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Full Length Article

Evaluation of Factors Affecting Embryo-like Structure and Callus Formation in Unpollinated Ovary Culture of Cucumber (*Cucumis sativus*)

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Abstract

The effects of various factors including genotypes of donor plants, induction and differentiation media, and thermal shock pretreatment were evaluated on embryo-like structure (ELS) and callus formation in an unpollinated ovary culture of cucumber. All five cucumber cultivars, which were used as donor plants, produced ELSs and calli, although their ELS and callus formation potentials varied significantly. The addition of 1 mg/L thidiazuron (TDZ) and 1 mg/L 6-benzylaminopurine (BA) into the induction medium resulted in the highest percentage of ELS formation, ranging from 42.3 to 91.4% with an average of 60.4%. However, the highest percentage of callus formation was observed in an induction medium containing 2 mg/L BA, 0.5 mg/L indole-3-acetic acid (IAA), 1 mg/L gibberellic acid (GA_3) and 32 mg/L putrescine (70.8%). By contrast, differentiation media had no significant effect on the formation potentials of both ELSs and calli. Thermal shock pretreatment reduced the percentage of ELS formation *ca.* 1.3-fold, but had no significant effect on callus formation. These results can be implicated for the efficient production of cucumber doubled haploids in the future.

Keywords: 6-Benzylaminopurine; Callus; Doubled haploid; ELS; Thidiazuron

Introduction

Cucumber (*Cucumis sativus* L.) is one of the most important economic vegetables in Thailand. It is widely grown throughout Thailand for fresh and processed consumption as well as for the pharmaceutical industry (Department of Agriculture, 2014). In cucumber, the production of inbred lines to be used as parents of F_1 hybrids or mutant selection can be accomplished by several generations of selfing; however, some heterozygosity may remain and it is time-consuming. Alternatively, true homozygous lines can be efficiently and rapidly obtained by *in vitro* culture of gametophytic cells, followed by spontaneous or induced chromosome doubling. These homozygous doubled haploids have been induced via either androgenesis (pollen or anther culture) or gynogenesis (ovule or ovary culture) processes, or induced by pollination with irradiated pollen (Islam, 2010; Chen *et al*., 2011; Lazaridou *et al*., 2011; Gałązka and Niemirowicz-Szczytt, 2013). In several plants, including sugar beet and gentians, *in vitro* culture of unpollinated ovules or ovaries have been reported to be more efficient in haploid and doubled haploid production than pollen or anther culture (Gürel *et al*., 2000; Chen *et al*., 2011; Doi *et al*., 2011). Haploid or doubled haploid plants are regenerated through either the formation of ELS directly on ovules or the formation of ELS or shoots on calli induced from ovules/ovaries (Bhojwani and Razdan, 1996; Reed, 2005).

Both *in vitro* androgenesis and gynogenesis have been induced in cucurbit crops for doubled haploid production (Metwally *et al*., 1998; Lotfi *et al*., 2003; Song *et al*., 2007; Gałązka and Niemirowicz-Szczytt, 2013). However, many reports show low frequency of gynogenesis and/or regeneration rates that limit their success in breeding programs. Several factors have been reported to affect the gynogenetic potential of ovules/ovaries in cucumber, including genotypes of donor plants, low/high temperature pretreatment, female gametophyte developmental stages, growth regulators or other components of media, and culture conditions (Gémes-Juhász *et al*., 2002; Suprunova and Shmykova, 2008; Diao *et al*., 2009; Chen *et al*., 2011; Gałązka and Niemirowicz-Szczytt, 2013; Mishra and Goswami, 2014). Diao *et al*. (2009) reported that the highest embryo formation frequency (72.7%) was obtained by adding 0.04 mg/L TDZ into the induction medium, and a thermal shock at 35°C for 3 days had a positive effect on the embryo formation. Similarly, the medium containing 0.04 mg/L TDZ gave the highest proportion of embryogenesis (32.2–48.1%) (Moqbeli *et al*., 2013).

