia' microspore mother cells: (**a**) Early-leptotene, (**b**) Late-leptotene, (**c**) Zygotene, (**d**) Pachytene, (**e**) Diplotene, (**f**) Diakinesis, (**g**) Metaphase I, (**h**) Anaphase I, (**i**) Telophase I, (**j**) Prophase II, (**k**) Metaphase II, (**l**) Anaphase II, (**m**) Telophase II, (**n**,**o**) Tetrad, (**p**) Microspore. Scale bar = 10 μm.

In short, when male flower buds were in stage I, with a transverse diameter of 1.4–2.0 mm, most of the microspores were in the pachytene to diakinesis stage of meiosis, which was the appropriate period for induction treatment.

3.2. 2n Pollen Induction and Identification

When the male flower buds were in the appropriate induction period, the male gametes of 'Shixia' were treated to induce 2n pollen, using colchicine and high-temperature treatments. In order to compare the induction efficiency of 2n pollen by different treatments, the induction rate of 2n pollen was calculated by means of the microscopic measurement and the abnormal separation in tetrad stage estimation.

The criterion to judge a 2n pollen was that the diameter of 2n pollen was generally 1.28 times the average diameter of normal pollen [35,36]. The pollen diameter in each treatment ranged from 15.5 to 33.2 μm, and the pollen diameters in the high-temperature treatment were the largest, reaching 23.8 μm (Table 2). The 2n pollen rates of the groups treated with colchicine or high temperature were significantly (6 to 14 times) higher than that of the untreated group. Among them, the 2n pollen rate obtained with the high-temperature treatment was the highest at 5.7%, while the 2n pollen rate obtained by soaking with 0.5% colchicine was 4.2%, which was the highest among all the colchicine

treatments. Therefore, the effect of high-temperature treatment was better than that of colchicine treatment.

Table 2. The 2n pollen rate in various treatments estimated based on microscopic measurement.

Treatment No.	Treatment	Treatment Time/Volume	Average Diameter of Pollen (μm)	Pollen Diameter Range (μm)	2n Pollen Rate (%)
1	control	-	21.2 ± 0.3 c	15.3~27.9	0.4 ± 0.0 e
2	soaking with colchicine 5%	5 d	21.7 ± 0.1 bc	16.0~29.2	4.2 ± 0.3 b
3	soaking with colchicine 7%	5 d	21.8 ± 0.1 bc	15.9~28.9	2.4 ± 0.1 d
4	injection with colchicine 5%	120 mL	23.6 ± 0.2 a	15.5~30.1	3.1 ± 0.2 c
5	injection with colchicines 7%	120 mL	22.6 ± 0.1 b	16.3~30.9	3.3 ± 0.3 c
6	high temperature (38 ± 2 °C)	10 d	23.8 ± 0.1 a	16.6~33.2	5.7 ± 0.2 a

The data in the table are presented as means ± SD. Different lowercase letters in the same column indicate significant differences between treatments at the $p < 0.05$ level according to Duncan's multiple comparison tests.

We also used the tetrad stage estimation method to estimate 2n pollen rate. The male flower buds induced by different treatments were used as materials to observe the stage of microspore mother cells, and the proportions of dyads and triads in the tetrad stage in each treatment were obtained. The results are shown in Table 3. The frequency of 2n pollen obtained from the treated groups was significantly (5 to 11 times) greater than that of the control group. Among them, 4.4% of 2n pollen was generated by cotton wool soaking with 0.5% colchicine. This method was the best among colchicine treatments. A higher 2n pollen rate of up to 5.5% was achieved by applying a high temperature of about 38 °C for 10 d. Therefore, the high-temperature treatment was the best way to induce 2n pollen production. There was no significant difference between the abnormal separation in tetrad stage estimation and microscopic measure method in estimating 2n pollen rate.

Table 3. The 2n pollen rate in various treatments estimated based on observation of abnormal split ratio in the tetrad period.

Treatment No.	Split Rate (%)			2n Pollen Rate (%)
	Dyad	Triad	Tetrad	
1	0	2.0 ± 0.1 d	98.0 ± 0.1 a	0.5 ± 0.0 e
2	1.4 ± 0.2 b	14.2 ± 0.6 a	84.4 ± 0.8 d	4.4 ± 0.2 b
3	1.0 ± 0.2 b	8.6 ± 0.4 c	90.4 ± 0.4 b	2.7 ± 0.1 d
4	1.4 ± 0.1 b	11.3 ± 0.9 b	87.3 ± 0.96 c	3.7 ± 0.3 c
5	1.4 ± 0.1 b	10.0 ± 0.6 bc	88.6 ± 0.6 bc	3.3 ± 0.1 cd
6	2.4 ± 0.4 a	15.9 ± 0.8 a	81.7 ± 1.1 d	5.5 ± 0.4 a

The treatment numbers represent the same treatments as those in Table 2. The data in the table are presented as means ± SD. Different lowercase letters in the same column indicate significant differences between treatments at the $p < 0.05$ level according to Duncan's multiple comparison tests.

