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BRIEF COMMUNICATION

Thidiazuron and silver nitrate enhanced gynogenesis of unfertilized ovule cultures of *Cucumis sativus*

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Abstract

Gynogenesis of Chinese long cucumber (*Cucumis sativus* L.) was induced in unpollinated ovules cultured on cucumber basal medium (CBM) supplemented with thidiazuron (TDZ) and in some experiments $AgNO_3$. High induction frequencies (7.85 - 12.14 %) were found in unpollinated ovules at the time of anthesis and at 0.03 - 0.07 mg dm^{-3} TDZ. Histological analysis indicated that embryo sacs developed completely at the time of anthesis. Further, the highest plant regeneration rate was achieved at CBM supplemented with 0.05 mg dm^{-3} *a*-naphthaleneacetic acid, 0.2 mg dm^{-3} 6-benzyladenine, and 5 - 10 mg dm^{-3} $AgNO_3$. Flow cytometry analysis showed that 80 % of the regenerated plants were haploid. Histological micrographs and ploidy level analyses clearly revealed initiation, development, and germination of embryos from the unpollinated ovules.

Additional key words: auxin, cucumber, cytokinin, embryogenesis, female gametophytes, haploid.

Cucumber (*Cucumis sativus* L.) is one of the most important vegetables in the world. Common cultivars produced in China are the slender and long type, commonly known as Chinese cucumbers which are largely hybrids. Due to their monoecious nature, development of hybrid cultivars through traditional breeding is a long process. Therefore, alternative approaches have been used for inbred line development; these include the production of doubled haploid (DH) plants by induction of androgenesis and gynogenesis. DH plants have been successfully used in breeding programs of a wide range of species (Palmer *et al*. 2005). In cucumber, the production of haploids has been reported by androgenesis (Kumar *et al*. 2003, Song *et al*. 2007) and from unfertilized ovules (Gémes-Juhász *et al*. 2002, Diao *et al*. 2009). However, the major hurdle in the use of unfertilized ovules is the low frequency in embryo induction and also low survey rates in embryo development and germination. Inducing embryogenesis and improving growth of plantlets depends on several factors, including plant genotypes, preculture conditions, pretreatment, medium components, embryo conversion, and photoautotrophic condition (Gémes-Juhász *et al*. 2002, Shalaby 2007, Park *et al*. 2011). To date, limited information is available regarding the unfertilized ovule embryogenesis in Chinese long cucumbers. Therefore, we conducted experiments to identify the optimal development stage of the female gametophyte for *in vitro* culture and examine the effects of different plant regulators on embryogenesis of unfertilized ovules.

Two Chinese long cucumber (*Cucumis sativus* L.) inbred lines IL57 and IL69 developed by the Cucumber Group of Horticulture College at Henan Agricultural University were used in this study. After 50 d planting in the spring, unfertilized ovaries were collected from plants in the greenhouse at the experimental plot at Henan

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Abbreviations: BA - 6-benzyladenine; CBM - cucumber basal medium; DBA - days before anthesis; DH - doubled haploid; NAA - α-naphthaleneacetic acid; TDZ - thidiazuron.
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Agricultural University. The developmental stages of female gametophyte were preliminarily determined by observing the female flowers. Unfertilized ovaries were obtained 3 d before anthesis (DBA), 2 DBA, 1 DBA, 12 h before anthesis, and at the time of anthesis. The ovaries were rinsed in distilled water and peeled. The peeled ovaries were surface-sterilized in 70 % ethanol for 40 s followed by 0.1 % $HgCl_2$ for 8 - 10 min and rinsed four times with sterile distilled water. Longitudinally sliced ovaries (1 cm in length) were cultured initially in Petri dishes containing a cucumber basal medium (CBM; Gémes-Juhász *et al*. 2002) supplemented with 40 g dm^{-3} of sucrose, 3 % agar, and four concentrations of thidiazuron (TDZ; 0.03, 0.05, 0.07, and 0.09 mg dm^{-3}). The same medium with no TDZ served as a control. The pH of the medium was adjusted to 5.8 - 6.0 before autoclaving at 110 °C for 20 min. The sliced ovaries were cultured in the dark at 35 °C for 4 d according to Gémes-Juhász *et al*. (2002).

