Studies on some factors affecting anther culture in six cucumber genotypes

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Abstract: Three experiments were conducted to develop an efficient cucumber (*Cucumis sativus* L., 2n= 2x=14) anther culture protocol. *The first experiment* was conducted to study the effect of genotypes, medium types and stage of male flower development from which anther will be cultured. Difference among cucumber genotypes was significant for their effect on frequency of anther culture development into callus, but callus size was not affected. Medium type (MS) positively affected the percentage of anther development into callus than B5 medium. Anthers from closed flower buds were significantly better in callus formation than those taken from open flowers. The best combination which affected anther growth was achieved using anther derived from closed flower buds of the genotypes Garawani, L82-57 or L88-83 when grown on solid MS medium +2,4-D at 2.0 mg/l+ BA at 1.0 mg/l. As indicated from histological examination, none of the tested treatments showed callus regeneration. *The second experiment* examined the effect of 24 combinations of glutamine and growth regulators types and concentration on callus growth from cold or non-cold treated anther of 6 cucumber genotypes. MS medium amended with 1.0 mg/l 2,4-D+0.5 mg/l BA+ 1.0 mg/l kin was the most effective on callus growth scale. Cold pre-treatment of anther at 4 °C for 48 hr resulted in more vigorous callus than non-cold. When callus was examined under microscope, embryo-like callus was observed in L 88-83 with the culture of cold-treated anther on MS medium + 0.5 mg/l 2,4-D + 0.5 mg/l BA, while shoot primordia appeared in medium containing 1.0 mg/l 2.4-D + 1.0 mg/l BA + 1.0 mg/l kin and 50 mg/l glutamine. Very visible shoot or root initials were observed in non-cold treated anther culture of the genotype WI 1701G in a medium devoid of glutamine, indicating difference among cucumber genotypes in their response to medium composition (PGR and glutamine) or thermal treatment. These results were confirmed by histological analysis. In *the third* experiment anther-derived callus from Exp.2 were sub-cultured into MS medium amended with 3 concentrations of ABA and two sucrose levels, to examine further differention of callus into regenerants. Microscopic examination of the resulted culture indicated shoot bud regeneration in a medium with 0.3 mg/l ABA+ 9% sucrose, while hairy roots were visible from sub culturing callus into 0.2 mg/L ABA+3% sucrose. Embryogenic like callus with side shoots was formed on MS medium + 0.1 mg/l ABA + 9% sucrose. Histological examination provided clear evidence of shoot bud formation in the above medium.

Keywords: Cucumis sativus L., anther culture, haploid plants, cold treatment, medium components.

INTRODUCTION

Cucumber is one of the most important cucurbit crops and has been grown worldwide for food at least since 3000 years. Therefore, improvement of this crop is urgently needed. The improvement of any genotype, especially open-pollinated ones, through conventional breeding, requires several years and adequate facilities. This led to more emphasis on the use of *in vitro* techniques for genetic improvement of particular traits. The cucumber is composed of several genotypes in which the genetics have been extensively studied. This knowledge, together with the ease in growing cucumber, makes it an excellent candidate for use in biotechnology research (Pierce and Wehner, 1990). Tissue culture techniques in cucumber are used primarily for embryo rescue, propagation, embryogenesis and shoot regeneration for genetic engineering system. One recent advance in biotechnology use in cucumber is the anther or pollen culture. The technique was used for improving some vegetable crops, such as asparagus, *Brassica* (Dias and Martins, 1999) and sweet pepper (Kristiansen and Andersen, 1993; Ercan *et al.,* 2006; Koleva-Gudeva *et al.,* 2007), but with limited success in cucumber (Kumar *et al.,* 2004).

First cucumber anther culture was reported in 1982 but without plantlet regeneration (Lazarte and Sasser, 1982). Kumar *et al.* (2003) studied the effects of temperature pretreatment (4 °C and 32 °C) and induction medium on androgenic capability and obtained haploid cucumber plants. Further experiments were carried out to study the effects of different sugars, amino acids (Kumar *et al.,* 2004), and polyamines in the induction medium (Kumar and Murthy, 2004) on anther culture response, but the low anther efficiency has limited its application.

