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RESEARCH ARTICLE

Haploid Induction *via In vitro* Gynogenesis in Tomato (*Solanum lycopersicum* L.)

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Abstract

In order to determine the potential for haploid induction *via in vitro* gynogenesis in tomato, the ovules and protoplasts of embryo sacs from the hybrids Zhongza 101 and Zhongza 105 were cultured. An efficient method of ovule isolation was established in this study. Using this method, 100-150 ovules could be isolated from one ovary. Isolated ovules were cultured on three induction media to induce gynogenesis *in vitro*. During culture, ovules were enlarged markedly, with opaque white color. When observed microscopically, there were cell divisions and cell clumps in embryo sacs. Subsequently, the cell clumps in embryo sacs ceased growth, likely because the integument grew faster than embryo sacs did and hindered the further development of embryo sacs. Therefore, subsequent callus morphogenesis might be originated from the integument. Thousands of calli from the two tomato varieties were obtained. Five diploid plants were regenerated after 15 months of subculturing. To eliminate the hindering effect of integument on embryo sac cells, the protoplasts of embryo sacs were prepared and cultured. After 48 hours of culture, the protoplasts of embryo sacs doubled in size and gradually formed clusters of cells. These results suggested that gynogenesis might be a potential way for haploid induction in tomato.

Key words: *Solanum lycopersicum*, ovule, protoplast of embryo sac, macrospore, *in vitro* gynogenesis, haploid

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the world's most important vegetable crops. Tomato haploids have a particular significance for tomato breeding, especially for the development of molecular markers, genetic map construction, gene mapping, and gene cloning. *In vitro* androgenesis and gynogenesis are two approaches often used for haploid induction in plants. The androgenesis approach, using anther and microspore cultures, has been widely employed in barley (Kuhlmann and Foroughi 1989), wheat (Touraev *et al*. 1996), maize (Gaillard *et al*. 1991), rice (Genovesi and Clint 1979), tobacco (Barinova *et al*. 2004), rape (Lichter 1982), cabbage (Arnison and Keller 1990), eggplant (Li *et al*. 2008), garlic (Suh and Park 1986), and watermelon (Wang 2008). Gynogenesis is defined that it comprises the development of sporophytes from an unfertilized element of the female gametophyte. Since the cells of the embryo sac are haploid, the plants derived from them are also haploid (Sant and Prem 2013). The gynogenesis approach includes the culture of non-fertilized ovaries, ovules, or female gametophytes. It has been used successfully in wheat (Zhu *et al*. 1981), rice (Zhou and Yang 1981), tobacco (Zhu

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and Wu 1979), sugar beet (Hosemans and Bossoutrot 1983), *Gerbera* (Sitbon 1981; Meynet and Sibi 1984), potato (Tao 1985), *Allium* (Campion and Alloni 1990; Keller 1990), carrot (Agnieszka and Adela 2010), and cotton (Stella and Demetrios 2009).

Despite the success of haploid production in many species, it has been difficult to achieve in tomato. Sharp *et al*. (1971, 1972) were the first experts who obtained haploid calli and roots through tomato anther culture. Gresshoff and Doy (1972) evaluated the androgenesis ability of 43 tomato lines. They could only obtain calli from three lines, and shoots from one line. Although subsequent researches have focused on the haploid induction of tomato, the results of using anther or microspore culture have remained unsatisfactory. They only obtained callus, or rarely shoots. Globular and heart-shaped embryos have been obtained from microspore culture (Yuan *et al*. 1999; Seguí-Simarro 2007). However, they failed to develop into plants.

The gynogenic approach is an alternative way to induce haploid plants (Chen 2011). However, there were few reports of gynogenesis in tomato (Bal and Abak 2007). San and Gilbert (1984) reported on the non-androgenic haploid induction through ovary culture. Their results indicated that induction of excess callus from the ovary wall hindered haploid development. To overcome this problem, Bal and Abak (2003a, b) pre-cultured ovaries from flower buds containing megaspores at the uninucleate stage, on a starvation medium at 10°C in the dark for 7 days. The ovaries were then cultured on half-strength Murashige & Skoog (MS) medium (Murashige and Skoog 1962) containing growth regulators and amino acids. Subsequently, the ovaries were subcultured on NLN medium (Lichter 1982) in the presence of casein hydrolysate and other substances. Their results indicated that somatic callus did not develop; ovary walls covering the ovules started to degenerate and exposed the ovules; and then the ovary walls on some parts of the ovaries completely degenerated. Morphologically, the ovules were two types: globular with spherical contours; and degenerated ovules with almost rectangular contours. Histological examination of the globular ovules revealed masses of cells in the embryo sacs. Thus, it appears that they relieved the hindering effect of the ovary reported by San and Gelebart (1984). However, no callus or shoot development was observed. Earlier research on *in vitro* gynogenesis concentrated only on ovary culture but the results obtained were not satisfactory (San and Gelebart 1984; Bal and Abak 2003a, b).

