

# Cytological mechanism of $2n$ pollen formation in Chinese jujube (*Ziziphus jujuba* Mill. ‘Linglingzao’)

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**Abstract** In order to elucidate the cytological mechanism of  $2n$  pollen formation in Chinese jujube, a cultivar named ‘Linglingzao’ ( $2n = 2x = 24$ ) which produces relative more  $2n$  pollens naturally was employed for microsporogenesis analysis. Chromosomes paired in 12 bivalents at diakinesis and the first meiotic division was normal, whereas, the second division was characterized by frequent abnormal spindle orientation (parallel and tripolar spindles). Perpendicular, tripolar, and parallel spindles at metaphase II accounted for 72.93, 17.22, and 9.85% respectively. Perpendicular and tripolar spindles led to tetrads and triads formation respectively. Two types of parallel spindles were observed and a significant association correlation ( $r = 0.84$ ,  $P < 0.05$ ) between parallel spindle II (fused spindles) and dyads was found which meant only fused spindles can form dyads, while parallel spindle I with a long distance between two sets of chromosomes seemed to lead tetrad formation. At tetrad stage, the observed frequencies of tetrads, triads and dyads were 80.99, 16.40, and 2.61% respectively. By the rule that each tetrad can form four  $n$  pollens, each triad can form one  $2n$  pollen and two  $n$  pollens, each dyad can form two  $2n$  pollens,

the frequency of  $2n$  pollen estimated was 5.71%. Based on the pollen diameter difference between  $n$  and  $2n$  pollens, frequency of  $2n$  pollen observed was 6.15%. No significant difference was observed between frequency of  $2n$  pollen estimated and that of observed. The results indicated that, tripolar spindles and fused spindles at metaphase II followed by more triads and few dyads formation at tetrad stage were responsible for the production of  $2n$  pollen in Chinese jujube.  $2n$  gametes observed correspond to first division restitution. Research on the cytological mechanism of unreduced pollen will provide a platform for unreduced pollen induction and polyploidy breeding in Chinese jujube.

**Keywords** Meiosis · Triads · Dyads ·  $2n$  gametes · Polyploidization

## Introduction

Polyploidization is widespread in plants and has played a major role in the evolution and diversification of the plant kingdom. Unreduced gametes are an important tool for polyploidization and the creation of genetic variation in plant breeding (Dewitte et al. 2009, 2010a, 2010b). Polyploidy is of great value to breeders (Dewitte et al. 2010a; Gallo et al. 2007). But among the rich germplasm resources of Chinese jujube (*Z. jujuba* Mill.), ‘Zanhuangdazao’ is the unique

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natural triploid identified up to now, which has the valued attributes of big fruit, high yield and good adaptation. Most of the cultivars of ber (*Z. mauritiana* Lam.), a close-related species of Chinese jujube, are tetraploid showing similar advantages. Considering the above-mentioned, polyploid induction of Chinese jujube should have attractive prospect.

Polyploidization may be asexual through somatic chromosome doubling and sexual through the formation of  $2n$  gametes following both intra- and inter-ploidy matings (Ton et al. 1977). Asexual polyploidization (traditional chromosome duplication) is performed through the use of harmful mitosis inhibitors like colchicine, oryzalin and trifluralin (Hancock 1997; Wan et al. 1991). The major drawback of this technique is that it largely disables the recombination between chromosomes of different parents in allopolyploids. As a consequence, this form of polyploidization offers limited possibilities to create genetic variability. Sexual polyploidization is far more efficient than somatic chromosome doubling. Sexual polyploidization effected by functioning of  $2n$  gametes in the parental species or in their hybrids has been got in ornamental plants, grain crops, potato, fruit-bearing trees and some other plants (Akutsu et al. 2007; Barba-Gonzalez et al. 2004, 2005; Carputo and Barone 2005; Carputo et al. 2003; Hayashi et al. 2009; Jauhar 2003; Lyrene et al. 2003; Ramanna et al. 2003; Sugiura et al. 2000; Van Laere et al. 2009; Van Tuyl and Lim 2003; Veilleux 1985; Yanagi et al. 2010; Zhou et al. 2008).