Anther culture involves the culture of immature anther (the pollen-producing organ of the plant) in order to generate plant from the pollen grains before the feundation process takes place, as the pollen grains have only one set of chromosomes, instead of two sets as in the all somatic cells of the plant. The pollen-derived plants are haploids with half the normal number of chromosome which can be doubled to the normal diploid state using chemicals (Gémes Juhász *et al.,* 2002; Yetisir and Sari, 2003; Szarejko and Forster, 2007). Anther culture therefore, offer the possibility of obtaining homozygous plants in a matter of months, rather than the years of breeding required by traditional breeding methods, (Collins and Genovesi, 1982). However, the use of anther culture for production of haploids has been limited. Even when production of haploid was possible, it was usually at low frequency (0.1-1%) for many vegetable crops, including cucurbits (Dias and Martins, 1999; Kumar *et al.,* 2004). Therefore, careful examinations of factors affecting anther culture success are needed. Those factors include genotype effects, stage of anther development in the

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donor plant, pre-conditioning treatments of anther before *in vitro* culture, medium components that affect callus formation and differentiation into complete plantlets. The present study aimed to: (1) evaluate different cucumber genotypes for their *in vitro* anther development, (2) examine several medium components, stage of anther development, cold pre-treatment of anther (pre-conditioning) on callus induction and differentiation and (3) histological analysis of anther-derived callus tissues.

MATERIALS AND METHODS

The following *in vitro* culture experiments were conducted at The Plant Tissue Culture Lab., Department of horticulture, Faculty of Agriculture, Suez Canal University to test the development of cucumber anther into callus, embryogenic callus, or callus differentiation into haploid plantlets under the examined conditions, during the period 2007-2010.

Materials and general procedures:

Seeds of five different cucumber breeding lines were obtained from Institute of Plant Genetic Resources Bulgaria in addition to the Egyptian variety Garawani. Seedlings were raised in foam trays for 3 weeks, and then transplanted into clay pots in the green house until flowering. Male flower buds (4.3 mm length for closed and 8mm for open flowers) were collected from healthy plants between 9:00 and 10:00 am on sunny days in sterile Petri dishes containing double layers of moist filter paper, then sealed with parafilm to maintain a high humidity, and transferred into the tissue culture laboratory. Flower buds were surface sterilized with 70% ethanol for 30 seconds and then with Clorox solution containing 5% sodium hypochlorite (20 ml) with two drops of Tween 20 as surfactant material for 5 min, followed by 3 rinses in sterile distilled water. Anthers were isolated from the male flower buds in a horizontal air-flow hood to be cultured onto the experimental media. In all the following experiments, media pH was set at 5.8 and solidified with 0.7% agar. The media were autoclaved at 121°C for 20 min, then distributed to small round jars (ca. 40 ml) with 10 ml medium per jar or baby food jars (ca. 150 ml) with 20 ml medium per jar.

To examine the stages of pollen grain developments before *in vitro* anther culture, anthers which collected from male flower buds were kept in humid Petri dishes, then covered and kept in refrigerator. Microscopic examinations of pollen were carried out with light microscope after squashing the anthers and staining with acetocarmine.

As all anther cultures produced callus, data were recorded on number of anther developing callus per jar, from which the % callus formation was recorded, and callus rating scale from 1-3 (where 1=small, 2=medium, 3=large callus). Observed callus color, friability and organ formation were also recorded, for each treatment combination using stereo-microscope at 10-20x and photographed using Sony Digital Camera.

