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SCREENING FOR CUCUMBER MOSAIC RESISTANT LINES FROM THE OVULE CULTURE DERIVED DOUBLE HAPLOID CUCUMBERS

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ABSTRACT

CMV is one of the major destructive viruses worldwide and commercial CMV resistant cucumber is very rare. Therefore we aimed to establish the ovule derived resistant lines used for the breeding program. Haploid plants of sixty-eight cucumber lines were successfully obtained by culturing the un pollinated ovaries harvested one day before an thesis on a modified MS medium supplemented with BAP and IAA at the ratio of 2:1 which was optimal to induce embryogenesis in most of the tested lines. For whole plant regeneration, another modified MS medium was used supplemented with a combination of 6-Benzylaminopurine (BAP) and Indole-3-Acetic Acid (IAA) (2:1) or BAP and IAA/6-(gamma, gamma-dimethylallylamino) purine (2ip) and IAA (5:1) and 5 ppm $AgNO_3$. Ploidy levels of the regenerants were determined by cytological analysis. Thirteen out of 42 clones derived from 14 accessions showed a chromosome number of n = 7 and chloroplast number of 6/pair of guard cell, 24 lines were auto-dihaploid with n = 14 and a chloroplast number of 11-12/pair of guard cell. Twenty-eight Double Haploid (DH) lines were mechanically inoculated with CMV and the level of resistance was evaluated by using DAS-ELISA. Ten highly Resistant lines (R) included $70S_2$, 91e, 91.1, $93S_4$-1, $93S_4$2-, 95S1-$_2$, $95S_2$ DHS_1, 117S2-1-3, 136.1 and $194S_1$ did not show any virus symptom and gave negative ELISA results. Twelve moderately resistant clones were identified including two clones from line 11, three clones from line 93, four clones from line 91 and one clone each from line $117S_2$ and 123, whereas clone 11.4 was moderately susceptible. Five DH clones; $117S_2$-1-1, $117S_2$-2, $117S_2$-4, $117S_2$-7 and $117S_2$-8were classified as highly susceptible.

Keywords: CMV Resistance, Cucumber Screening, DAS-ELISA, Ovule Culture, Double Haploid Cucumber

1. INTRODUCTION

Cucumber (*Cucumis sativus*) is one of the most economically important vegetable crops in Thailand where it is grown intensively all year around. In 2008, the total cultivated area of cucumber was 43,063.5 hectares producing 193,170 tons, for an average of 4.45 ton/acre. Most of the cucumbers in Thailand are produced for fresh consumption and seed sold in our country and foreign countries. In 2012, a total of 87.82 tons was exported to other countries and made the income of 260.19 million baths which less than 2011 (105.47 tons, 298.47 million baths) (OAE, 2014). The OAE data, indicates that the quantity of exported seed has decreased each year, primarily due to outbreaks of many diseases in the cucumber production areas. Cucumber varieties are severely affected by several diseases caused by fungi, bacteria, nematodes and particularly viruses. The Cucumber Mosaic Virus (CMV) is one of the important viruses in temperate, tropic and sub tropic regions of the world which could cause crop

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losses on average of 10-20% (Zitter and Murphy, 2009). Most of the commercial cucumber cultivars are susceptible to CMV and show mosaic, mottling and distortion of leaves and fruits. Moreover, CMV is one of the most widely spread virus in the world infecting over 1,000 plant species belonging to more than 85 families (Roossinck, 2002). This virus is transmitted by several species of aphid in a non-persistent manner which is an easy way to transmit the virus from the infected plant to healthy ones. Moreover, CMV can also be transmitted by mechanical means. Due to the wide host range, rapid spread by vectors and lack of suitable resistant hosts, management of CMV is difficult by cultural practices alone (Munshi *et al*., 2008). Unfertilized ovaries could be cultured on suitable medium for haploid plant production and chromosomes doubled by an *in vitro* treatment with colchicines (Claveria *et al*., 2005). DH lines produced from ovule culture are obtained in a shorter time and show greater variability than those obtained by self-pollination. Moreover, homozygous double haploid lines from new cucumber accessions could be used to accelerate breeding for new resistant commercial varieties. Therefore, the purpose of this study are to establish Double Haploid (DH) plant lines by ovule culture and to screen DH lines.