Compared with androgenesis approach, the gynogenic approach has the following advantages (Zhou 1981). The gynogenic approach is an alternative way to induce haploid plant for the varieties with low frequency of haploid induction. The gynogenic approach increases the frequency of green plant regeneration and has low frequency of aneuploidy and character variation. In this study, we explored the possibility of haploid induction through gynogenesis.

RESULTS

The suitable stage of flower buds for ovule culture

Our initial attempts at ovule culture clearly indicated that the stage of ovule development was very important. Most of the ovules obtained from the flower buds at stages E-H (Fig. 1) from Zhongza 105 did not form calli, because they gradually turned brown and died. Only a limited number of ovules, obtained from flower buds at stage E, expanded and subsequently produced calli. However, ovules obtained from flower buds at stages C and D began expansion after 8-12 h in culture, with the rate of callus induction reached 40-60%. Therefore, the buds at stages C and D (Fig. 1) were most suitable for gynogenesis induction. We observed that the flower buds at these two stages contained ovules at the embryo sac formation stage. The diameters of ovule and the lengths of sepal separations at each stage of flower bud development are listed in Table 1. The length of sepal separation for buds at stages C and D ranged from 0 to 3 mm, and the buds were approximately 3 to 4 days before anthesis. The 0 to 3 mm length of sepal separation was easy to identify, and no obvious differences were observed among the different tomato materials. Therefore, the length of sepal separation was a very reliable indicator of ovule development. It was used throughout the remainder of our research.

Induction culture of isolated ovules

Isolated ovules enlarged markedly after 8-12 h culture

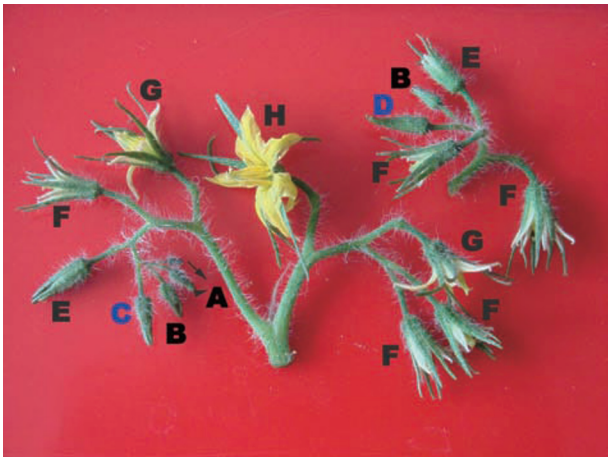

Fig. 1 The classification of bud development stages from A to H in Zhongza 105.

on three induction media, with an opaque white color. After 1 week, the ovules doubled in size, and more than 40% of ovules had an opaque white globular appearance (Fig. 2-A). Calli began to form after 10-15 days. After 20 days calli grew to 2 to 3 mm in diameter. Usually, 40-50 calli were produced in each centrifuge tube. No differences were noted in the performance of ovules obtained from the two tomato hybrids cultured on the three evaluated media (Table 2).

Plant regeneration and ploidy identification

Calli, deriving from isolated ovule culture on induction media, were cultured on six differentiation media sep-

Table 1 Bud lengths, anther lengths, expected flowering time, length of sepal separation, and ovule sizes for eight stages of bud development

Bud stage	Bud length (mm)	Anther length (mm)	Expected flowering time (d)	Length of sepal separation	Ovule size (equatorial diameter× longitudinal diameter, mm×mm)
A	<5	<3	7-8	0 mm	-
B	5-7	3-5	6-7	0 mm	0.05×0.07
C	7-9	5-6	5-6	0-2 mm	0.06×0.08
D	8-10	6-7	4-5	1-3 mm	0.06×0.09
E	10-12	7-8	3-4	3 mm-1/2 of bud	0.07×0.09
F	11-13	7-9	2-3	1/2-3/4 of bud	0.07×0.10
G	12-13	8-9	1-2	3/4-all of bud	0.08×0.11
H	12-14	9-10	0	Open	0.08×0.12