After induction, the sliced ovarum explants were transferred onto CBM containing 30 g dm^{-3} sucrose, 3 % agar, 0.05 mg dm^{-3} *a*-naphthaleneacetic acid (NAA), and 0.2 mg dm^{-3} 6-benzyladenine (BA). The medium pH was adjusted to 5.8 - 6.0 before autoclaving. The cultures were maintained at 26 °C, irradiance of 33 µmol m^{-2} s^{-1} (cool white fluorescent tubes), and 16-h photoperiod. The embryos developing from the ovules were not removed from the ovaries but transferred to the regeneration medium to promote embryo development every two weeks. Matured and germinated embryos produced plantlets. The plantlets with radicals were separated from the ovaries, transferred to the CBM medium without growth regulators, and cultured under the same conditions. Based on the experiments above, another experiment was carried out by culturing induced embryos on the CBM with $AgNO_3$ (5, 10, and 15 mg dm^{-3}) under the same environmental conditions. The same medium without $AgNO_3$ was used as control.

A completely randomized design was used for all experiments. For each treatment, 20 explants were cultured (four sliced ovaries per Petri dish and five replicates per treatment) and all the experiments were repeated three times. The cultures were observed periodically and anatomical changes were microscopically examined at regular intervals. The number of induced embryos and regenerated plantlets was calculated at 6 and 12 weeks after induction of experiments. The percentage of induced embryos and the percentage of plant regeneration were calculated on the quotient of the total number of regenerated embryos or plants against the total number of cultured ovaries. Data were transformed with arcsine transformation [arcsin ($X^{1/2}$)] before they were used for statistical analysis. The data were subjected to analysis of variance (*ANOVA*) using the fixed model and mean values were separated based on Duncan's multiple test using the *SAS v. 9.1* software program (*SAS Institute Inc.*, Cary, NC, USA).

The selected ovary fragments were fixed in formalin : glacial acetic acid : 70 % ethanol (1:1:18; FAA) at room temperature for at least 24 h. Afterwards, the fragments were dehydrated through a graded ethanol series and embedded in resin. The sections of 0.5 µm thick were cut, stained with Delafield's hematoxylin (Sharma and Millam 2004) and observed under a light