Advantages of  $2n$  pollen in polyploidization have been proposed. Firstly, usage of  $2n$  pollen can conquer homogeneity increasing, severe recession and chimeras occurrence resulted from asexual polyploidization. Secondly,  $2n$  pollen can simply transfer advantage genes from wild species and part-cultivars into cultivars that can be used in breeding and production directly. Thirdly,  $2n$  gametes can transmit the parental heterozygosity to the offspring. The first division restitution (FDR) and the second division restitution (SDR) gametes can transfer at least 75–80% and 33–35% heterozygosity respectively (Camadro and Peloquin 1980; Peloquin et al. 2008). FDR gametes are particularly valuable in plant sexual polyploidization breeding programs (Bretagnolle and Thompson 1995; Ramanna 1983). Fourthly, using  $2n$  gametes can improve self-fruitfulness much as well as the pollen fertility of triploids. Fifthly, endosperm balance number (EBN) can be conquered by  $2n$  gametes, most

breeding experiences showed that usage of  $2n$  gametes can conquer this barrier directly and shorten the period of breeding (Johnston et al. 1980). In tuber-bearing *solanums*, it was reported that EBN in combination with  $2n$  gametes maintains the ploidy integrity of diploid ancestral species, while proving the flexibility for either unilateral or bilateral sexual polyploidization (Carputo et al. 2003). Sixthly, seedless germplasm can be obtained by using  $2n$  gametes. In citrus breeding,  $2n$  gametes have been successfully used for the production of seedless variety, triploid from  $2n$  female gametes (Geraci et al. 1975).

Unreduced gametes are of importance for basic studies in cytogenetics as well as applied plant breeding and facilitate the production of new polyploidy species. According to the previous observation on 223 Chinese jujube cultivars, only four cultivars ('Linglingzao', 'Yingluozao', 'Zanhuangchangzao', 'Zhongyangmuzao') produce relative high percentage of  $2n$  pollens and it was proposed that the diameter of  $2n$  pollen was 1.5 times of  $n$  pollen (Peng 2008). The cytological basis for the formation of  $2n$  pollen in Chinese jujube has not been clearly understood. The aim of this research was to elucidate the cytological mechanisms that lead to  $2n$  pollen formation and provide new insights on utilization of  $2n$  pollen in polyploidy breeding of Chinese jujube.

## Materials and methods

### Plant material

'Linglingzao' ( $2n = 2x = 24$ ) which can produce relative high frequency of  $2n$  pollen naturally was used for cytological analysis of meiosis. All the sampled trees were grown under regular management in the field.

### Cytological analysis of meiosis

During May 15 to 22, 2009, flower buds were collected and fixed in ethanol:acetic acid (3:1) fixative for 24 h, then transferred into 70% ethanol and stored in refrigerator at 4°C. Before observation, anthers were dissected from the buds with a dissecting needle, then microsporocytes were prepared by squashing anthers with a nipper, and stained with propanoic acid-iron-hematoxylin-chloral hydrate (PIHCH). Chromosome number was determined at

diakinesis and all phases of meiosis were observed with light microscope OLYMPUS. Photomicrographs were taken under binocular light microscope ( $\times 1000$  magnification) with image-forming system Stk 'MDC200(LY-HPCCD)'.

#### Observation of $2n$ pollen

Anthers from opening flowers were collected and squashed in a drop of 0.5% triphenyltetrazolium chloride (TTC) solution on a microscope slide. After a minimum of 1 h, the preparation was observed under a binocular light microscope ( $\times 100$  magnification). Viable pollen grains were stained into full red, whereas aborted pollen grains were unstained. More than 5000 pollen grains were measured. Pollen which is 1.5 times of  $n$  pollen in diameter was regarded as  $2n$  pollen.

#### Estimation of the frequency of $2n$ pollen

The number of dyads, triads, and tetrads were counted respectively. The number of observed dyads, triads, and tetrads during meiosis enabled an evaluation of the theoretical frequency of  $2n$  pollen. Based on the rule that each dyad led to two unreduced pollen grains, each triad produced one unreduced pollen grain and two reduced pollen grains, and each tetrad gave rise to four reduced pollen grains, the frequency of  $2n$  pollen (F) was estimated as below:

$$F = \frac{2D + Tr}{2D + 3Tr + 4Te} \times 100\%$$

$D$  = number of dyads,  $Tr$  = number of triads,  $Te$  = number of tetrads.

#### Statistical analysis

Correlation analysis was carried out with Statistical Product and Service Solutions version 16 (SPSS Inc, USA).