2. MATERIALS AND METHODS

2.1. Plant Materials and Ovary Preparation

Sixty-eight varieties of cucumber seeds from different origins as shown in **Table 1**. Seeds of these varieties were planted in seed trays. Seedlings at the cotyledon stage were transferred to 30 cm pots and kept under greenhouse conditions. Un-pollinated ovaries were harvested 1 d before an thesis. Petals and styles were removed and surface sterilized twice using 10% calcium hypochlorite and 3% sodium hypochlorite for 20 min. Each, followed by rinsing three times with sterile distilled water.

2.2. Ovule Culture

Ovaries were aseptically sliced in a laminar air flow cabinet and cultured on the Cucumber Basal Medium (CBM medium) supplemented with 5 ppm $AgNO_3$ for 1 month. After that, embryoids were transferred to a modified Murashigeand Skoog (MS) medium supplemented with kinetin,6-Benzylaminopurine (BAP)/6-(gamma, gamma-dimethylallylamino) purine (2ip): Indole-3-Acetic Acid (IAA) at a ratio of 2:1, 3:1 and 4:1 ppm for 2 months. The green embryos were transferred to MS medium supplemented with BA/2ip: IAA at a ratio of 2:1, 3:1, 4:1 and 5:1 ppm. The cultures were incubated at 25°C with 16 h day and 8 h night. The regenerants were cultured on MS medium for rooting.

2.3. Ploidy Level Determination

2.3.1. Chromosome Number

Tendrils or root tips of growing plantlets were used for chromosome number counting according to the method described.

2.3.2. Guard Cell Chloroplast Number

Fully expanded leaves from the top of the ovule-derived plants were collected. Epidermal strips were peeled from the lower leaf surface using tweezers. The epidermal strips were and mounted in sterile distilled water on microscope slides. The guard cell chloroplast numbers were observed under the light microscope and pictures taken for the later counting.

Table 1. Origins and accession codes of cucumbers used for DH production

Countries of origin	Number	Accessions
India	2	PI 197086 and PI 209065
China	6	PI 432854, PI 432860, PI 432868, PI 432875, PI 432895 and PI 464873
USA	3	Tender green, space master and green dragon
Netherland	1	PI 422182
Philippines	1	PI 426169
Japan	2	PI 432852 and PI 279466
Pakistan	1	PI 250147
Malaysia	1	PI 358813
Thailand	51	CSL 0001, CSL 0002, CSL 0003, CSL 0005, CSL 0006, CSL 0007, CSL 0008, CSL 0009, CSL 0010, CSL 0011, CSL 0012, CSL 0013, CSL 0014, CSL 0015, CSL 0021, CSL 0023, CSL 0026, CSL 0033, CSL 0035, CSL 0037, CSL 0040, CSL 0043, CSL 0044, CSL 0045, CSL 0048, CSL 0050, CSL 0051, CSL 0052, CSL 0053, CSL 0054, CSL 0055, CSL 0056, CSL 0060, CSL 0063, CSL 0071, CSL 0074, CSL 0075, CSL 0076, CSL 0078, CSL 0079, CSL 0080, CSL 0081, CSL 0084, CSL 0085, CSL 0086, CSL 0093, CSL 0097, CSL 0098, CSL 0099, TOT1974, F_1Meechai and F_1 Junior6

2.4. CMV Resistant DH Lines Screening

Twenty-eight lines of DH lines plantlets were grown in pots under the insect proof nursery conditions. Leaves at 2-3from top were lightly dusted with carborundum (400 mesh) and rub-inoculated with virus-infected sap (approximately 1:5 dilution leaf material: 0.1 M phosphate buffer pH 7.0). After 15 min, the inoculated leaves were rinsed with tap water and kept under insect proof net house condition. Inoculated plants were monitored at 4 weeks after inoculation; symptoms and disease severity were recorded and tested for virus using DAS-ELISA. Resistant levels based on the ELISA reading were modified from Daryono *et al*., (2003).

