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In Vivo Fast Induction of Homogeneous Autopolyploids via Callus in Sour Jujube (*Ziziphus acidojujuba* Cheng et Liu)

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Abstract

Polyploidization has been demonstrated as a very effective approach in fruit tree improvement. Sour jujube (*Ziziphus acidojujuba* Cheng et Liu) is a promising diploid wild, traditional fruit species (2n = 2x = 24) that is rich in vitamin C, which is the main rootstock of Chinese jujube (*Z. jujuba* Mill.). The novel method we developed for rapid *in vivo* induction of homogeneous autopolyploids (IVIHA) via callus in Chinese jujube was first applied and further optimized in sour jujube. Under optimized conditions, an average of one pure autotetraploid shoot could be regenerated from one treated branch, thereby indicating a relatively high efficiency rate. A total of 9 pure autotetraploid genotypes were created, and one of these was released as a new cultivar named 'Zhuguang' in 2015. Moreover, unexpected octoploids and hexaploids were also simultaneously created and detected. The leaves of tetraploids were thicker, broader, and darker in color than those of the original diploids, whereas the leaf sizes of octoploids were much smaller compared to that of diploids. However, stoma size increased with the occurrence of ploidy, mainly from diploid to octoploid. The well grown ploidies of jujube included diploids, triploids, and tetraploids. Anatomical observation indicated that adventitious buds/shoots emerged from the callus that formed on the cut, which was then followed by the development of connective vascular tissues between the adventitious bud and the stock plant tissue. This study demonstrates the universality of the IVIHA method that was initially developed in Chinese jujube, as well as provides a foundation for high-efficiency pure polyploid induction in sour jujube.

Keywords: sour jujube; *in vivo*; callus; homogeneous autopolyploid; tetraploid; hexaploid; octoploid

1. Introduction

Polyploidization is a highly effective method of fruit tree improvement. Polyploid fruit cultivars generally possess favorable horticultural characteristics such as large fruit size, sturdiness, high productivity, better disease-resistance, and minimal to negligible seeds (Predieri, 2001; Gu et al., 2005; Reforgiato et al., 2005; Shi et al., 2012). Tetraploids have been induced in a number of fruit trees such as grape (*Vitis vinifera* L.) (Yang et al., 2006), citrus (*Citrus sinensis* Osbeck) (Zeng et al., 2006), and Chinese jujube (*Ziziphus jujuba* Mill.) (Jiang and Liu, 2004; Gu et al., 2005; Wang and Liu, 2005), and most tetraploids show better economically significant characters.

Sour jujube or wild jujube/acid jujube (*Ziziphus acidojujuba* Cheng et Liu–*Z. jujuba* Mill. var. *spinosa* Hu) is the wild ancestral species and the main rootstock of Chinese jujube (*Z. jujuba* Mill.), which is an important cultivated fruit tree native to China (Liu, 2006; Liu and Wang, 2009). Compared to Chinese jujube, the fruit of sour jujube is much smaller but much richer in organic acids, particularly vitamin C. The vitamin C content of sour jujube fruit on average is 5.54 mg · g^{-1} FW and could reach 13.93 mg · g^{-1} FW, which is > 100 fold higher than that of common fruits (Yang et al., 2002; Liu et al., 2009). In addition, sour jujube has outstanding fruit setting ability and adaptability. Tetraploid and octoploid sour jujube have been obtained through *in vitro* shoot induction with colchicine (Wang and Liu, 2005; Lu et al., 2010). However, this method also has some disadvantages, i.e., unavoidable formation of mixploids, time-consuming purification of mixploids, and an extended period of development starting from a test-tube plantlet to blooming in the field. To circumvent these problems, we developed a system for rapid *in vivo* induction of homogeneous

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autopolyploids (IVIHA) in Chinese jujube, after several years of explorations (Shi et al., 2015a, 2015b). The novel IVIHA method addresses the problem of mixploid purification and generates pure polyploid shoots at around 1 month in the open field that later bloom within the experimental year. In the present study, we successfully applied and further optimized the novel method in sour jujube, and obtained pure tetraploids from 9 diploid genotypes. Furthermore, we compared the characteristics of leaves with different ploidies and performed a preliminary anatomical observation of adventitious bud regeneration during polyploid induction. This study further demonstrates the universality of the IVIHA method, which was initially developed using Chinese jujube, as well as provides a foundation for high-efficiency pure polyploid induction in sour jujube.