The formation of dyads and triads is related to the abnormal orientation of the spindle, which leads to abnormal chromosome orientation in anaphase II and in turn affects their arrangement and distribution in telophase II, resulting in the abnormal division [37]. The normal spindle orientation involved the formation of parallel spindles and perpendicular spindles, while the abnormal spindle orientation caused 'Eight-type' spindles (tripolar spindles) and fused spindles (Figure 5a–d). Among them, the parallel spindles formed a 'Matts-type' tetrad, and the perpendicular spindles formed a 'Cross-type' tetrad (Figure 5i,j). The tripolar spindles formed a triad, and the fused spindles formed a dyad (Figure 5k,l). The results showed that the 2n pollen formed from various treatments were mainly caused by abnormal spindles.

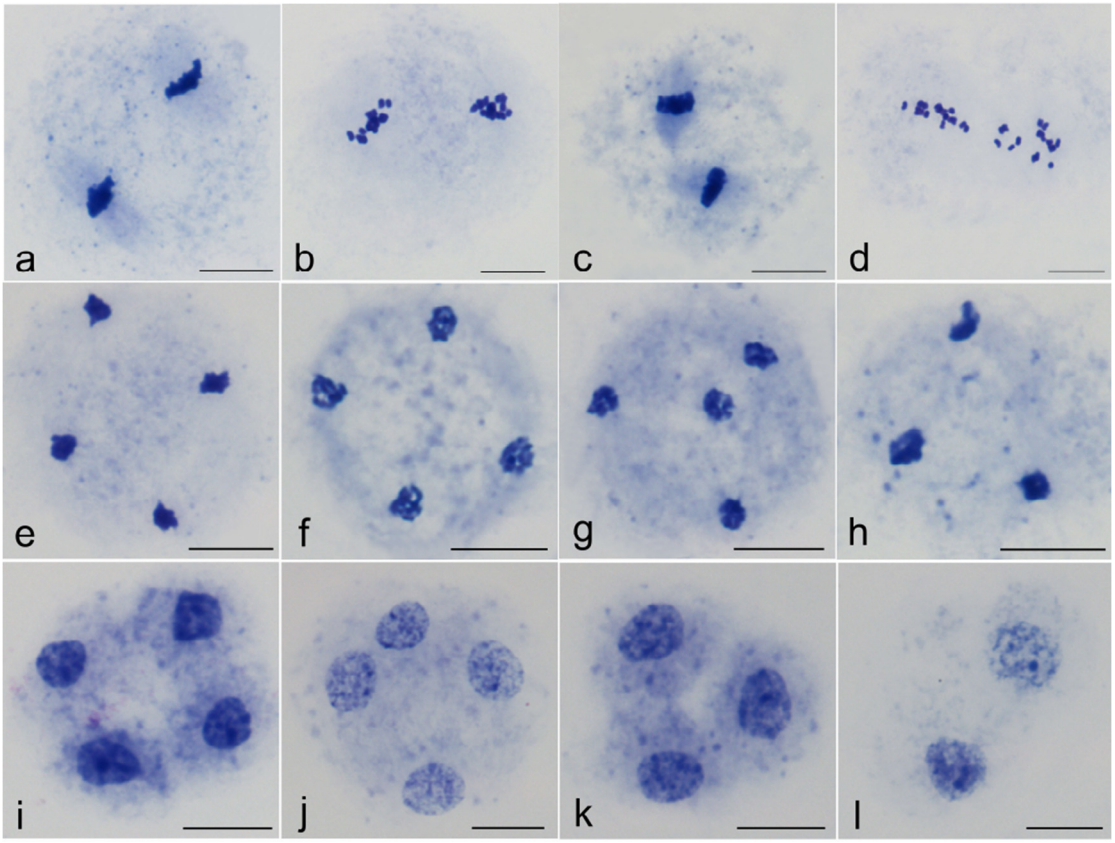

Figure 5. Observation of abnormal meiotic division of microspore mother cells. (**a**–**d**) Metaphase II showed parallel spindles, perpendicular spindles, 'Eight-type' spindles (tripolar spindle), and fused spindles, respectively. (**e**,**f**) Telophase II formed by parallel spindles. (**g**) Telophase II formed by perpendicular spindles. (**h**) Telophase II formed by tripolar spindles. (**i**) 'Matts -type' tetrads as a result of parallel spindle formation. (**j**) 'Cross-type' tetrads as a result of perpendicular spindle formation. (**k**) Triads as a result of tripolar spindle formation. (**l**) Dyads as a result of fused spindle formation. Scale bar = 10 μm.