## Results

#### Microsporogenesis

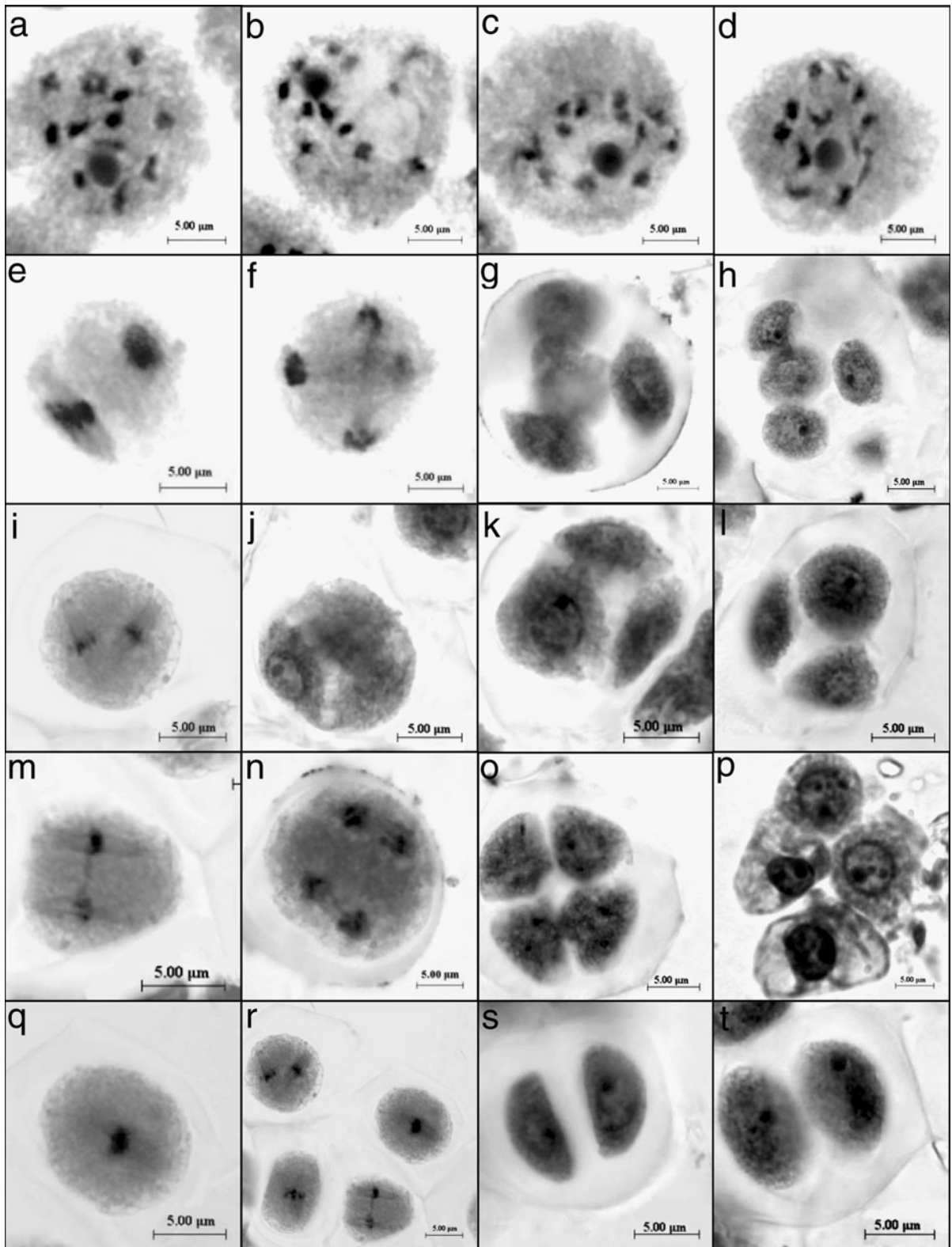
During microsporogenesis, chromosomes paired in 12 bivalents at diakinesis (Fig. 1a–d) and first

meiosis was normal. However, abnormal spindle orientation (parallel and tripolar spindles) were observed at metaphase II (Fig. 1i, m, q). The frequencies of perpendicular, tripolar and parallel spindle were 72.93, 17.22, and 9.85% respectively (Table 1). While the frequencies of tetrad (Fig. 1g, h), triad (Fig. 1k, l) and dyad (Fig. 1s, t) were 80.99, 16.40, and 2.61% respectively at tetrad stage (Table 1). The correlation analysis showed that there was a significant association ( $r = 0.98$ ,  $P < 0.01$ ) between the frequency of tripolar spindle and that of triad (Table 2), which indicated that tripolar spindles resulted in triads. The correlation between parallel spindle and dyad and that between perpendicular spindle and tetrad were not significant, which indicated that not all the parallel spindles resulted in dyads formation.