3. RESULTS

3.1. Cucumber Ovule Culture

Ovaries from 68 cucumber accessions from various origins were collected 1 d before an thesis (**Fig. 1A**). After surfaced sterilization, thin ovary sections were cultured on a CBM medium and subsequently transferred to induction and differentiation medium respectively. One week after culture, the growth of ovules protruded from the slices and globular embryogenic tissue formed (**Fig. 1B**). The tissues were subsequently transferred to MS plus BAP and IAA at a 2:1 ratio for the further regeneration. The first visual shoot-like organogenesis appeared as cotyledon-like structures (**Fig. 1C**) found at 2 weeks after setting on the medium. New shoot, mostly 2-5 elongated ones, developed from each callus (**Fig. 1D**). These plantlets rooted after transfer to MS medium (**Fig. 1E and F**) and were ready to be transplanted into the pots within 2 weeks (**Fig. 1G and F**). Forty-two clones from fourteen accessions were obtained namely: 1 clone from PI 197086 (India), 11 clones from PI 209065 (India), 1 clone from PI 464873 (China) and 29 clones from the Thai accessions which consisted of 1 clone from CSL 0006, 7 clones from CSL 0011, 4 clones from CSL 0013, 2 clones from CSL 0015, 1 clone from CSL 0021, 9 clones from CSL 0037 and one clone from each of CSL 0043, CSL 0052, CSL 0056, CSL 0059 and 1.F1 Meechai (**Table 2**).

Induction and regeneration of cucumber plantlets were successfully achieved when ovule slices were cultured on the MS medium supplemented with cytokinin (BA, 2ip or kinetin) and auxin (IAA), however the number of the derived clones varied with the different ratios of cytokinin and auxin used. The accessions PI 197086 (India) and PI 209065 (India) showed good sprouting of the embryo when cultured on MS medium plus BA: IAA ratio of 2:1 and new plants were obtained after transfer to MS plus BA: IAA at a ratio of 4:1. New plants were obtained from the China accession 70 PI 464873 when ovules were cultured on a medium with a ratio of 2:1 BA: IAA in both the induction and regeneration stages. The Thai accessions, e.g., 86 S_2 CSL 0006, 91 CSL 0011and 93S_1 CSL 0013 responded best to different hormone ratios in the induction and regeneration stages. It could be concluded that cucumber ovules reacted differently to the hormonal concentration and ratios based on genetic background as shown in **Table 2**.

3.2. Ploidy Level Determination

Direct chromosome counting of the plants derived from the ovule culture showed abasic chromosome number of n = 7 in all haploid plants which was correlated strongly with the chloroplast number in the guard cell (6 chloroplast/pair of guard cell). From all 42 clones derived from 14 accessions, 11 had a chromosome number of n = 7 and were haploid plants, 24 clones were identified as auto-diploid plants with a chromosome number of 2n = 14 and the chloroplast number of guard cell were 11-12 chloroplast/pair of guard cell. The other seven clones had slightly lower chromosome numbers than the diploid plant (**Fig. 2 and Table 3**). Besides the difference in chromosome numbers of the haploid and auto-dihaploid plants, different phenotypes were observed. The plantlets after transplanted and kept under the nursery conditions showed slower growth and had smaller size than the normal parent line plants which later one characterized as haploid plants. For the auto-dihaploid plants, they developed into normal plants like the parental lines.