2. Materials and methods

2.1. Plant material

A total of 37 genotypes of sour jujube (Table 1) were employed to evaluate the feasibility of the rapid IVIHA, which was initially developed using Chinese jujube (a very close relative of sour jujube) for sour jujube. Of these, 'Xingtai 0605' was chosen for further optimization of factors influencing polyploid induction efficiency. All the sample trees were routinely managed at the Xianxian Jujube Experimental Station of the Agricultural University of Hebei, China, which is located at a 38°48' north latitude, with an average annual temperature of 12 °C, and an annual precipitation of 550 mm.

2.2. Optimization of in vivo polyploid induction technique

The procedure of IVIHA that was initially developed using Chinese jujube was as follows: select strong branches and clip these during the growing season. Add 2 mL of a solution containing 4 mg · L^{-1} TDZ and 2 mg · L^{-1} $AgNO_3$ onto the cambium of the cut section and then cover the cut section with plastic film. After 14 h, replace the cover with humid mud and plastic film to retain moisture to induce the regeneration of adventitious calluses and buds. After five days, treat the newly formed calluses with colchicine dissolved in 1% DMSO for 14 h. Remove the cover once the regenerated new shoots reach a height of 2 cm (Shi et al., 2015a).

Based on the procedure of IVIHA, two tests were conducted to further optimize the technique for sour jujube.

Test A: Branches of 1 to 3-year-old and 4 to 5-year-old trees were respectively employed to induce callus. On the 5th day after callus induction, 2 mL of 0.050% colchicine dissolved in 1% dimethylsulfoxide (DMSO) was added onto the calluses that were regenerated from the cambium of branch cut section.

Test B: On the 5th day after callus induction, when tiny calluses could be observed around the cambium of the cut section, 2 mL of colchicine at a concentration of 0, 0.050%, or 0.075% and dissolved in 1% DMSO was respectively added drop-wise onto the calluses.

In Tests A and B, each treatment has 9–12 replicates. After colchicine treatment, the calluses were covered with black plastic film. Fourteen hours later, the calluses were rinsed with distilled water to remove the un-absorbed colchicine and then covered with humid mud and plastic film to retain moisture. The coverings were removed about 50 days after callus induction in case the length of the new shoots that regenerated from the callus had reached more than 2 cm. Another 20 days later, the number and morphological characteristics of the regenerated young shoots were observed and recorded.

2.3. Ploidy detection and comparison among materials with different ploidies

New shoots regenerated from calluses were sampled by mixing up their secondary shoot apex. Its ploidies were detected by flow cytometry (FCM) as reported elsewhere (Jiang and Liu, 2004; Wang and Liu, 2005). The ploidy data were processed with MoFlo™ XDP (Beckman Coulter, Inc., Brea, CA, USA).

The characteristics of the mature leaves, including color, length, width, and shape index (length/width), were visually examined and measured using a ruler. The stomatal characteristics, including density, length, width, and shape index, were analyzed by using the method described by Shi et al. (2015a).