The effects of several factors on embryo formation is,

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however, shown to be genotype-dependent, and may need to be determined individually for each cultivar to maximize efficiency (Gürel *et al*., 2000; Burbulis *et al*., 2007; Moqbeli *et al*., 2013). Moreover, recently it was reported that combinations of TDZ and BA enhanced embryogenesis of rice-bean and led to more prolific callus, ELS and shoot formation than using TDZ singly in *Arachis paraguariensis* (Aina *et al*., 2012; Saini and Chopra, 2012). Therefore, it is possible that supplementation of both TDZ and BA into an induction medium may result in higher ELS and callus formation efficiencies in unpollinated ovary culture of cucumber. In Thailand, success in the gynogenic induction of haploid/doubled haploid cucumber is still limited. No plant regeneration was observed from ovule-derived calli of a Thai cucumber cultivar in a study by Sampaokaew (Sampaokaew *et al*., 2009).

The objective of this study was to examine the effects of several factors affecting *in vitro* gynogenesis including genotypes of donor plants, induction and differentiation media, and thermal shock pretreatment on ELS and callus formation in the unpollinated ovary culture of five cucumber cultivars in Thailand that are productive and/or moderately resistant or resistant to downy mildew (*Pseudoperonospora cubensis*). The optimized ovary culture conditions will be useful for efficient haploid/doubled haploid production in the future.

Materials and Methods

Plant Materials

Five genotypes of cucumber, cv. 'Chai Lai' (Chia Tai Co., Ltd.), 'Big C' (East-West Seed Co., Ltd.), 'Saifa 185' (Metro Seed Agricultural Co., Ltd.), 'Meechai' (Advance Seeds Co., Ltd.) and 'Mini Kings' (Syngenta Seeds Co., Ltd.) were used as donor plants in this study. Plants were grown in cement blocks at Suranaree University of Technology, Nakhon Ratchasima, Thailand, and maintained using standard horticultural practices. 'Chai Lai' and 'Saifa 185' were determined to be resistant to downy mildew, while 'Big C' and 'Meechai' were moderately resistant to downy mildew (Tantasawat *et al.*, unpublished data). All five cultivars produce relatively high yields and are used for commercial production in Thailand.

Unpollinated Ovary Culture

Female flowers were harvested from donor plants for only three weeks after the first female flower appeared. Unpollinated ovaries were obtained from female flowers one day prior to anthesis. Petals, sepals and styles were removed, and ovaries were surface sterilized in 70% ethanol for 1 min, followed by a 30 min incubation period in 25 mg/L streptomycin sulfate solution, a 15 min incubation period in 0.1% (w/v) mercuric chloride solution, and three rinses of sterile distilled water. The peels of sterile ovaries were removed, and the ovaries were further sterilized in 3% (v/v) hydrogen peroxide for 30 s. Ovaries were then sliced longitudinally into two equal halves under sterile conditions and placed on five different induction media. All induction media had Murashige and Skoog (1962; MS) as basal medium, supplemented with 800 mg/L glutamine and solidified with 0.3% (w/v) gelrite. However, they differed in composition of growth regulators, putrescine and sucrose concentrations: I1 (0.02 mg/L thidiazuron [TDZ], 4% (w/v) sucrose), I2 (1 mg/L TDZ, 1 mg/L 6-benzylaminopurine [BA], 3% (w/v) sucrose), I3 (0.18 mg/L BA, 0.1 mg/L kinetin [KIN], 0.2 mg/L 2,4-dichlorophenoxyacetic acid [2,4-D], 3% (w/v) sucrose), I4 (0.5 mg/L KIN, 1 mg/L 4-chlorophenoxyacetic acid [CPA], 3% (w/v) sucrose) and I5 (2 mg/L BA, 0.5 mg/L indole-3-acetic acid [IAA], 1 mg/L gibberellic acid [GA_3], 32 mg/L putrescine, 3% (w/v) sucrose). Ovaries were kept on induction media in the dark at either 25°C for 2 weeks or at 35°C for 3 days followed by 25°C for 11 days, and then transferred to three different differentiation media. All differentiation media had MS as basal medium, supplemented with 3% (w/v) sucrose, 20 mg/L ascorbic acid, 100 mg/L proline and solidified with 0.3% (w/v) gelrite, but varied in growth regulators and silver nitrate: D1 (3 mg/L BA, 2 mg/L 1-naphthalene acetic acid [NAA]), D2 (0.2 mg/L BA, 0.05 mg/L NAA), and D3 (0.1 mg/L KIN, 0.1 mg/L IAA, 2 mg/L silver nitrate). The pH of all induction and differentiation media was adjusted to 5.7 before autoclaving at 121°C for 20 min. After being transferred to differentiation media, ovary pieces were cultured at 25°C under a 16/8 h (light/dark) photoperiod. Four weeks after being transferred to differentiation media, the percentages of ELS or callus formation were calculated from the number of ovary pieces forming ELSs or calli/total number of ovary pieces × 100. Percentage values were transformed with arcsine transformation before statistical analysis.