3.3. CMV Resistant DH Lines Screening

The 28 DH clones were screened by mechanical inoculation with virulent CMV Ion cucumber leaves at the second to third true-leaf stage. CMV resistant levels were determined by using DAS-ELISA; it was found that DH cucumbers had different levels of resistance to the virus as shown in **Table 4**. Ten clones that showed no CMV I symptoms 30 days after inoculation and had negative ELISA compared to the healthy control plants and the negative control probe, were identified as a highly Resistant (R) group which included 70S_2, 91e, 91.1, 93S_4-1, 93$S_4$2-, 95S1-$_2$, 95S_2DHS_1, 117S3-1-$_2$, 136.1 and 194S_1. Of twelve Moderately Resistant (MR) clones, 91a, 91b, 91c, 91d, 93S2, 93.1,93.3 and 117S_2 -1-2 showed mild mosaic, while 11a, 11.3, 123 and 117S_2-5 showed no sign of virus infection but had ELISA readings slightly higher than the healthy control plants and negative control probe. Clone 11. 4was classified as Moderately Susceptible (MS) and exhibited mild to severe mosaic symptoms and malformation (**Table 4 and Fig. 3**). Five clones, 117S2-1-1, 117S2-2, 117S2-4, 117S2-7 and 117S2-8, were classified as Susceptible (S).

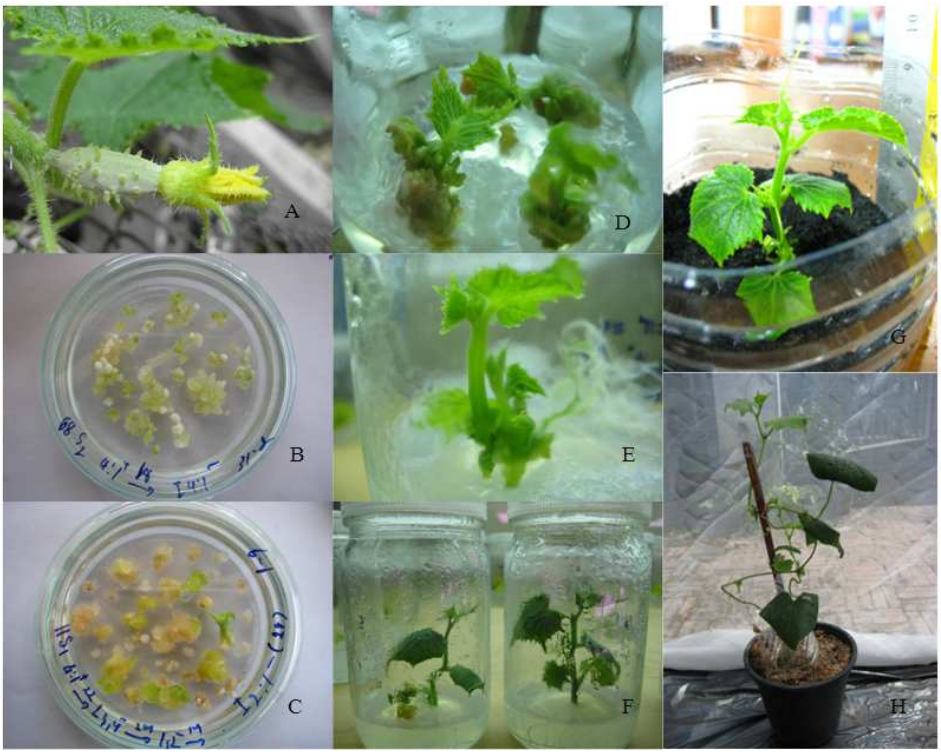

Fig. 1. Embryogenesis and regeneration of plantlets derived from ovule culture (A) Un pollinated ovary of cucumber 1 day before an thesis; (B) sprouting embryo (C) shoot-like organogenesis; (D) elongated shoots (E and F) regenerated plantlets; (G) *in vitro* grown regenerated plants; (H) ovule derived plant after transfer to the insect proof net house