3.3. Triploid Production

During the flowering period of female flowers of 'Shixia', the induced 2n pollen grains obtained with high-temperature treatment were used to artificially pollinate the stigma with a brush. After pollination, 884 seeds of 'SX (n)' × 'SX (2n)' were harvested, and 629 seedlings were obtained, with a germination rate of 71.2%. Four triploid plants were identified after double identification by chromosome number counting and flow cytometry (Figure 6). The induction rate of triploid was 0.6%, which was about three times that of 'SX (n) '× 'SX (n)' (Table 4). The chromosome number of the diploid 'Shixia' longan showed 30 (2n = 2x = 30) (Figure 6a), while it was 45 (2n = 3x = 45) (Figure 6b) in the triploid plants. The plant growth, leaf, and stomotal characteristics of four triploid plants (named Triploid 1, 2, 3 and 4, respectively) are shown in Supplemental Figures S1–S3 and Supplemental Table S2. There were significant differences in some leaf characteristics between triploid and diploid plants, such as petiole length, leaflet length, leaflet width, and leaflet thickness. Among them, these leaf characteristics of Triploid 3 and Triploid 4 were generally greater than that of diploid plants, which reflected the gigantism of polyploidy. However, there was no significant difference in stomatal length and width between triploid and diploid leaves. Furthermore, the chloroplast number of stomatal guard cells in leaves of the triploids was about 1.4 times that of the diploid longan (Supplemental Figure S4 and Supplemental Table S3). This index could be used as a basis for auxiliary identification of longan germplasm ploidy.

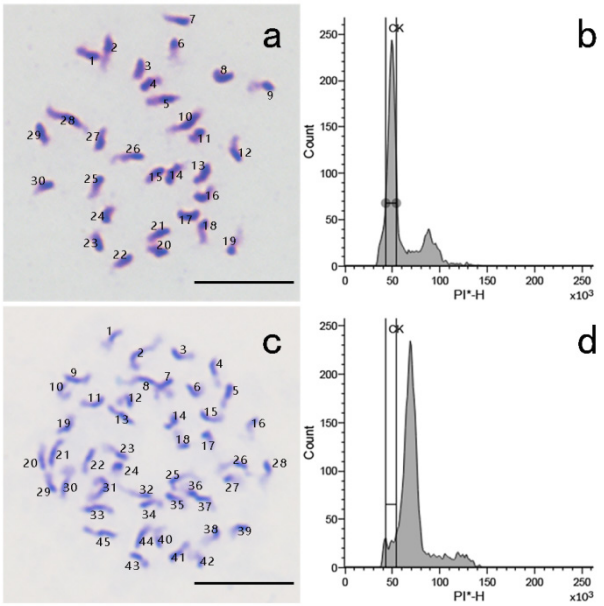

Figure 6. Detection of the ploidy level of the progenies of 'Shixia' longan generated from artificial pollination with 2n pollen. (**a**) Chromosome number of diploid root tip cells (2n = 2x = 30). (**b**) The ploidy level of diploid young leaves were detected by flow cytometry. (**c**) Chromosome number of triploid root tip cells (2n = 3x = 45). (**d**) The ploidy level of triploid young leaves was detected by flow cytometry. Scale bar = 10 μm.

Table 4. The production of triploids from artificial pollination with 2n pollen.

Female Gamete Type	Male Gamete Type	Pollinated Female Flower Number	Seed Number	Fruit Set Rate (%)	Seedling Number	Germination Rate (%)	Triploid Number	Triploid Production Rate (%)
SX (n)	SX (n)	1500	1070	71.3	950	88.1	2	0.2
	SX (2n)	1500	884	58.9	629	71.2	4	0.6

4. Discussion

Sexual polyploidization is the main way to produce polyploids in plants. After the discovery of 2n gametes, artificial sexual polyploidization has become an important means for germplasm innovation in plants. Although asexual polyploidization using microtubule inhibitors is a common method for obtaining polyploidy, it generates homogeneity of plant and leads to decline. Asexual polyploidization often induces chimeras, and the isolation of polyploids from the chimeras is complicated. In addition, some plants, such as fruit trees, do not easily undergo chromosome doubling in somatic cells with colchicine. The value of 2n gametes has been demonstrated in many genetic breeding studies of polyploid plants [36]. Studies have shown that sexual polyploidization of 2n gametes has more biological advantages, such as the higher induction frequency and higher genetic heterozygosity of polyploids than that of asexual polyploidization. The 2n gametes also have special value in transmitting heterozygosity and epistasis [17]. The 2n gametes include 2n male gametes (2n pollen) and 2n female gametes. Although the rate of induced 2n pollen is low, the use of 2n pollen can transfer high heterozygosity and favorable genes to progeny, enhancing their heterosis.