A completely randomized design (CRD) in a factorial experiment with four factors (genotypes [5 cultivars], thermal shock pretreatment [25 and 35°C], induction media [I1–I5] and differentiation media [D1–D3]) was conducted with 6 replications (ca. 1,800 ovary pieces/replication). One way analysis of variance (ANOVA) was used to evaluate the effects of genotypes, thermal shock pretreatment, induction media and differentiation media as well as their interactions on ELS and callus formation using SPSS Software version 14 (Levesque and SPSS Inc., 2006). The means were compared by Duncan's multiple range test (DMRT; $P < 0.05$).

Results

ELSs and calli began to appear on the surface of ovules at approximately 2 to 3 weeks after culture initiation. Genotypes of donor plants, thermal shock pretreatment, and induction media significantly affected the percentages of ELS formation in cucumber ($P<0.01$), while percentages of callus

formation were only influenced by genotypes of donor plants and induction media (P<0.05 and P<0.01, respectively). No significant effect of differentiation media was observed on either ELS or callus formation. The interactions among the four factors studied on ELS and callus formation were not significant except for the interaction between temperature pretreatment and induction media, which had a significant effect on ELS formation (P<0.05).

Effect of Genotypes of Donor Plants

After 4 weeks of culture on various differentiation media, numbers of ovary pieces with clearly observed ELS and/or calli were recorded and calculated for percentages of ELS/callus formation. Genotypic variation was evident among the five cucumber cultivars. 'Chai Lai', 'Big C' and 'Saifa 185' had significantly higher ELS formation abilities than 'Mini Kings'. Similarly, callus formation abilities of 'Chai Lai' and 'Big C' were significantly higher than 'Meechai' and 'Mini Kings' (Table 1).

Effect of Thermal Shock Pretreatment

Thermal shock pretreatment at 35°C for 3 days significantly reduced the percentages of ELS formation *ca.* 1.3-fold. However, the percentages of callus formation were similar under both 25 and 35°C (Table 2).

Effect of Induction Media

When five induction media (I1, I2, I3, I4 and I5) varying in growth regulators, putrescine and sucrose concentrations were evaluated on all five cultivars and at two pretreatment temperatures, it was found that there were strong differences among induction media in ELS inducing ability. I2 was the best induction medium for ELS formation (60.4%), followed by I1 (51.4%), I5 (37.6%), I3 (37.0%), and I4 (19.3%), respectively. The percentage of ELS formation on I2 medium was signicantly higher than those on other media, and was 3-fold higher than that on I4. However, the highest percentage of callus formation was achieved on I5, which was significantly higher than the other media (Table 3). Although there was a significant interaction between induction media and thermal shock pretreatment (P < 0.05), I2 was found to be the best medium for ELS induction at both 25 and 35°C (data not shown). The percentages of ELS formation observed on I1 and I2 ranged from 38.4 to 64.6% and 42.3 to 91.4%, respectively (Table 4).