Table 1 Polyploid induction of 37 sour jujube genotypes

Genotype	Number of buds regenerated per branch	Ratio of polyploid buds /%	Genotypes	Number of buds regenerated per branch	Ratio of polyploid buds /%
Xingtai 0605	8.33	24.00 (4x, 6x, 8x, 2x + 3x + 4x)	Beiqi 6	0.33	0
Xingtai 0604	6.86	6.25 (4x)	Suanzao 8	0.33	0
Xingtai 0641	5.50	4.55 (4x)	Xingtai 0630	0.25	0
Suanzao 21	5.00	0	Xingtai 0602	0.25	0
Suanzao 17	4.50	5.56 (4x)	Ziyuanpu Y	0.20	0
Ziyuanpu D	4.33	23.07 (2x + 4x)	C16	0	0
Beiqi 11	4.00	25.00 (4x)	D12	0	0
Xingtai 0648	4.00	0	Jin 3	0	0
Xingtai 0610	3.00	16.67 (4x, 2x + 4x)	Suanzao 14	0	0
Xingtai 0608	3.00	16.67 (4x)	Suanzao 15	0	0
Xingtai 0618	2.60	7.69 (4x)	Suanzao 20	0	0
Ziyuanpu 8	2.33	28.57 (4x)	Suanzao 28	0	0
Suanzao 3	1.50	33.33 (2x + 4x)	Suanzao 38	0	0
Suanzao 6	1.50	0	Suanzao 45	0	0
Xingtai 0601	1.40	0	Xingtai 0619	0	0
Beiqi 4	1.33	0	Xingtai 0701	0	0
Suanzao 27	1.00	0	Ziyuanpu 2	0	0
Suanzao 11	1.00	0	Ziyuanpu 28	0	0
Suanzao 12	0.50	0			

Fig. 1 The process of *in vivo* polyploid induction in sour jujube 'Xingtai 0605'
(A) Callus (arrow) initiated from a branch cut section; (B) a callus grew and buds (arrow) regenerated; (C) new shoots grew out of the callus; (D) diploid shoot/leaf; (E) tetraploid shoot/leaf; (F) hexaploid shoot/leaf; (G) octoploid shoot/leaf (arrow).

2.4. Anatomical observations of bud regeneration

Anatomical observations were conducted to determine how adventitious buds originated from callus tissues using the method of Shi et al. (2015a). Paraffin sections were examined and imaged under an Olympus BX41 microscope.

2.5. Data analysis

The data were analyzed using the SPSS 17.0 statistics package. Duncan's multiple range test was used to assess differences among treatments, with significance defined as $P < 0.05$.

3. Results

3.1. Morphogenesis of autopolyploids during in vivo induction

Calluses that regenerated from the cambium of the cut sections were observed at 5 days after callus induction (Fig. 1, A). Then the calluses rapidly developed until about 20 days after callus induction, and tiny yellow buds (Fig. 1, B) were visible on callus circle. The adventitious buds rapidly grew, and the regenerated new shoots grew to an approximate length of 2 cm another 20 days later (Fig. 1, C). As the adventitious buds developed into shoots, some of these underwent morphological differentiation (Fig. 1, E–G) to the diploid control (Fig. 1, D). Compared to Chinese jujube, sour jujube regenerated buds 3–5 days earlier.

3.2. The influences of genotype on polyploid induction

A total of 37 genotypes of sour jujube were employed to induce polyploid using the IVIHA approach that was developed using Chinese jujube. The results (Table 1) showed that calluses could regenerate from cut sections of branches in all genotypes tested, but the genotype largely influenced adventitious bud regeneration (0–8.33 buds per branch) and polyploid induction ratio (0–33.33%). Table 1 shows that nearly 64.9% of the genotypes could regenerate adventitious buds from calluses, 29.7% of the genotypes produced polyploids, and 24.3% of the genotypes resulted in pure tetraploids. Further comparison indicated that among the 11 genotypes that regenerated polyploids, approximately 81.8% produced pure tetraploids. These results indicated that the IVIHA approach has the highest universality on callus induction among the genotypes (calluses were observed in all tested genotypes), followed by adventitious bud induction, polyploidy induction, and homogeneous polyploid induction.