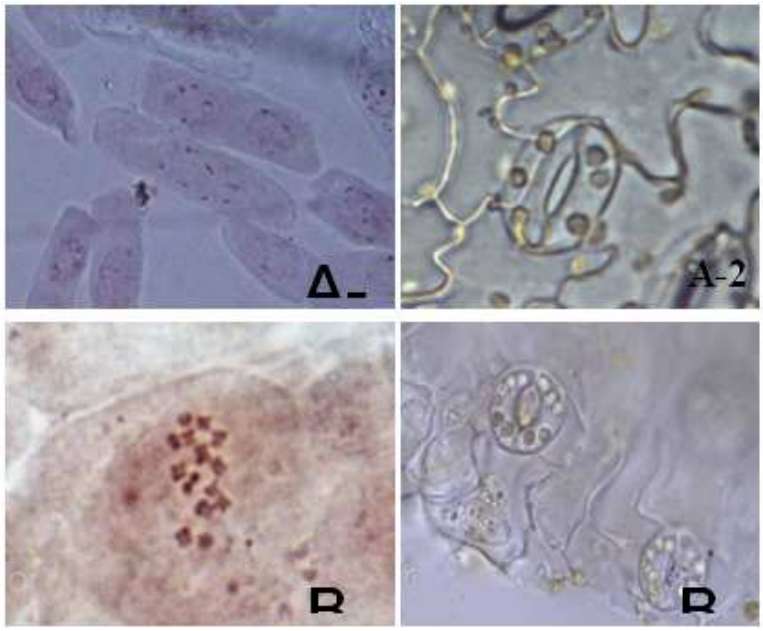

Fig. 2. Chromosome and chloroplast number of haploid and auto-dihaploid cucumber plants derived from ovule culture A 1-2 Chromosome (n = 7) and chloroplast (6) of haploid. B 1-2 Chromosome (2n = 14) and chloroplast (12) of auto-dihaploid

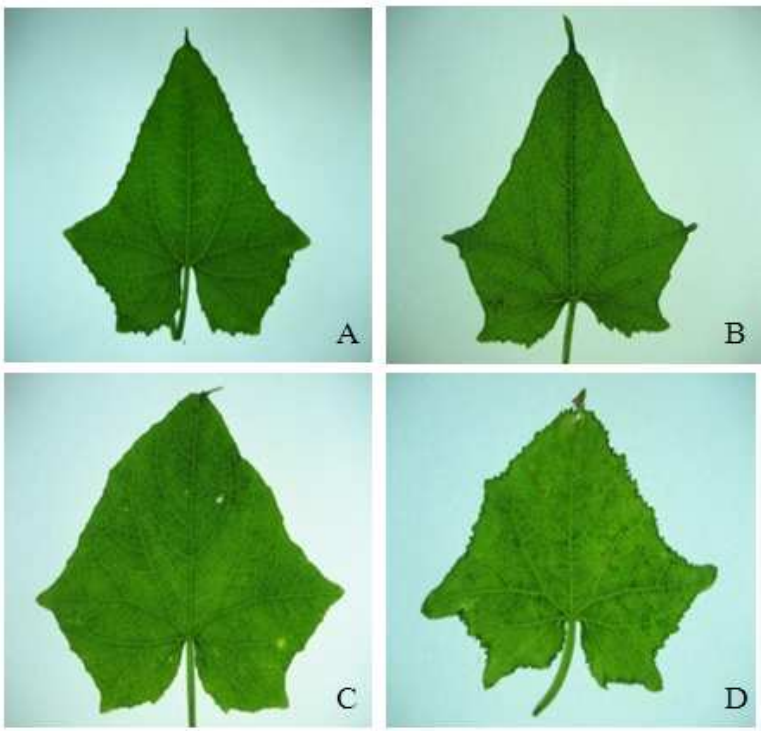

Fig. 3. Different resistant levels of double haploid cucumbers as infected by CMV I. A = Resistant B = Moderately resistant C = Moderately susceptible D = Susceptible

Table 2. Number of clones derived from the ovule culture of different cucumber accessions and ratios of hormones used in different culture stages