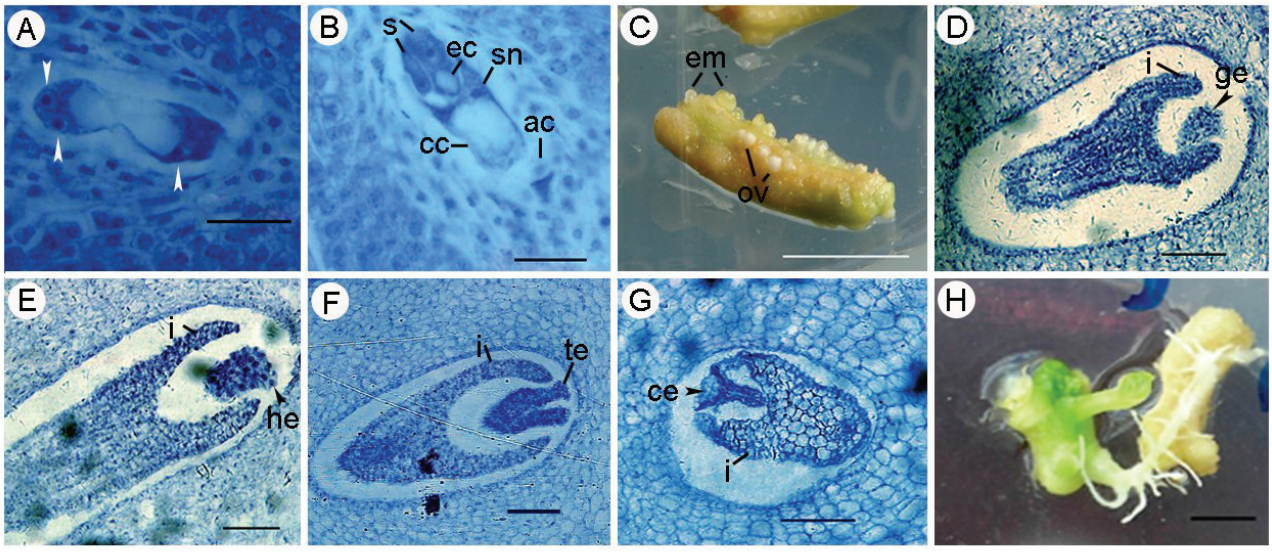

Fig. 1. Embryogenesis and plant regeneration in cultured unpollinated ovules of cucumber IL69. *A* - Four-nucleate embryo sac, the *arrows* indicate 3 of 4 nuclei. *B* - Eight-nucleate ES: the synergids (s), egg cell (ec), secondary nucleus (sn), central cell (cc), and antipodal cell (ac) are marked. *C* - Embryos (em) and ovules (ov) after 4 weeks of culture. *D, E, F, G* - Transverse section of unpollinated ovule with induced embryo at globular stage, early heart shape stage, torpedo stage, and cotyledon stage, respectively: ge - globular embryo; he - heart embryo; i - integument; te - torpedo embryo; ce - cotyledonary embryo. *H* - Plantlet resulted from directly germinating embryo. *Scale bars* 50 µm in *A*, *B*, *D-G*; 0.5 cm in *C* and *H*.

Table 1. Effects of TDZ concentrations and the ovary development stages (days before anthesis, DBA) on embryo induction frequency (total number of embryos/total number of ovaries) in cucumber. Means ± SE of 20 replicates per treatment in three repeated experiments. Means with the same superscript letters within columns are not significantly different at $P > 0.05$. All data were taken after 6 weeks of culture.

TDZ [mg dm^{-3}]	DBA [d]	Frequency of embryo induction [%] IL69	IL57
0.00	0	0.16 ± 0.16^{hi}	$0.10 \pm 0.10h^{i}$
	1/2	0.06 ± 0.06^{hi}	0^{i}
	1	0^{i}	0^{i}
	2	0^{i}	0^{i}
	3	0^{i}	0^{i}
0.03	0	12.14 ± 1.35^{a}	9.97 ± 0.85^{ab}
	1/2	2.52 ± 0.67^{cde}	2.59 ± 0.49^{efgh}
	1	1.96 ± 0.56^{def}	2.24 ± 0.60^{efghi}
	2	0.10 ± 0.10^{hi}	0.35 ± 0.16^{hi}
	3	1.80 ± 0.54^{def}	1.35 ± 0.24^{ghi}
0.05	0	7.85 ± 1.09^{ab}	6.21 ± 0.64^{c}
	1/2	3.97 ± 1.08^{cd}	4.60 ± 0.54^{cde}
	1	3.56 ± 0.74^{cd}	4.11 ± 0.42^{cdef}
	2	$0.18 \pm 0.15^{gh}i$	2.47 ± 0.55^{efghi}
	3	1.46 ± 0.46^{def}	0.61 ± 0.23^{hi}
0.07	0	8.98 ± 1.23^{ab}	11.11 ± 0.98^{a}
	1/2	0.44 ± 0.29^{fghi}	4.56 ± 0.57^{cde}
	1	4.93 ± 1.57^{bc}	5.36 ± 0.76^{cd}
	2	1.84 ± 0.52^{def}	2.17 ± 0.44^{fghi}
	3	1.50 ± 0.59^{def}	0.86 ± 0.30^{hi}
0.09	0	7.25 ± 0.93^{b}	8.64 ± 1.01^{b}
	1/2	1.53 ± 0.94^{efg}	3.23 ± 0.46^{defg}
	1	2.17 ± 0.53^{cdef}	4.14 ± 0.47^{cdef}
	2	1.25 ± 0.57^{efgh}	1.92 ± 0.25^{fghi}
	3	2.06 ± 0.61^{cdef}	1.89 ± 0.31^{fghi}