3.3. Optimization of the in vivo polypoid induction technique

To determine the optimal branch age for *in vivo* polyploid induction, the regeneration potential of branches of different ages were compared. Table 2 shows that it was relatively easier to regenerate adventitious buds and obtain polyploids using branches of 1 to 3-year-old trees compared to those of 4 to 5-year-old

Table 2 The effects of branch age on polyploid induction in sour jujube 'Xingtai 0605'

Branch age	Average diameter of branch /mm	Number of buds regenerated per branch	Ratio of ploid-varied buds /%	Number of ploid-varied bud per branch	Pure tetraploid buds per branch
4 to 5-year-old branch	31.70	1.33	25.00	0.33	0.33
1 to 3-year-old branch	16.17	5.67	41.20	2.33	1.00

Table 3 Effects of colchicine concentrations on polyploid induction in sour jujube 'Xingtai 0605'

Colchicine concentration /%	Average number of buds per branch	Percentage of buds with different ploidies /%				
		Diploid (2x)	Tetraploid (4x)	Hexaploid (6x)	Octoploid (8x)	Mixploid (2x + 3x + 4x)
0.050	8.33	76.0	8.0	4.0	8.0	4.0
0.075	4.00	66.7	16.7	0	16.7	0
0	7.00	100.0	0	0	0	0

Note: Average number of buds per branch was recorded at 50 days after colchicine treatment; ploidy was identified via flow cytometry.

branches. Young branches of 1–3 years of age regenerated more than 5 buds per branch, which was almost 4 fold higher than that observed in 4 to 5-year-old branches. Furthermore, the ratio of ploid-varied bud was much higher in younger branches (41.20%) compared to that in older branches (25.00%). On average, one pure tetraploid shoot could be acquired from one section of a 1 to 3-year-old branch. These findings indicate that branch age largely influenced both number of regenerated buds/shoots and polyploid induction efficiency, which in turn might be caused by differences in nutrition or hormone levels among branches of different ages.

Colchicine concentration played an important role in *in vivo* polyploid induction. The two colchicine treatments (0.050% and 0.075%) induced a relatively high percentage of polyploids (24.0% and 33.4%). Compared to 0.075% colchicine, 0.050% colchicine was less toxic and regenerated a higher number of buds (8.33 buds per branch). On the other hand, 0.075% colchicine showed a better induction percentage of polyploids. Therefore, the average number of pure tetraploid buds per branch after 0.050% colchicine treatment was similar to that of 0.750%. Colchicine treatment of the calluses also unexpectedly regenerated tetraploids, octoploids, hexaploids, and mixploids (2x + 3x + 4x) (Table 3). A higher level of ploidy variation was obtained from the treatment with 0.050% colchicine compared to that with 0.075% colchicine.

Based on the results of the present study, the optimal conditions of the *in vivo* polyploid induction approach were refined by choosing 1 to 3-year-old strong branches and using 0.050%–0.075% colchicine.

3.4. Comparison of different ploidies

The ploidies were detected by FCM (Fig. 2). Tetraploid shoots showed normal growth patterns in the field, whereas hexaploids and octoploids presented a slower growth rate and were unable to undergo lignification. Compared to diploids, tetraploids developed bigger and broader leaves (Fig. 3, Table 4). Hexaploids showed smaller leaves than diploids (Fig. 3), and the leaves of octoploids were relatively smaller (Fig. 3). However, the size of the stomata was significantly bigger with higher ploidy, namely, from diploid to octoploid (Table 4, Fig. 4).