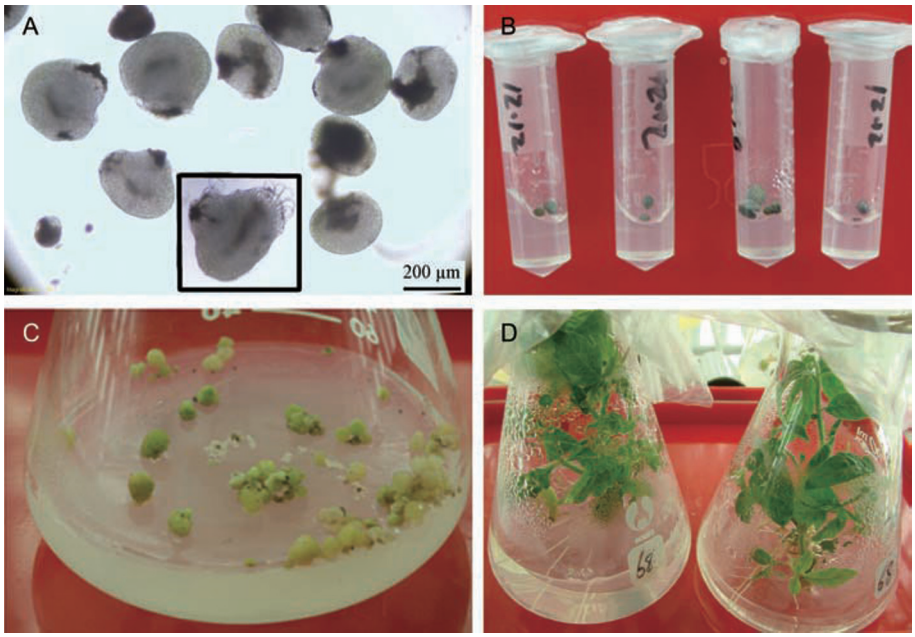

Fig. 2 Ovule culture and plant regeneration in tomato. A, the appearance of ovules cultured for one week on induction medium in centrifuge tubes. Even at this early stage, a few ovules showed signs of callus formation (inset, lower center). B, callus in centrifuge tubes, approximately three weeks after inoculation of ovules on induction medium. C, callus in a flask on induction medium approximately 4-5 weeks after inoculation. D, rooted plantlets arising from callus obtained from isolated ovules. Bar in A=200 μm.

Table 2 Percentage of ovules from two tomato varieties that produced callus on each of the three induction media

Variety	Callus production (%)		
	Medium B5-I1[1)]	Medium B5-I2[2)]	Medium B5-I3[3)]
Zhongza 105	44.6	45.6	43.4
Zhongza 101	45.8	43.0	45.8

[1)] B5+2.0 μmol L^{-1} 2,4-D+1.0 μmol L^{-1} 6-BA

[2)] B5+9.04 μmol L^{-1} 2,4-D+5.0 μmol L^{-1} NAA+1.0 μmol L^{-1} 6-BA+1.0 μmol L^{-1} IBA+1.0 μmol L^{-1} kinetin

[3)] B5+2.0 μmol L^{-1} 2,4-D+2.0 μmol L^{-1} NAA+1.0 μmol L^{-1} 6-BA+1.0 μmol L^{-1} TDZ

arately (Table 3). After one week in culture, the calli enlarged. Subsequently the surface of the callus became brown and then began to turn green after three weeks in culture (Fig. 2-B and C). The performance of callus growth was similar on all of the six media. After a period of two to three months, the calli became fully green.

Shoot regeneration occurred in Zhongza 105 when its calli were transferred to the differentiation medium B5+IAA 0.01 mg L^{-1}+zeatin 2.0 mg L^{-1} after subculturing 10-12 times over a 15-month period. Five shoots, from five calli, were produced. Roots were initiated one week after the shoots were transferred to rooting media (Fig. 2-D). The chromosome number of the root tips was 2n=24. Therefore, the ovule culture did not result in a haploid generation from the embryo sac cells in our study.