Effect of Differentiation Media

Three differentiation media (D1, D2, D3) differing in types of cytokinin and auxin as well as silver nitrate were evaluated on ELS and callus formation potentials. It was found that more ELSs developed after transferring to the differentiation medium, however, all differentiation media led to equivalent percentages of ELS and callus formation

Table 1: Effects of cucumber genotypes on percentages of embryo-like structure (ELS) and callus formation

Cultivars	ELS formation (%)	Callus formation (%)
Chai Lai	*44.74±3.13a	58.36±3.05ab
Big C	44.60±3.54a	61.56±3.39a
Saifa 185	41.82±3.30a	50.94±3.24bc
Meechai	39.50±3.12ab	46.45±3.06c
Mini Kings	32.02±2.94b	48.02±3.10c

*Data are presented as means ± SE. Data not followed by the same letter in a column are significantly different (P < 0.05) based on DMRT

Table 2: Effects of thermal shock pretreatment on percentages of embryo-like structure (ELS) and callus formation

Temperature (°C)	ELS formation (%)	Callus formation (%)
25	*46.27±2.08a	54.00±2.06
35	35.22±1.96b	52.31±1.98

*Data are presented as means ± SE. Data not followed by the same letter in a column are significantly different (P < 0.05) based on DMRT

Table 3: Effects of induction media on percentages of embryo-like structure (ELS) and callus formation

Induction media	ELS formation (%)	Callus formation (%)
I1	*51.36±2.86b	43.10±3.08c
I2	60.40±3.06a	55.99±3.43b
I3	37.03±3.47c	46.90±3.58bc
I4	19.27±2.53d	52.05±2.64b
I5	37.62±3.14c	70.76±2.73a

[a]Data are presented as means ± SE. Data not followed by the same letter in a column are significantly different (P < 0.05) based on DMRT

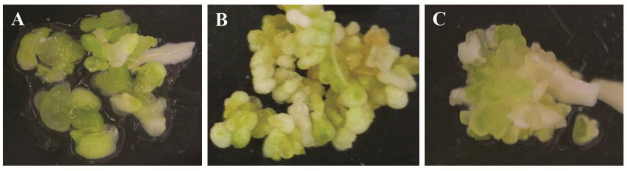

Fig. 1: Embryo-like structures (ELSs) formed on various differentiation media (A) D1, (B) D2 and (C) D3. Most ELSs derived from ovules on D2 was greenish white or light green, opaque and compact. Some ELSs derived from ovules on D1 and D3 had the same appearance as those on D2, but some ELSs appeared green and translucent

(Table 5). Nevertheless, the morphology of ELSs differed on various differentiation media. The appearance of most ELSs derived from ovules on D2 was greenish white or light green, opaque and compact. This type of ELSs also arose from ovules on D1 and D3, but many ELSs on these two media appeared green and translucent (Fig. 1).

Discussion

Our study showed that genotypes of donor plants significantly affected ELS and callus formation abilities among five Thai cucumber cultivars. This is not surprising

since the effects of genotypes on ELS and/or callus formation have been demonstrated in ovule/ovary culture of numerous plants such as sugar beet, cucumber, summer squash, *Nicotiana rustica* etc. (Katoh and Iwai, 1993; Gürel *et al*., 2000; Shalaby, 2007; Suprunova and Shmykova, 2008; Moqbeli *et al*., 2013). Nevertheless, Diao *et al*. (2009) reported that embryo formation frequencies of six cucumber cultivars were not variable.

Temperature shock (high or low temperature) is recommended to improve gynogenesis by diverting normal gametophytic development into a sporophytic pathway resulting in haploid embryo formation (Shalaby, 2007; Chen *et al*., 2011). Recently, it has been shown that thermal shock pretreatment of ovary slices at 35°C for 2–4 days significantly induced the embryo formation of six Chinese cucumber cultivars (Diao *et al*., 2009). Gémes-Juhász *et al*. (2002) also reported that 35°C thermal shock pretreatment was effective in the cucumber ovary culture of five parthenocarpic breeding lines and a hybrid variety. Using six cucumber hybrids, it has been reported that the highest proportion of embryogenesis was obtained with 3 days of 35°C pretreatment (Moqbeli *et al*., 2013). Similarly, pretreatment of summer squash ovaries at 4 or 32°C for 4 days also produced a better embryogenic response than untreated control (Shalaby, 2007). However, this effect is likely to be genotype-dependent since we did not observe any beneficial effect of thermal shock pretreatment on the ELS formation of the five Thai cultivars. Insensitivity to thermal shock pretreatment on several plants has also been reported (Metwally *et al*., 1998; Gugsa *et al*., 2006). It was shown that squash ovules without cold pretreatment at 4°C produced a better embryogenic response than the ones treated at 4°C for 2, 4 or 8 days (Metwally *et al*., 1998). Similarly, pretreatment at 4°C for up to 9 days or at 32°C for 1 day did not improve gynogenic development of tef pistil culture (Gugsa *et al*., 2006).