3.5. Anatomical observation of bud regeneration from callus

Compared to Chinese jujube (Shi et al., 2015a), the adventitious buds of sour jujube underwent earlier differentiation from the callus. At about 5 days after callus induction, the callus was almost uniform and some vascular tissues were observed (Fig. 5,

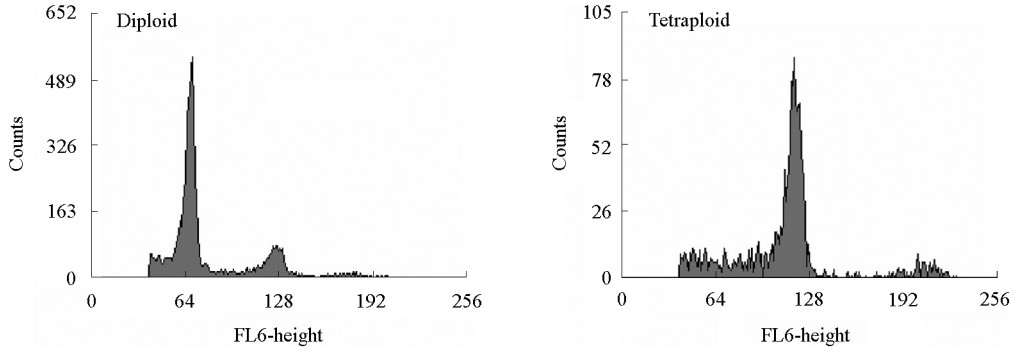

Fig. 2 Ploidy detection of diploid and tetraploid sour jujube 'Xingtai 0605' by FCM

Table 4 Comparison of leaves of diploid, tetraploid, hexaploid, and octoploid sour jujube 'Xingtai 0605'

Character	Leaf color	Leaf length/cm	Leaf width/cm	Leaf index (length/width)	Length of stomata /μm	Width of stomata /μm	Stoma index (length/width)
Diploid	Green	4.30 ± 0.45 b	2.29 ± 0.27 b	1.88	29.41 ± 2.87 d	21.89 ± 1.46 d	1.34
Tetraploid	Dark green	5.31 ± 0.69 a	3.76 ± 0.62 a	1.41	43.01 ± 4.32 c	27.47 ± 1.48 c	1.57
Hexaploid	Dark green	4.12 ± 0.04 b	2.50 ± 0.03 b	1.65	46.06 ± 5.45 b	32.43 ± 2.39 b	1.42
Octoploid	Light green	2.14 ± 0.01 c	0.97 ± 0.04 c	2.21	50.92 ± 8.27 a	36.92 ± 7.42 a	1.38

Note: Different letters in the same column indicate difference at 5% level.

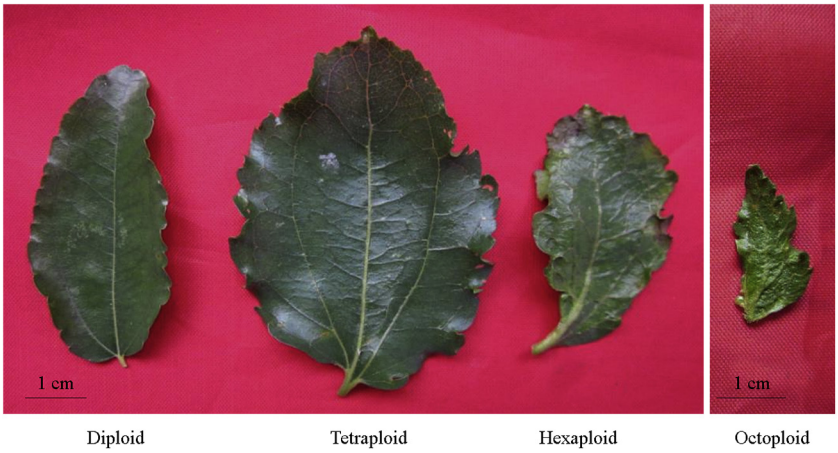

Fig. 3 Leaf of a diploid, tetraploid, hexaploid, and octoploid sour jujube 'Xingtai 0605'