Histological observation of ovules

Microscopic observations on ovule sections were carried out to identify whether the plants originated from haploid cells in the embryo sac or from diploid somatic cells.

Observations revealed that the best stage for ovule induction was 4-5 days before anthesis. At this stage, the ovule usually contains eight nuclei in the embryo sac, and approximately eight integument layers. Polar nucleus and antipodal nuclei were observed separately on different planes of the same ovule (Fig. 3-A and B).

Table 3 Media used for differentiation of callus obtained from ovule culture

Basal medium	Auxin and cytokinin concentrations
B5	NAA 0.11 μmol L^{-1}+zeatin 4.56 μmol L^{-1}
B5	NAA 0.5 μmol L^{-1}+zeatin 10.0 μmol L^{-1}
B5	NAA 21.6 μmol L^{-1}+2,4-D, 4.5 μmol L^{-1}+kinetin 3.7 μmol L^{-1}
B5	IAA 0.06 μmol L^{-1}+zeatin 9.12 μmol L^{-1}
MS	IAA 1.14 μmol L^{-1}+6-BA 8.88 μmol L^{-1}
DBM	NAA 0.5 μmol L^{-1}+zeatin 10 μmol L^{-1}

Following a two-day culture, ovules enlarged with an opaque white color. At this point, embryo sac cells at the chalazal end of ovules were induced to divide (Fig. 3-C and D). After ovule culture for 4 days, living cell clumps could be clearly seen in the embryo sacs of the tomato ovules, and separated from ovule wall cells (Fig. 3-E and F). It was thought that the cell clumps in the embryo sac originated from egg apparatus (egg or synergid), in that the cell clumps grew from the chalazal end of the ovule.

After 6 days of culture, ovules doubled in size, and had an opaque white globular appearance. The cell clumps in the embryo sac stopped growing when the embryo sac was full of cells. While, the cell of ovule wall continued to divide and increased the number of integument layers from eight to fifteen (Fig. 3-G). These integument layers ultimately formed calli. Therefore, the cell clumps in the embryo sac were taken over by excess callus developed from integument, which likely hindered gynogenic development.

The isolation and culture of embryo sac protoplasts

As mentioned previously, during the ovule culture, the integument hindered the growth of haploid cells in the embryo sacs. However, the culturing of embryo sac protoplasts could eliminate the hindering effect of integument on embryo sac cells, because there was no integument on embryo sac protoplasts.

Living embryo sac protoplasts were successfully released from ovules mechanically. Dissociation for six hours was suitable for releasing living embryo sac protoplasts (Fig. 4-A). After 48 h of culture, most of the embryo sac protoplasts were doubled in size (Fig. 4-B). In order to evaluate the viability of released embryo sac protoplasts, they were stained with fluorescein diacetate (FDA) and then observed under fluorescence microscopy. Embryo sac protoplasts fluoresced brightly, indicating that they had strong viability (Fig. 4-C). And globule-like tissues were occasionally observed in the transparent embryo sacs (Fig. 4-D). Following 3 days of culture, two or more globule-like tissues, similar in appearance to cells, were released from the embryo sac protoplasts, and then divided into more tissues of various