ELS and callus formation potentials varied significantly when using different induction media. I2 was the best induction medium for ELS formation, while I5 was the best induction medium for callus formation. Although some researchers were successful in obtaining haploid/doubled haploid plants from ovule-derived calli (Wei *et al*., 2006; Diao *et al*., 2009; Pathirana *et al*., 2011), we were unable to regenerate any plantlets from calli. In all of our experiments, haploid/doubled haploid plants only developed from ELSs (Tantasawat *et al.*, unpublished data). Therefore, I1 and I2, which induced the highest ELS formation, were considered the best induction media for efficient haploid/doubled haploid production. It is interesting to note that both I1 and I2 contained TDZ as growth regulators either singly or in combination with BA. TDZ has been frequently reported as the most efficient growth regulator for gynogenesis induction in cucurbit crops (Gémes-Juhász *et al*., 2002; Suprunova and Shmykova, 2008; Diao *et al*., 2009; Malik *et al*., 2011; Li *et al*., 2013). The percentages of ELS formation obtained

Table 4: Effects of cucumber genotypes, thermal shock pretreatment and induction media on percentages of embryo-like structure (ELS) and callus formation

Cultivars	Temperature (°C)	Induction media	ELS formation (%)	Callus formation (%)
Chai Lai	25	I1	58.43 ± 9.36*	44.85 ± 10.41
		I2	60.13 ± 9.66	60.08 ± 10.32
		I3	56.59 ± 11.03	66.67 ± 11.89
		I4	31.22 ± 7.89	52.20 ± 5.86
		I5	50.65 ± 10.51	70.51 ± 8.54
	35	I1	47.13 ± 8.31	49.03 ± 10.98
		I2	68.60 ± 12.90	81.03 ± 9.97
		I3	38.16 ± 11.23	51.45 ± 9.84
		I4	18.70 ± 7.81	52.76 ± 8.38
		I5	30.85 ± 8.92	68.95 ± 8.82
Big C	25	I1	59.97 ± 11.58	64.33 ± 12.29
		I2	67.22 ± 9.60	51.43 ± 10.67
		I3	46.56 ± 16.25	37.50 ± 18.48
		I4	26.09 ± 10.59	59.41 ± 10.52
		I5	58.96 ± 12.11	90.76 ± 4.66
	35	I1	47.45 ± 10.16	63.27 ± 10.65
		I2	63.74 ± 10.49	62.55 ± 9.74
		I3	51.88 ± 11.70	53.13 ± 12.47
		I4	22.92 ± 9.96	62.73 ± 9.74
		I5	19.67 ± 4.76	65.40 ± 7.81
Saifa 185	25	I1	59.55 ± 7.28	34.96 ± 8.36
		I2	91.43 ± 5.58	70.91 ± 12.23
		I3	26.92 ± 9.11	46.65 ± 9.88
		I4	8.69 ± 4.91	50.51 ± 10.13
		I5	57.92 ± 10.31	62.79 ± 10.63
	35	I1	64.60 ± 8.89	47.57 ± 8.13
		I2	54.91 ± 6.97	43.03 ± 11.85
		I3	36.67 ± 11.33	54.22 ± 12.07
		I4	5.27 ± 2.10	41.76 ± 7.65
		I5	18.50 ± 8.85	70.06 ± 11.05
Meechai	25	I1	45.01 ± 9.29	37.59 ± 9.81
		I2	58.87 ± 9.37	46.87 ± 10.58
		I3	45.18 ± 11.37	48.50 ± 11.42
		I4	23.17 ± 8.85	57.23 ± 6.93
		I5	61.09 ± 9.13	72.94 ± 7.74
	35	I1	41.98 ± 7.74	26.64 ± 6.70
		I2	55.20 ± 11.44	51.72 ± 11.36
		I3	16.46 ± 6.99	21.75 ± 7.77
		I4	23.11 ± 9.47	54.11 ± 7.84
		I5	26.25 ± 6.17	64.76 ± 8.66
Mini Kings	25	I1	53.08 ± 8.81	41.28 ± 10.41
		I2	46.68 ± 8.27	48.46 ± 10.35
		I3	25.77 ± 8.98	33.25 ± 9.34
		I4	19.57 ± 8.93	46.72 ± 9.17
		I5	32.75 ± 9.74	73.26 ± 8.00
	35	I1	38.41 ± 9.69	34.42 ± 8.96
		I2	42.33 ± 9.89	52.59 ± 10.23
		I3	27.41 ± 9.65	49.23 ± 10.96
		I4	12.86 ± 5.96	42.93 ± 7.43
		I5	21.19 ± 9.10	71.36 ± 8.38