microscope (*Olympus BX 51 DP 71*, Tokyo, Japan). Ploidy levels of regenerated plants were examined using a flow cytometer (*Partec D-48161*, Münster, Germany) equipped with a mercury arc lamp (435 - 500 nm, 15 mW) according to Aleza *et al*. (2010). Leaves (50 mg) from each young regenerated plant were chopped with a razor blade in a plastic Petri dish containing 0.4 cm^3 of *Partec HR-A* buffer. The supernatant was filtered through a 30 µm filter (*Partec*, *Celltrics*^{TM}) and stained with 0.8 cm^3 of *Partec HR-B* buffer. Every sample from each treatment was replicated three times using diploid cucumber plants as the control.

To determine megagametogenesis in unfertilized ovaries, the ovaries were harvested on 3, 2, or 1 DBA, 12 h before anthesis, and at the time of anthesis. On 3 DBA, ovules contained female gametophytes at the one-nucleate embryo sac stage. The functional megaspore undergoes a round of mitosis producing a two-nucleate cell with a large central vacuole (data not shown). In the next stage, each of two nuclei divided into two, forming a four-nucleate embryo sac. The four-nucleate embryo sac had a large vacuoles determining dimensions of the embryo sac (Fig. 1*A*). At the time of anthesis, the nuclei became completely surrounded by cell walls, resulting in the formation of the eight-celled female gametophyte with an egg apparatus at the micropylar pole, the secondary nucleus (two polar nuclei fused) in the central cell situated close to the egg cell, and three degenerated antipodals at the chalazal pole (Fig. 1*B*).

Significant differences in the initial embryo induction rate were observed among the unfertilized ovaries at different development stage of female gametophyte (Table 1). The highest frequency of embryo induction was from the ovaries at the time of anthesis, which was significantly higher than those obtained in the other developmental stages (Table 1), indicating that the completely developed embryo sac was optimal explant to induce *in vitro* gynogenesis. A similar finding was reported by Gémes-Juhász *et al*. (2002) in pickling cucumber. They identified 6 h before anthesis as optimum for haploid induction, at this stage, the cell membranes in the embryo sacs had already developed but the cells had not completely formed their final shape. This variation may be explained by the different genotypes between pickling cucumber and Chinese long cucumber.

Segments of unfertilized ovaries were cultured on embryo induction medium in the dark at 35 °C for 4 d. After one week of culture, the first sign of enlarged ovules could be observed on some explants surface. Embryos appeared and developed into globular embryos (Fig. 1*C*,*D*) in 2 - 4 weeks. After transferring to the regeneration medium, many globular embryos developed into heart-shaped embryos 4 - 6 weeks later (Fig. 1*E*). The heart-shaped embryos further differentiated into torpedo-shaped embryos (Fig. 1*F*) and then embryos with cotyledons appeared after 6 - 8 weeks culture (Fig. 1*G*). Abnormal embryos, such as translucent, fused embryos, fused cotyledons, and multiple cotyledons were also observed in this study. In contrast, the normal mature embryos germinated and grew into plantlets with an elongated hypocotyl, cotyledons, and single tap root at the regeneration medium within 12 - 14 weeks (Fig. 1*H*).

To stimulate embryo formation, the induction media supplemented with different concentrations of TDZ were tested. The results suggested that all four concentrations of TDZ improved embryo formation (Table 1) as the frequencies of induced embryos were significantly higher than that of the control. The highest embryo induction frequency for IL69 was 12.14 % with 0.03 mg dm^{-3} TDZ and for IL57 11.11 % with 0.07 mg dm^{-3} TDZ using the unfertilized ovaries at the time of anthesis (Table 1). These results are in agreement with some of previous reported work that TDZ is the most effective cytokinin for embryo induction (Sauer and Wilhelm 2005, Diao *et al*. 2009, Ma *et al*. 2010, Elviana *et al*. 2011).