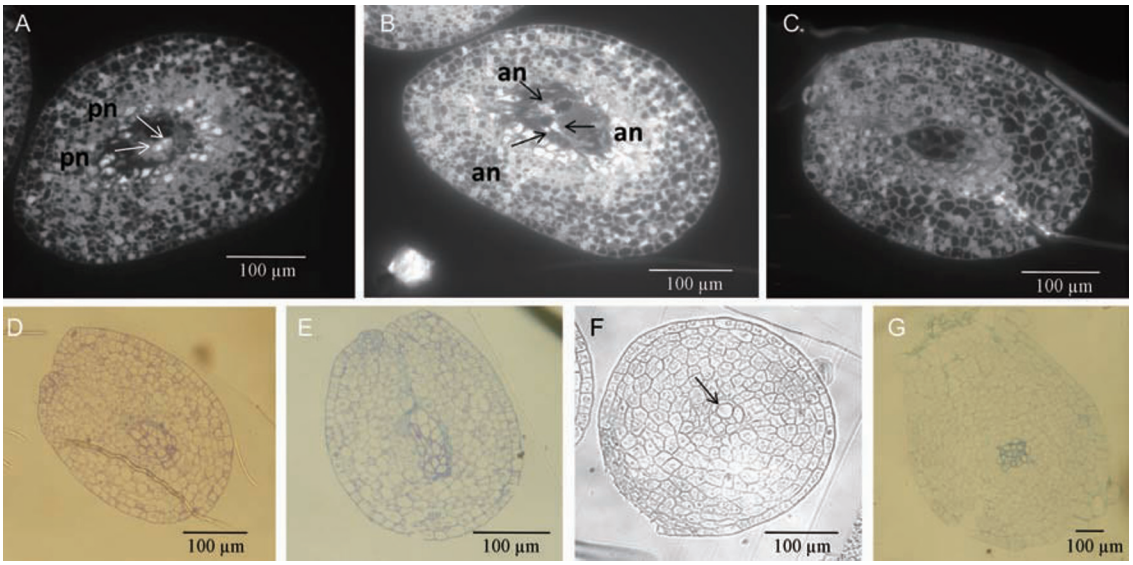

Fig. 3 Gynogenesis in tomato ovules. A, epifluorescence microscopy of polar nucleus (pn) presented in the embryo sac in the ovule 3 days before anthesis. Labeled arrowheads indicate the polar nucleus (pn). B, epifluorescence microscopy of antipodal cells (an) presented in the different layers of embryo sac of the same ovule as shown in (A). Labeled arrowheads indicate the antipodal cells (an). C, epifluorescence microscopy of a cell clump in the embryo sac of an ovule cultured for 2 days. D, light microscopy of an ovule cultured for 2 days and then stained with toluidine blue. The cell clump originated from the chalazal end of the ovule. E, light microscopy of an ovule stained with toluidine blue. The cell clump has separated from ovule wall cells. The ovule was cultured *in vitro* for 4 days. F, cross section of an ovule displaying a cell clump separated from the ovule wall, ovule cultured for 4 days. G, light microscopy of an ovule cultured for 6 days stained with toluidine blue. Outer integument of the ovule grew instead of cell clump within the embryo sac. Bar=100 μm.

sizes (Fig. 4-E). These tissues then formed clusters with different appearance (Fig. 4-F and G). However, browning and shrinkage of these small clusters was observed after ten days of culture. The clusters stopped growing gradually, so ultimately no callus was obtained.

DISCUSSION

The development of a rapid, efficient and genotype-insensitive method for tomato haploid production is a highly desired goal for tomato breeders. Haploid production would also facilitate research on molecular markers, genetic map construction, gene mapping, gene cloning and numerous other studies in tomato. It has been 42 years since Sharp (1971) reported successful androgenesis in tomato. Subsequently, androgenesis has been attempted by several research groups (Chlyah *et al*. 1990; Poonam *et al*. 2004; Bal and Abak 2007) with minimal success. Their lack of success provides impetus to examine other ways that lead to haploid production, such as gynogenesis.

Gynogenesis has been examined in seed plants of at least 40 species, belonging to 20 families. Gynogenic haploids have been successfully obtained in 25 of these species, belonging to 10 families. In tomato, San and Gelebart (1984) examined ovary culture, and their results indicated that the excess callus from the ovary wall hindered gynogenetic haploid development. Bal and Abak (2003a, b) pre-cultured ovaries on a starvation medium, and then subcultured on NLN medium. This approach relieved the hindering effect of the ovary reported by San and Gelebart (1984). However, they did not report further callus and shoot development. Our findings agree with the observation that the presence of ovary tissue may hinder ovule development in culture, because only ovules were induced to form calli successfully in our study and explants were relatively free of ovary tissue.

Researchers did not attempt to culture detached ovules of tomato (San and Gelebart 1984; Bal and Abak 2003a, b), perhaps because of the difficulty of ovule isolation from their tomato lines. The isolation of ovules from plant ovaries is a critical and often time-consuming step in many plant haploid induction procedures. This is especially true for studies of *in vitro* gynogenesis induction, cytology research, or histology research, because hundreds or even thousands of ovules need to