Accessions	Code	Induction stage (mg/L)	Regeneration stage (mg/L)	Number of clone(s)
PI 197086	10	BA:IAA(2:1)	BA:IAA(4:1)	1
PI 209065	11	BA:IAA(2:1)	BA:IAA(2:1)	1
PI 209065	11	BA:IAA(4:1)	BA:IAA(2:1)	10
PI 464873	70	BA:IA (2:1)	BA: IAA(2:1)	1
CSL 0006	86S2	BA;:AA(2:1)	2ip: IAA(5;1)	1
CSL 0011	91	BA;:IAA(2:1)	BA: IAA(2:1)	5
CSL 0011	91	2ip 1mg	BA: IAA(2:1)	2
CSL 0013	93S1	BA:IAA(2:1)	BA:IAA(2:1)	2
CSL 0013	93S4-2	Kinetin 2mg	2ip:IAA(5:1)	2
CSL 0015	95S2	BA:IAA(2:1)	Kinetin 2mg	1
CSL 0015	95 S1	BA: NAA(2:1)	2iP: IAA(5:1)	1
CSL 0021	101	BA:IAA(4:1)	BA: IAA(4:1)	1
CSL 0037	117S2	Kinetin 2mg	BA 1mg	4
CSL 0037	117S2	BA1mg	BA1mg	5
CSL 0043	123a	BA: IAA(2,1)	BA: IAA(5:1)	1
CSL 0052	132 S2	BA :IAA(8:1)	kinetin 0.5mg	1
CSL 0056	136	BA;IAA(3.5,1)	BA: IAA(5:1)	1
CSL 0059	139	BA:IAA(4:1)	BA:IAA(3:1)	1
F1Meechai	194S1	Kinetin 1 mg	Kinetin : IAA (5:1)	1

Table 3. Chromosome and chloroplast numbers of regenerated cucumber plants derived from ovule culture

Clones	Origin	Chromosome number	Chloroplast number (pairs of guard cell)
10 a	India	ND	6±1.15
11.1	India	ND	7.9±0.74
11.2	India	ND	8.80±1.03
11.3	India	14±0	10.30±0.68
11.4	India	8.50±1.18	6.10±0.57
11.8	India	ND	7±0.677
11.9	India	13.20±1.79	7.20±0.79
11.10	India	ND	8.10±0.88
11.19	India	ND	8.70±0.82
11.20	India	ND	8.40±0.70
11A	India	8.57±0.98	7±0.67
11B	India	ND	6.10±0.88
$70S_2$	China	14±0	10.40±0.84
$86S_2$	Thailand	14±0	14.50±1.43
$91S_1$a	Thailand	14±0	10.50±0.53
$91S_1$b	Thailand	14±0	10.90±0.32
$91S_1$c	Thailand	14±0	9.90±0.74
$91S_1$d	Thailand	14±0	11±0
$91S_1$e	Thailand	14±0	10.30±0.82
91.1	Thailand	8.25±0.71	7±0.47
91.2	Thailand	ND	10.10±1.37
$93S_4$	Thailand	13±1.00	10.80±0.42
$93S_4$-2	Thailand	14±0	11±0
93.1	Thailand	12±1.05	11±0
93.3	Thailand	12.60±0.74	11±0
$95S_2$	Thailand	14±0	11±0
$95S_2$-1	Thailand	14±0	11±0
101 S2	Thailand	13.70±0.48	12±1.33
$117S_2$1-1	Thailand	14±0	11±0
$117S_2$1-2	Thailand	14±0	11±0
$117S_2$1-3	Thailand	14±0	11±0
$117S_2$-2	Thailand	14±0	10.80±0.63
$117S_2$-3	Thailand	14±0	11±0
$117S_2$-4	Thailand	14±0	11±0
$117S_2$-5	Thailand	14±0	11±0
$117S_2$-7	Thailand	14±0	11±0
$117S_2$-8	Thailand	14±0	11±0
123a	Thailand	14±0	13.5±0.97
$132S_2$-5	Thailand	14±0	12.6±1.27
$136S_1$	Thailand	14±0	11.7±0.95
139 S_1	Thailand	ND	10.8±0.63
$194s_1$	Thailand	14±0	11.1±0.99