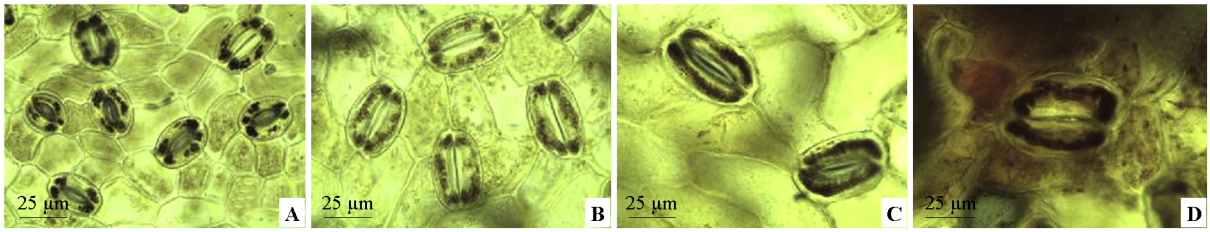

Fig. 4 Stoma of a diploid (A), tetraploid (B), hexaploid (C), and octoploid (D) sour jujube 'Xingtai 0605'

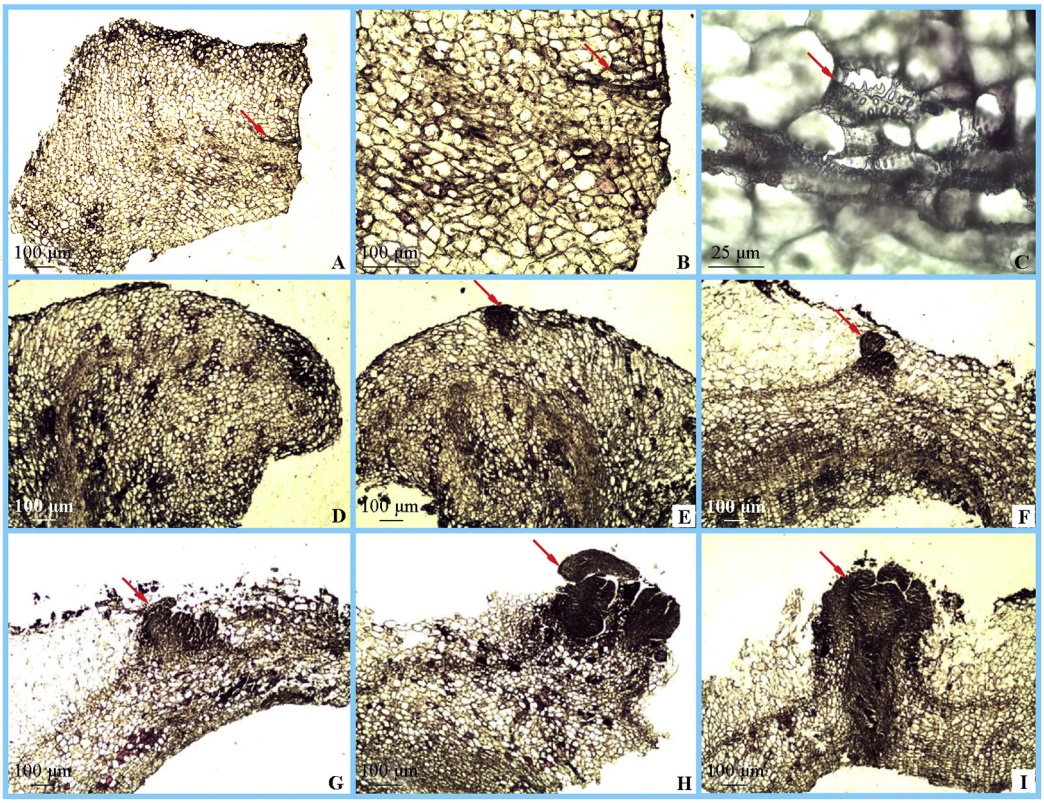

Fig. 5 Paraffin sections of regenerating adventitious buds of sour jujube 'Xingtai 0605'

(A–C) Five days after callus induction, vascular tissues (showed by arrows) emerged; (D) 10 days after callus induction, vascular tissues showed distinct structural organization, whereas other cells showed initial signs of differentiation; (E) 10 days after callus induction, a few adventitious buds (shown by arrow) emerged at the surface of the callus; (F, G) adventitious bud (shown by arrow) derived from the inner callus at 15 days after callus induction; (H) vascular tissues developing from the adventitious bud (shown by arrow) to stock plant; (I) adventitious buds (shown by arrow) totally connected to the stock plant via vascular tissues.