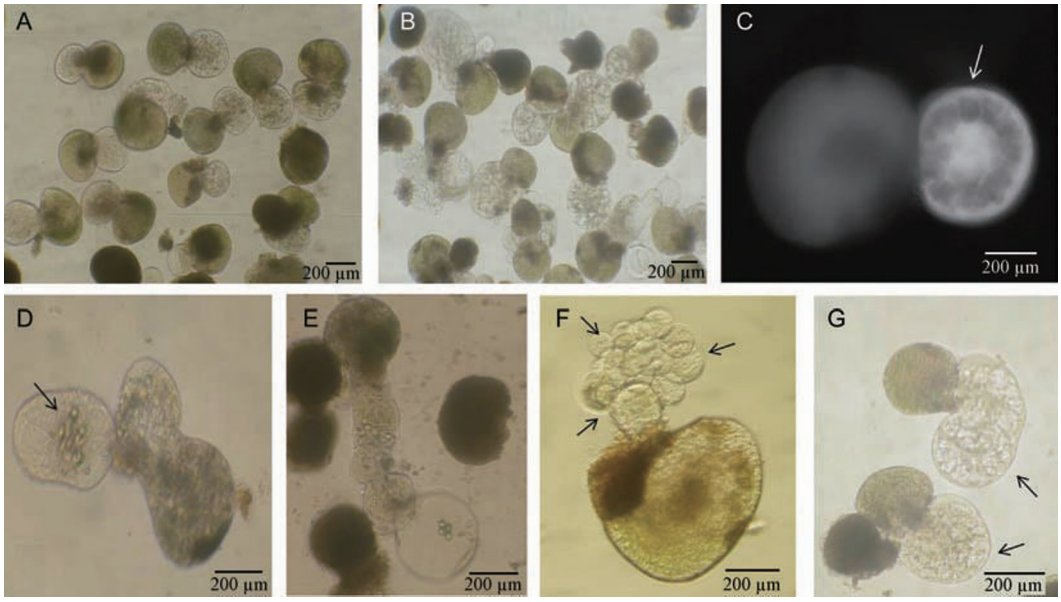

Fig. 4 Embryo sac protoplasts. A, a population of embryo sac protoplasts released from ovules. B, a population of embryo sac protoplasts doubled in size after 48 h of culture. C, embryo sac protoplast stained with FDA showing strong viability after 48 h of culture. D, small tissue presented in embryo sac protoplast after culturing for 48 h. E, F, G, different appearances of cell clusters arising from embryo sac protoplast cultured for three days. Bar=200 μm.

be isolated in a short time. Many protocols of ovules isolation are laborious and inefficient with regard to the production of a large number of ovules. In previous research, ovules were removed individually by needle under the microscope, which expended a lot of time and effort. In this study, an efficient protocol was established for the isolation of a large quantity of ovules from ovaries. Using this protocol, one person can isolate several thousands of ovules in approximately 30 minutes. The yield is approximately 100-150 ovules per ovary, so the number of ovules can be maximized by using more ovaries. This ovule isolation protocol may become the first step of *in vitro* gynogenesis induction of plants in the future. This protocol not only speeds up the progress of gynogenesis research in tomato, but also may provide a useful method in the gynogenesis, embryology and biology research of other plant species.

In this research, sampling criteria were developed to select the flower buds at the appropriate stage for ovule isolation and culture. Relationships between bud size, anther size, color, and ovule size differ among tomato genotypes, which make it difficult to select ovules at the appropriate stage. The use of the length of sepal separation as an indicator of ovule development considerably enhanced our ability to harvest ovules at the appropriate stage required for producing cell clumps in the embryo sacs. Additionally, our results confirmed the reports of Li (2008), who successfully used a similar technique to identify ovule development stage in eggplant.

This study also established an ovule micro-culture method employing 2 mL centrifuge tubes. Compared with the conventional methods, in which ovaries or ovules are inoculated directly into flasks or dishes, use of small tubes has the advantage of reducing contamination rate. In a bud, the number of ovules is fewer than the number of microspores. Therefore, the ovules extracted from 20 buds can be inoculated into 2-3 flasks or dishes, while 5-6 flasks or dishes for microspores. In our experience, the 2-3 flasks or dishes usually had 2-3 bacterial lesions in each flask or dish. The contamination rate can reach 80% or more; and it can be 100% when employing buds from tomatoes in the autumn. However, when the ovules from 20 buds were inoculated into 20-30 2-mL centrifuge tubes, only 4-6 tubes were usually contaminated. Furthermore, this method required less culture medium, while its culture efficiency was markedly increased.