Actually, two types of parallel spindles were observed. For parallel spindle I (Fig. 1m), a long distance between the two sets of chromosomes led to tetrads formation (Fig. 1o). Differed with the regular perpendicular tetrads, the tetrads resulted from the parallel spindle I were arranged at the same division plane. For parallel spindle II (fused spindle), the two sets of chromosomes were very close, even integrated with each other (Fig. 1q). Fused spindles were similar to spindles at metaphase I. However, fused spindles could be distinguished based on the synchronization of meiosis (Fig. 1r). The correlation analysis showed a significant association ( $r = 0.84$ ,  $P < 0.05$ ) between the frequency of parallel spindle II and dyads (data not show). This result further indicated that fused parallel spindles (Fig. 1q) led to dyads formation (Fig. 1s, t).

#### $2n$ pollen formation

Each triad gave rise to one  $2n$  microspore and two  $n$  microspores, while each dyad produced two  $2n$  microspores.  $2n$  microspore formed  $2n$  pollen grain. The frequencies of  $2n$  pollen from different types of sporads were estimated (Table 3). The average frequency of  $2n$  pollen from both dyad and triad was estimated as 5.71%, which was not significantly different with the observed one, 6.15% (Table 3). It indicated that the  $2n$  pollens in 'Linglingzao' are resulted from the formation of triads and dyads at tetrad stage which originated from the abnormal spindle orientation at metaphase II.



◀ **Fig. 1** Dyads and triads formation of Chinese jujube (*Ziziphus jujuba* Mill. ‘Linglingzao’). Bar 5  $\mu\text{m}$ . **a–d** Diakinesis with 12 bivalents. **e** Normal perpendicular spindles. **f** Anaphase II with two sets of chromosomes being pulled to four poles. **g–h** Tetrads as a result of perpendicular spindles formation. **i** Tripolar spindles at Metaphase II. **j–l** Triads as a result of tripolar spindles formation. **m** Parallel spindle I with a distance between two sets of chromosomes. **n** Anaphase II with sets of chromosomes being separated. **o** Tetrads resulted from parallel spindle I. **p** Tetraspore. **q** Parallel spindle II (Fused spindle). **r** Tripolar, parallel and fused spindle. **s–t** Dyads following the formation of fused spindles

## Discussion

The formation of  $2n$  gametes is a common phenomenon in the plant kingdom.  $2n$  gametes may result from a variety of different cytological abnormalities (Bretagnolle and Thompson 1995; Harlan and De Wet 1975; Veilleux 1985).

Five main cytological mechanisms of  $2n$  gametes formation were summarized: (i) premeiotic chromosome doubling during the process from mitosis to meiosis, disturbances appeared and resulted in chromosomes of archesporial cell being doubled followed by  $2n$  gametes formation; (ii) meiosis disturbances, most  $2n$  gametes were formed by this reason. Meiosis abnormalities led to  $2n$  gametes of different types, second division with crossing-over (SDR-CO), first division with crossing-over (FDR-CO), first division

restitution without crossing-over (FDR-NCO), transmitting  $\pm 40$ ,  $\pm 80$ , 100% of the parental heterozygosity to the offspring, respectively (Crespel et al. 2002; El Mokadem et al. 2002); (iii) abnormal cytokinesis, in *Paspalum*, 52 accessions presented failure of cytokinesis during first or second meiosis and resulted in dyads and triads formation respectively, followed by  $2n$  pollen formation (Pagliarini et al. 1999). In *Brachiaria*, it was reported that the failure of cytokinesis at metaphase I and total absence of cytokinesis resulted in unreduced pollen formation (Gallo et al. 2007); (iv) desynaptic mutants, desynaptic mutants of diploid potato ( $2n = 2x = 24$ ) were found to produce a fairly high frequency of fertile  $2n$  pollen grains (Ramanna 1983); (v) abnormal spindles of meiosis, this often happened in simultaneous dicotyledonous plants. Parallel or tripolar spindles during metaphase II of microsporogenesis were responsible for the production of  $2n$  pollen. Triads formation resulted in  $2n$  pollen formation in Brazilian ginseng (Taschetto and Pagliarini 2003) and rose (Crespel et al. 2006). In *Populus tomentosa* Carr, parallel, fused and tripolar spindles were considered to be leading to  $2n$  pollen formation (Zhang and Kang 2010; Zhang et al. 2009). Parallel and tripolar spindles have been reported leading to  $2n$  pollen formation in the wild potato *Solanum okadae* (Camadro et al. 2008).