(ND = Not determine due to no root formation)

4. DISCUSSION

The production of DH lines can be effectively used for the development of homozygous varieties in breeding program by the doubling of a set of chromosome from haploid plants to produce a completely homozygous individual. DHs can facilitate the selection of desired (e.g., disease-resistance) genotypes for breeding. Lotfi *et al*. (2003) used a hybrid melon which showed resistance to various viruses as a donor for the production of DH melons. DH cucumber is produced by culturing the ovaries at one day before an thesis to induce new plants through cytogenesis. We found that this stage is suitable for harvesting as reported by Bhagyalakahmi (1999). Ovaries with yellow stigma were the best explants for direct shoot regeneration, while explants from blooming flowers fail to respond.

Table 4. Evaluation of CMV resistance in double haploid cucumber plants generated via ovule culture

Code	Symptoms[1]	Infection percentage (30 days after inoculation)	ELISA[3] (30 days after inoculation)	Resistance level[2]
11.3	0	0	+	MR
11aS1	0	0	+	MR
11.4	M, Mm, Ma	33.33	++	MS
70S2	0	0	-	R
91a	Mm	25	+	MR
91b	Mm	25	+	MR
91c	Mm	25	+	MR
91d	Mm	14.29	+	MR
91e	0	0	-	R
91.1	Mm	13.63	-	R
93.1	Mm	33.33	+	MR
93.3	Mm	75	+	MR
93S2	Mm	40	+	MR
93S4 -1	0	0	+	R
93S4-2	0	0	+	R
95 S2-1	0	0	-	R
95S2 DHS1	0	0	-	R
$117S_2$-1-1	Mo	100	+++	S
$117S_2$-1-2	Mo, Mm	50	+	MR
$117S_2$-1-3	0	0	+	R
$117S_2$-2	M	100	+++	S
$117S_2$-4	Ma	100	+++	S
$117S_2$-5	0	0	+	MR
$117S_2$-7	Mo	68.18	+++	S
$117S_2$-8	Mo	100	+++	S
123	0	0	+	MR
136.1	M,Ma	60	-	R
194 S1	0	0	-	R
Positive	-	-	2.65	-
Negative	o	0	0.31	-

[1)] Symptom on leaf 0 = No symptom, M = Mosaic, Mm = Mild mosaic, S = Stunt, Y = Yellow, IV = Interveinal-chlorosis, D = Distortion, Ma = Malformation

[2)] DAS-ELISA reading at 405 nm of extracts of inoculated leaves -: S≤ H, +: S>H<2xH, ++: S>2x<3xH +++ S>3xH (S = tested sample sap, H = healthy plant sap)

[3)] Reaction R= resistant MR = moderately resistant MS = moderatelysusceptible S = susceptible

Using the combination of calcium hypochlorite and sodium hypochlorite for the surface sterilization was found to be an effective means to eliminate bacteria and fungi from tissue culture, most likely due to synergism. Moreover, calcium ions helped harden the ovary tissue which made it easy to get thinner slices from which the ovules could contact to the medium directly. Moreover, the culture medium composition was critical for the induction of embryogenesis and could be separated by formula into induction and regeneration media (Chen *et al*., 2010). Gynogenesis consists of two stages, firstly, the induction stage, in this stage ovaries required a low level of growth regulators and were kept in the dark; and secondly, the regeneration stage, in this stage ovules were transferred to a medium with a high concentration of growth regulators and kept under light. Ovule cultures from 69 varieties of cucumber resulted in 42 clones from 14 accessions that were successfully induced to form whole plants. Each of the varieties showed a different response to the culture media. We found that an induction medium consisting of MS supplemented with BAP and IAA ratio 2:1 was the most suitable medium for embryogenesis in13 clones. Some clones required a medium with a high hormone concentration (BAP: IAA at a ratio of 5:1) for regeneration, while some accessions were regenerated using the same low hormone level (BAP: IAA at a ratio of 2:1) used for the induction stage. Chen *et al*. (2010) also reported that the donor genotype was one of many factors important for unfertilized ovary/ovule culture;