In this research, thousands of calli of Zhongza 101 and Zhongza 105 were reliably induced at a high frequency. Because of its high frequency of callus induction, the developed approach meets the requirements of tomato haploid culture technology. In our experience, callus

induction rate for anther culture of the two varieties used in this research was less than 1%. It was considerably less than the ~45% induction rate for ovules from these varieties. Although the induction of calli was reliable, only five plants were induced. All of them were from the variety Zhongza 105.

Bal and Abak (2003b) reported that a mass of cells are developed inside the embryo sacs in the cultured ovaries, but the origin of cell clumps remained in question. Because the calli may originate from embryo sac cells or other somatic cells, histological research was carried out to define the origin of ovule calli in this study. Histological research revealed that embryo sacs cells could be induced to divide and grew from the chalazal end (egg cells or synergids) of embryo sacs, and then formed cell clumps. The cell clumps separated from integument of ovules, so these cell clumps might originate from the egg apparatus. Nevertheless, the hindering effect of somatic cells (ovule wall, tapetal cells, and the nucellus) on haploid cell development could be avoided by in vitro embryo sac protoplast culture. In our study, the protoplasts of embryo sacs were released form ovules successfully and induced to form small tissues, but no callus was obtained. Kranz (1995) found that a non-fused egg cell could divide *in vitro* with the supplementation of high amounts of 2,4-D. However, no effect of 2,4-D on the division of embryo sac cells was found in our study.

In this study, the ovules in tomato were successfully isolated and cultured. However, in the process of ovule culture, the integument cells grew faster and covered embryo sac. To eliminate integument cells, living embryo sac protoplast were successfully isolated and cultured, but failed to induce callus or embryoid formation. Therefore, how to induce the division and growth of embryo sac protoplast will be the missing link for the haploid induction *via* gynogenesis intomato.

CONCLUSION

In our study, thousands of calli and five regeneration plants were obtained through ovule culture. We developed the method for selection of appropriate bud stages and optimized induction media, because they are critical to enlarge ovules and produce cell clumps in embryo sacs. We also developed an efficient protocol for ovule isolation. This protocol may be helpful not only for *in vitro* gynogenesis induction, but also for the cytology and histology research of tomato. Furthermore, the protoplasts of embryo sacs were successfully released from ovules, and cell clusters with distinct appearances were observed during culture. In conclusion, our findings provided potential for further research in the haploid induction of tomato.

MATERIALS AND METHODS

Stages of bud development, and plant material

Eight stages of bud development were evaluated in this research. Stages and their designations are depicted in Fig. 1. Buds of the appropriate stages were excised from plants growing in plastic tunnels or greenhouses at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences. Management and fertilizer application followed locally accepted practices (Du *et al*. 2005). This research was conducted over several seasons and years (2005 to 2012). In the initial stages of research about thirty different cultivars of tomato were used to survey the responsiveness of explants to a range of proliferation media. Once potential proliferation media had been identified, research centered on two tomato cultivars, Zhongza 101 (Du *et al*. 2005) and Zhongza 105. These hybrids were released by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences in 2005 and 2006, respectively, and are large, pink, fresh market tomatoes.

Ovule preparation

Buds at the appropriate stage (stages C-D, Fig. 1) were excised from plants and immediately taken to the laboratory. To evaluate the extent of bud development, bud size, anther length, and expected flowering time, were noted for each sampled bud. Additionally, for each stage of bud development the ovule size, and the length of sepal separation, from the tip of the sepal to the bottom of the separation formed between two sepals, were measured. Ovaries were then removed from each bud, and surface disinfected in 10% sodium hypochlorite solution for 16-20 min; they were then rinsed with sterile distilled water 3-4 times. All subsequent operations took place under sterile conditions.

Ovules were prepared by placing 20 intact tomato ovaries (stages C and D) into a 60×15 (Φ×H mm) glass Petri dish and then adding 5-6 mL of sterile deionized H_2O. One side of the dish was then raised so that the water covered about half of the bottom of the dish. Ovaries were then moved near the edge of the water, pressed gently several times with a small pestle, releasing the ovules into the water. Remaining ovary

tissue was then removed from the dish, and excess water was removed with a pipette. Ovules, now concentrated in a small amount of water were carefully removed with a pipette, and transferred to appropriate media. Using these procedures, 100-150 ovules could be isolated from one ovary.