**Table 1** Frequency of different type spindles and sporads in ‘Linglingzao’

Observations	Different spindles at metaphase II				Different sporads at tetrad stage			
	Total MMC analyzed	Parallel spindle (%)	Tripolar spindle (%)	Perpendicular spindle (%)	Total sporads analyzed	Dyad (%)	Triad (%)	Tetrad (%)
1	714	8.54	18.49	72.97	755	2.52	17.88	79.60
2	150	9.33	16.00	74.67	251	2.39	15.14	82.47
3	1,200	10.75	17.17	72.08	1,048	2.77	16.51	80.72
4	1,046	10.80	17.21	71.99	542	2.77	16.05	81.18
Average	649	9.85	17.22	72.93	5,333	2.61	16.40	80.99

MMC microspore mother cell

**Table 2** Correlation between the frequencies of different types of spindles and sporads

Correlation coefficient	Dyad	Triad	Tetrad
Parallel spindle	0.84	−0.40	0.25
Tripolar spindle	0.25	0.98**	−0.98**
Perpendicular spindle	−0.95*	−0.45	0.58

\*  $P < 0.05$ ; \*\*  $P < 0.01$

**Table 3** Frequency of  $2n$  pollen observed and estimated

Observations	Estimated frequency of $2n$ pollen (%)			Total pollens observed	Observed frequency of $2n$ pollen (%)
	From dyads	From triads	From (dyads + triads)		
1	1.33	4.74	6.07	5,333	7.20
2	1.26	3.98	5.24	5,150	6.07
3	1.46	4.37	5.83	5,233	5.85
4	1.46	4.24	5.70	5,616	5.47
Average	1.38	4.33	5.71	5,333	6.15

Unreduced gametes formation of Chinese jujube was due to the abnormal configuration of spindles according to this research. Detailed studies have been made on pollen mother cells (PMCs) at all stages of meiosis to determine whether or not meiotic disturbances were responsible for  $2n$  gametes formation. During the observation of microsporogenesis of Chinese jujube, chromosomes paired in 12 bivalents at diakinesis and the first meiotic division was normal, whereas the second division was characterized by frequent abnormal spindle orientation (parallel and tripolar spindles), as have been reported in some plants (Bretagnolle and Thompson 1995; Camadro et al. 2008; Veilleux 1985; Xu et al. 2008). However, the occurrence of parallel spindles at the second division does not necessarily mean that  $2n$  pollen has been formed (Ramanna 1979; Tavoletti et al. 1991). The ‘fused spindle’ at metaphase II has been associated with the formation of dyads (Ramanna 1983; Veilleux et al. 1982; Xu et al. 2008). In this study, a significant association correlation ( $r = 0.84$ ,  $P < 0.05$ ) between fused spindles and dyads was found. This meant only parallel spindle II (fused spindle) can form dyads thereby result in  $2n$  gametes, while parallel spindles I (with a long distance between two sets of chromosomes) seemed to be leading tetrad formation followed by  $n$  gametes in Chinese jujube.

Most researches indicated that formation of  $2n$  gamete was controlled by genes (Calderini and Mariani 1997; Katsiotis and Forsberg 1995). d’Er-furth et al. (2008) isolated and characterized a gene (*AtPSI*) involved in the abnormal orientation of spindles at meiosis II that controlled  $2n$  gamete formation apparently in *Arabidopsis thaliana*. It was reported that the mechanism of  $2n$  pollen formation of *Solanum* is under control of Mendelian recessive *ps* (Camadro et al. 1980). Jumbo pollen formation in

alfalfa is dependent on the homozygous recessive condition of the gene *jp* that causes complete failure of post-meiotic cytokinesis which results in 100% of  $4x$  pollen (Tavoletti et al. 2000). Desynapsis was found to be controlled by a single recessive gene *ds* (Ramanna 1983). Although the production of  $2n$  gametes is under genetic control, it can be also affected by environmental conditions. The variation of  $2n$  pollen production could be related to environmental fluctuations (Crespel et al. 2006).

Depending on the cytological mechanisms involved in the production of  $2n$  gametes in Chinese jujube, such gametes are generally categorized as being equivalent to FDR. FDR gametes could transmit at least 75% of parental genotype to their progenies. Further research is needed to reveal the actual level of heterozygosity of the unreduced gametes and their contributions to the progeny.

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