A–C). At about 10 days after callus induction, the callus showed faster growth. Furthermore, the vascular tissues apparently showed more distinct structural organization, whereas the other cells presented initial signs of differentiation (Fig. 5, D). A few adventitious buds formed at the surface of callus (Fig. 5, E). At about 15 days after callus induction, some adventitious buds emerged from the inner callus (Fig. 5, F–G). Then the connective vascular tissue developed further between the adventitious bud and the stock plant tissue (Fig. 5, H). Eventually, the adventitious bud established a complete connection with the stock plant (Fig. 5, I). The anatomical observations of bud regeneration indicated that no vascular tissue was initially present between the adventitious bud and the stock branch. The adventitious buds were generated from the induced callus cells instead of the original stock tissue. The adventitious buds formed 10 days after callus induction in sour jujube 'Xingtai 0605', which was 5 days earlier than that observed in 'Dalilongzao' (Shi et al., 2015a). In sour jujube, adventitious buds emerged from both inner and surface of the callus, whereas in Chinese jujube, these originated only from surface of the callus (Shi et al., 2015a).

4. Discussion

Conventional polyploid induction approaches face various challenges such as the formation of mixploids and the expensive and time-consuming process of mixploid purification (Shao et al., 2003; Yang et al., 2006; Li et al., 2007). We developed a rapid system for the IVIHA in Chinese jujube, which circumvents the formation of mixploids (Shi et al., 2015a). In the present study, 9 pure tetraploids (accounting for 24.3% of the total genotypes tested) were obtained from 37 genotypes of sour jujube using IVIHA, and one of these was released as a new cultivar named 'Zhuguang' in 2015 (JI S-SV-ZJ-015-2015). To date, we have generated pure polyploids from 12 cultivars of Chinese jujube, 9 genotypes of sour jujube and 1 genotype of elm (data not shown) using this novel method, indicating that the IVIHA approach may be applicable to other species/genotypes.

In the present study, octoploid sour jujube, in addition to tetraploids, was obtained. Hexaploids were also detected, which were not observed in our previous investigation involving Chinese jujube polyploid induction. Hexaploids have been reported in *Citrus* polyploid breeding (Zeng et al., 2006). We once obtained pentaploids during polyploid induction from a diploid in Chinese jujube (data not shown). Triploids were also detected in the polyploid breeding process in oil palm (Madon et al., 2005), pear (Sun et al., 2009), and Chinese jujube (data not shown). Normal mitosis could not explain this phenomenon. It is possible that abnormal mitosis occurred during polyploid induction, which in turn resulted in cells losing or gaining an entire chromosome set, thereby forming polyploids.

Both hexaploid and octoploid sour jujube buds showed slow growth rates and died around 1 month later. Diploids are considered as the predominant ploidy state, and tetraploids show normal growth patterns in both Chinese jujube (Liu et al., 2010) and sour jujube (Wang and Liu, 2005). The two natural triploid cultivars, Chinese jujube 'Zanhuangdazao' and 'Pingguozao', also show superior cultivation characteristics. Tetraploids and diploids naturally predominate in Indian jujube (*Z. mauritiana* Lam.), which is another important species of genus jujube (*Ziziphus* Mill.). The findings of the present study indicate that diploid, triploid, and tetraploid sour jujubes could be potentially used as routine propagated cultivars.

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