*Data are presented as means ± SE

Table 5: Effects of Differentiation media on percentages of embryo-like structure (ELS) and callus formation

Differentiation media	ELS formation (%)	Callus formation (%)
D1	41.23 ± 2.52[a]	53.37 ± 2.57
D2	40.63 ± 2.56	55.29 ± 2.45
D3	40.01 ± 2.41	50.76 ± 2.41

[a]Data are presented as means ± SE

on I1, which contained 0.02 mg/L TDZ (38.4 to 64.6%) was comparable to that reported by Diao *et al*. (2009) using 0.02 mg/L TDZ with six Chinese cucumber cultivars (20 to 65.7%), confirming the TDZ efficiency of the ovary culture of cucumber. However, at higher TDZ concentration (0.04 mg/L), they found up to 72.7% embryo formation. With two different Chinese cucumber inbred lines, the highest embryo induction frequencies (11.1–12.1%) were achieved using 0.07 mg/L TDZ (Li *et al*., 2013). In melon, 0.04 mg/L TDZ could also induce embryo formation of a Chinese cultivar up to 76.6% when combined with 4°C pretreatment for 4 days (Malik *et al*., 2011). When using I2, which contained a higher TDZ concentration (1 mg/L) together with 1 mg/L BA, we were able to induce higher ELS formation efficiency (up to 91.4%). These results suggested a synergistic effect of TDZ and BA on embryogenesis, which was in consistent with those reported by Aina *et al*. (2012) and Saini and Chopra (2012). This new induction medium will be very useful for haploid/doubled haploid production in the future.

Previous work has shown that differentiation of cucumber ovule tissues required an appropriate ratio of cytokinin and auxin (Lotfi *et al*., 2003; Suprunova and Shmykova, 2008; Plapung *et al*., 2014). In this study, we found no effect of differentiation media on percentages of ELS and callus formation although the morphology of ELSs on various differentiation media appeared different. The regenerability of those ELSs into plantlets is currently being evaluated. It is likely that the induction of gynogenic competence and formation of ELSs was predetermined early by an induction medium, and the differentiation medium might play a later role in the differentiation of ELSs into complete plantlets.

Conclusion

We have developed improved procedures for the induction of ELSs from the unpollinated ovary culture of cucumber. The percentages of ELS formation of cucumber depended on genotypes of donor plants, temperature pretreatment and induction media, while differentiation media affected ELS appearance but had no influence on the percentages of ELS formation. By contrast, only genotypes and induction media had an effect on callus formation. The best induction medium for ELS formation was I2, and the optimal temperature was 25°C. Of the five different cucumber cutivars, 'Chai Lai' and 'Big C' had the highest ELS formation potentials. High percentages of ELS formation were achieved in several Thai cucumber cultivars, which are very promising for the future production of haploid/doubled haploid plantlets.

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