The induction frequency of 2n gametes is very low in most fruit trees under natural conditions. It is thus difficult to be directly applied to practical breeding. Therefore, researchers have used different methods to increase the induction frequency of 2n gametes, including chemical and physical treatments. An appropriate induction period is the key to improve the efficiency of gamete chromosome doubling [38]. Relevant studies have shown that the optimal treatment period for inducing 2n male gametes is from the pachytene

to diakinesis stage of microsporocyte meiosis [34]. Therefore, it is very important to accurately and easily distinguish the meiotic stage of pollen mother cells so as to obtain a high proportion of 2n gametes by doubling induction treatment. In this study, through the observation of the external morphology and anatomical structure of the male flower buds of 'Shixia' longan, the male gamete development stage could be determined by the size and morphological characteristics of the male flower buds. It was found that the appropriate induction period was when the male flower buds were in stage I and light green in color, with a diameter of 1.4–2.0 mm, sepals completely closed, and stamens and pistils clearly visible. At this stage, the microspores were in the pachytene to diakinesis stage of meiosis. This study clarified the optimal induction period and the corresponding morphological characteristics of male flower buds in longans. In addition, the 2n male gamete induction rate was affected by the synchronization of microspore meiosis. The longan flower cluster belongs to conical cyme. There are hundreds to thousands of flower buds on the main inflorescence and side inflorescence, leading to a great difference in the time of microspore mother cells entering meiosis among the male flowers. Therefore, the induction method and treatment time need to be optimized. This study showed that 2n pollen could be obtained by colchicine treatment. However, if the colchicine treatment time was too long, the flower buds would be greatly damaged and fall off. Compared with chemical treatments, high temperature has the advantages of good consistency of treatment conditions, simple operation, large number of flower buds treated at one time, and less damage to flower buds. The study showed that the 2n pollen frequency increased from 0.26% (the control) to 2.64%, induced by the treatment of heat shock of 30 °C for 12 h in loquats [39]. In our study, a high 2n pollen rate (up to 5.7%) could be obtained by applying a high-temperature treatment in longans, extending treatment time appropriately and causing less damage to flower buds.

2n pollen has two important differences from normal pollen. It has a nuclear DNA content twice that of normal pollen and a cell volume of 1.28–1.5 times larger than that of normal pollen [36,40–43]. The traditional method for identifying 2n pollen is to measure the diameter of the pollen grains under a microscope, for which the results are relatively accurate [7,43]. At the same time, the proportion of 2n pollen production can be calculated indirectly by counting the number and proportion of dyads, triads, and tetrads in the meiosis stage of the microspore mother cells [31]. Theoretically, a dyad can form two 2n pollen, a triad can form one 2n pollen and one normal pollen, and a tetrad can form four normal pollen. In this study, a large number of tetrads, as well as some dyad and triads, were observed in the meiosis process of microspore mother cells. The proportion of tetrads, triads, and dyads induced by each treatment ranged from 81.7% to 98.0%, form 8.6% to 15.9%, and from 1.0% to 2.4%, respectively, and the proportion of triads was 5–10 times higher than that of dyads, indicating that 2n pollen were mainly formed by triads. The formation of dyads and triads was related to the abnormal spindle orientation [37], which led to abnormal orientation of chromosomes in anaphase II, which in turn affected the arrangement and distribution of nuclei in telophase II, resulting in abnormal divisions. A previous study showed that after colchicine treatment, the formation of 2n pollen in 'Linglingzao' jujube was controlled by two mechanisms: abnormal cytokinesis and abnormal spindle positioning [31]. It has been found that the formation of 2n gametes is related to the abnormal spindle in peaches, European strawberries, persimmons, etc. [44,45]. Our results indicate that tripolar spindle and fusion spindle lead to the formation of 2n male gametes. This finding is important for elucidating the formation mechanism of 2n male gametes for artificial induction of 2n gametes and for polyploidy breeding in longans.

5. Conclusions

This study reported for the first time the obtaining of 2n male gametes by high-temperature treatment, which was more efficient than colchicine treatment. Through artificial pollination with induced 2n pollen, four longan triploid plants were successfully created. In addition, the development process of longan microspore mother cells was

characterized in relation to the size and morphological characteristics of male flower buds, and the optimal treatment period for the induction of 2n gametes was determined for longans. These findings are of great significance for producing longan triploids and breeding seedless varieties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8050437/s1, Figure S1: The growth of triploid and diploid plants; Figure S2: The leaf morphology of triploid and diploid plants; Figure S3: Observation of stomatal characteristics and distribution of triploid and diploid leaves; Figure S4: Observation of chloroplast number in stomatal guard cells of triploid and diploid leaves; Table S1: The morphological characteristics of male flower buds according to the meiotic process of microspore mother cells; Table S2: The leaf characteristics of triploid and diploid plants; Table S3: Determination of chloroplast number in stomatal guard cells of triploid and diploid leaves.

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