To achieve the high plant regeneration rate *via* unfertilized ovules gynogenesis, $AgNO_3$ was used for

Table 2. Effects of $AgNO_3$ upon cucumber embryo induction (total number of embryos/total number of ovaries) and plant regeneration (total number of plantlets/total number of ovaries) on CBM medium supplemented with 0.05 mg dm^{-3} NAA, 0.2 mg dm^{-3} BA and 30 g dm^{-3} sucrose. Means ± SE of 20 replicates per treatment in three repeated experiments. Means with the same superscript letters within columns are not significantly different at $P > 0.05$. All data were taken after 12 weeks of culture.

$AgNO_3$ [mg dm^{-3}]	Frequency of embryogenesis [%]		Frequency of plant regeneration [%]	
	IL69	IL57	IL69	IL57
0	10.78 ± 1.21^a	8.62 ± 1.40^a	0.36 ± 0.09^b	0.34 ± 0.10^b
5	8.85 ± 1.54^a	7.95 ± 1.24^a	0.42 ± 0.13^b	0.87 ± 0.24^a
10	9.69 ± 1.37^a	8.37 ± 1.40^a	0.94 ± 0.19^a	0.38 ± 0.10^b
15	8.78 ± 1.37^a	6.91 ± 0.96^a	0.38 ± 0.10^b	0.42 ± 0.12^b

improving embryo conversion and plantlet regeneration. The results indicated that $AgNO_3$ in the range of 5 to 10 mg dm^{-3} was effective in inducing embryo germination and plant growth for both IL69 and IL57 (Table 2). These results concur with those reported by Leroux *et al*. (2009) that ethylene biosynthesis inhibitors significantly increased embryo occurrence. Moreover, ethylene has been considered to exert a negative control on primary events that trigger the embryogenesis of isolated microspores and somatic embryogenesis *in vitro* (Agarwal *et al*. 2006, Kumar *et al*. 2007, Leroux *et al*. 2009). In this study, we have found for the first time that $AgNO_3$ could promote embryo germination and plantlets regeneration of Chinese long cucumber. The use of ethylene biosynthesis inhibitors, such as $AgNO_3$, may represent an additional way of improving somatic embryogenesis and plant regeneration of cucumbers.

Furthermore, some of the completely formed embryos left in or transferred to the induction media did not grow further whereas many callus formed again on the explants surface. The conversion percentages varied widely from 0.34 to 0.94 % (Table 2) depending on the composition of basal medium, genotypes, and the concentration of growth regulators used. The best result was obtained in our study with CBM medium supple-mented with 5 to 10 mg dm^{-3} $AgNO_3$, 0.05 mg dm^{-3} NAA, 0.2 mg dm^{-3} BA, and 30 g dm^{-3} sucrose.

In the flow cytometric analysis, we used the fresh leaves of diploid IL69 as the control and adjusted the peak of their ploidy level to 100. Analysis of the regenerated plants showed that the peaks of 80.0 % of the plantlets were close to 50 which indicated that these plantlets were haploid plants and confirmed their haploid cell origin. The peaks of 16.7 and 3.3 % plantlets were near 100 and 50 - 100, respectively, so these plants were diploid or mixoploid. This finding was in accordance with some previous studies showing that the very low rates of spontaneous doubling happened at early stage in haploid production in cucumber (Gémes-Juhász *et al*. 2002, Kumar *et al*. 2003, Song *et al*. 2007). The further work is needed to distinguish spontaneously formed doubled haploid or diploid cucumber plants.

In conclusion, gynogenesis was induced from unpollinated ovules of Chinese long cucumber. The best induction treatment was CBM medium containing 0.03 - 0.07 mg dm^{-3} TDZ and the optimum explants were mature female gametophytes or unpollinated ovules at anthesis. Mature embryos were able to germinate and convert into plantlets at the regeneration medium supplemented with 5 - 10 mg dm^{-3} $AgNO_3$. However, the conversion rate of embryos into plantlets was low. Further work is needed to elucidate the biochemical and molecular regulation of cucumber embryo development with the aim of adjusting maturation and germination treatments for the production of high quality doubled haploid plants.

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