gynogenesis efficiency in plants was highly dependent on the variety used. Moreover, the quality of donor material and the growth condition of plants affected ovule gynogenesis. Abde1-Maksoud *et al*. (2009) also reported that growth hormone concentration affected the anther culture response traits in cucumber.

The ploidy level of the regenerated plants was easily and rapidly evaluated by counting chloroplast number instamatic guard cells (Jacobs and Yoder, 1989). However, chromosome counting from root tips was the most reliable and precise method (Shengli *et al*., 2002). Forty-two clones of 14 lines were obtained via ovule culture, eleven clones were haploid, the chromosome number was n = 7 and chloroplast number of 6/guard cells, while 24 clones were direct doubled haploids, having a chromosome number of 2n = 14 and chloroplast number of 11-12/guard cells. Another seven clones had chromosome and chloroplast numbers less than the diploid plants. Our research agreed with that of Gémes-Juhasz *et al*. (2002) and Diao *et al*. (2009) who reported that cucumber ovary slice culture was successfully used to directly produce doubled haploids.

Of 28 clones from 9 lines of DH cucumber plants were screening for resistance to CMV, 10 clones were found to be highly Resistant (R) based on the absence of virus symptoms and negative ELISA results. The R cucumbers included 1 clone of accession 70, 2 clones of each 91, 93 and 95 and 1 clone of each $117S_2$, 136.1 and 194. Twelve clones were classified as moderately resistant including 2 clones of 11, 3 clones of 93, 4 clones of 91 and 1 clone of each $117S_2$ and 123, while clone 11.4 was classified as moderately susceptible. Five clones of DH were highly susceptible including $117S_2$-1-1, $117S_2$-2, $117S_2$-4, $117S_2$-7 and $117S_2$-8. The different levels of resistance to CMV exhibited by the regenerants could reflect different levels of defensive reactions. Mandadi and Scholthof (2013) reported that during a viral infection, the Hypersensitive Response (HR) of the resistant plants would be initiated by Avr/Rprotein. The reaction induced metabolic changes which include levels of such phytohormones as Salicylic Acid (SA), Jasmonic Acid (JA) and Nitric Oxide (NO). Moreover, the accumulation of oxygen species such as, O^{2-} and hydrogen peroxide both in the infected and non-infected tissues could differ between regenerated cucumbers. Furthermore, during the HR process, vacuolar processing enzymes are activated which act as effectors of cell death or necrosis (Mur *et al*., 2008).Research is needed to understand the mechanism (s) involved in the resistance of cucumber to CMV infection that we have observed.

5. CONCLUSION

Factors affecting the success of the cucumber ovule culture were identified as the genotypes of the donor plants and the hormonal balance of auxin and cytokinin. A modified MS medium supplemented with BAP and IAA at the ratio of 2:1 was optimal for the ovule culture of most cucumber lines used in the current research and embryo induction was successfully obtained by transfer of the cultured ovules to MS plus BAP and IAA or 2ip and IAA at the ratio of 5:1. After transplantation the plantlets were kept under the nursery conditions and had a slower growth rate were smaller than the normal parent lines which are characteristics of the haploid plants. For the auto-dihaploid plants, they developed normally like the parent lines. Most notably, 10 cucumber clones were found to be highly resistant to CMV showing no symptom of virus infection and negative ELISA results, while 12 clones were moderately resistant to the virus. Future research should include screening the resistant lines for field resistance and their inclusion in the cucumber seed cluster program of the National Center for the Science and Technology Development and make available to the private companies.

6. ACKNOWLEDGMENT

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