Culture on induction media

Three culture media for induction were based on Gamborg's B5 medium (Gamborg *et al*. 1968): 1) B5+2.0 μmol L^{-1} 2,4-D+1.0 μmol L^{-1} 6-BA (B5-I1). 2) B5+9.04 μmol L^{-1} 2,4-D+5.0 μmol L^{-1} NAA+1.0 μmol L^{-1} 6-BA+1.0 μmol L^{-1} IBA+1.0 μmol L^{-1} kinetin (B5-I2). 3) B5+2.0 μmol L^{-1} 2,4-D+2.0 μmol L^{-1} NAA+1.0 μmol L^{-1} 6-BA+1.0 μmol L^{-1} TDZ (B5-I3). All media contained sucrose (20 g L^{-1}) and agar (8 g L^{-1}). In all cases, the pH of the medium was adjusted to 5.8-6.0 before autoclaving, heat labile compounds were filter sterilized and added after autoclaving.

Ovules were cultured in 2-mL centrifuge tubes (Xygen Lab Supplies, Beijing) containing 400 μL of induction medium. Each centrifuge tube was inoculated with 100-200 ovules. Tubes were incubated at 28°C in the dark, and checked every 48 hours for signs of growth and development of the explant. If there was no growth, tubes were discarded. The percentage of induction was calculated on a per embryo basis, determined from photographs taken at intervals.

Subculture on differentiation media

Once calli had developed on induction media, which took approximately 20 days, they were transferred to differentiation media. Six differentiation media were evaluated (Table 3). All media contained sucrose (20 g L^{-1}) and agar (8 g L^{-1}). Different growth regulator combinations were added to the basal media for evaluation. The pH of the media was adjusted to 5.8-6.0 before autoclaving. Heat labile compounds were filter sterilized and added after autoclaving. Calli, approximately 2 mm in diameter, were transferred to 2 mL centrifuge tubes containing 400 μL of media. Tubes were then incubated under conditions of 16-h light (90 μmol m^{-2} s^{-1})/8-h dark cycles, at 28°C. When the callus diameter was greater than 5 mm, it was inoculated into a 100-mL flask that contained differentiation medium. Shoot production was the desired endpoint.

Rooting of shoots, and counting of root tip chromosomes

Once the shoots had developed 2 to 3 leaves, they were placed on root induction medium (MS with the addition of 1.14 μmol L^{-1} IAA, 2% (w/v) sucrose and 0.8% (w/v) agar). After the shoots had rooted, root tips were excised, and chromosomes were counted in tips with the aid of a light microscope (Zeiss Axiovert 40 CFL), using the method described by Zhu (1982).

Histological observation of ovules

Ovules were removed from ovaries at the optimum stage (4-5 days before anthesis), and cultured on induction media (Table 2). After culture for 0-7 days, enlarged ovules were fixed in 0.7 mol L^{-1} phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde for 24 h. Following dehydration by a graded ethanol series, the samples were embedded in Spur resin. Sections of 2 μm thickness were cut using glass knives and then observed using fluorescence microscopy (Leica DM 5000 B). The sections were also stained with toluidine blue (Sigma, USA) and observed using a bright field microscope (Leica DM 5000 B, Germany).

Embryo sac protoplast culture

Ovules, isolated from tomato ovaries (stages C and D in Fig. 1), were incubated in a solution containing 1.5% cellulase R-10, 0.5% macerozyme R-10, 0.1% pectase, 13% sugar, 10 mmol L^{-1} $CaCl_2$, and a concentration of 0, 20, or 40 mg L^{-1} 2,4-D, respectively, at pH 5.8 for 6 h. After cleaning three times with 13% sucrose, slight pressure with a micropipette was used to release living embryo sacs, which were incubated in three types of liquid induction media (Table 2) containing 13% sugar and no agar, at room temperature, and kept in the dark for further examination and manipulation.

Newly isolated embryo sacs were stained with 1 μg mL^{-1} fluorescein diacetate (FDA) to test cell viability. The cultured embryo sacs were stained with Calcofluor white ST (Sigma, USA) for detection of the cell wall, and propidium-iodide (PI) for localization of the nucleus. The embryo sacs were observed and photographed by fluorescence microscopy (Leica DM 5000 B) and light microscopy (Zeiss Axiovert 40 CFL).

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