For histological analysis, anthers and the calluses were fixed in formalin-acetic acid – ethanol (FAA). Tissues were dehydrated in alcohol, infiltrated and embedded in pure paraffin wax (m.p. 56-58 °C) as described by Johansen (1940). A rotary microtome was used to prepare serial sections (10μ), which were then stained with safranin and light green or hematoxilin. Stained sections were examined and photographed using a Zeiss Microscope.

Treatments and experimental design:

First experiment:

This experiment was conducted to test the effect of genotypes, medium components and stage of male flower development on *in vitro* anther development.

Collected male flower buds were divided into two groups: closed buds and partially open buds to test suitable stage of anther development for tissue culture response.

In this experiment, two media were tested, the first was MS (Murashige and Skoog, 1962) basal salts and vitamines containing 3% sucrose, 2,4-dichlorophenoxy acetic acid (2,4-D) at 2.0 mg/l, plus Benzylaminopurine (BA) at 1.0 mg/l. The second tested medium was B5 medium (Gamborg *et al.*, 1968) containing the same plant growth regulators (BA and 2,4-D) as the first medium. Anther derived from the two flower stages were inoculated as five anthers per jar, and incubated in the growth room under dark condition and 24± 2 °C for six weeks after inoculation. A factorial experiment in randomized complete block design (RCB) with 10 replications was used. The cultures were observed weekly. Morphological changes on callus production were recorded after 45 days.

Second experiment:

This experiment was carried out to test the response of anther culture to different media and anther pre-treatment (cold vs. non-cold anther treatments). Anther were collected from close male flower buds which contain microspores at the mid-to late-uni-nucleate stage as revealed by microscopic tests of anthers stained with 1% acetocarmine. The collected flower buds were kept either in refregirator at 4°C for 48 hr or kept under room temperature at 25 °C.

In this experiment, MS basal salts and vitamins was utilized and amended with 3% sucrose and the following plant growth regulators: 2,4-D at 1.0 and 2.0 mg/l, BA at 0.0, 0.5 and 1.0 mg/l, kinetin at 0.0 and 1.0 mg/l, in addition to glutamine at 0.0 or 50 mg /l (Table 1). Cultures were incubated in a growth chamber under dark condition at 24±°C for two weeks, and then at 16 hr photoperiod of 2000 lux light provided by white fluorescent tubes until the termination of the experiment (45 days). A factorial experiment in RCB design with 5 replications, in each, two anthers were cultured per jar.

Third experiment:

The sequence of experiments with cucumber anther culture were continued to seek further or better callus differentiation into organs. The current experiment was conducted to test the effect of stress imposed by the plant growth hormone abscisic acid (ABA) or high concentration of sucrose, on the frequency of callus differentiation from previously induced callus in experiment 2.

Callus from one cucumber genotype (Garawani) that was grown on MS medium +0.5 mg/l 2,4-D + 0.5

mg/l BA and pretreated with 4 °C cold treatments in the previous experiment were used for subculture into sucrose × ABA media. The media tested included MS basal salts and vitamins amended with ABA at 0.1, 0.2 or 0.3 mg/l in combination with sucrose at 30 g/l or 90 g/l, resulting in six medium combinations. Before the addition of agar, ABA was filter sterilized to avoid degradation by autoclaving and poured into media at the tested concentrations. Cultures were incubated in a growth chamber under dark condition at 24±°C for two weeks, and then at 16 hr photoperiod from fluorescent lamps providing light of 2000 lax.

Data statistical analysis

Data were statistically analyzed using ANOVA/MANOVA of Statistica 6 software (Statsoft, 2001) with mean values compared using Duncan's multiple range test with a significance level of at least $p \leq 0.05$.

Table (1): Media formulations for experiment (2)

Medium No.	PGR (mg/l)			L-Glutamine (mg/l)
	2,4-D	BA	Kinetin	
1	1.0	0.0	0.0	0.0
2	1.0	0.5	0.0	0.0
3	1.0	1.0	0.0	0.0
4	1.0	0.0	1.0	0.0
5	1.0	0.5	1.0	0.0
6	1.0	1.0	1.0	0.0
7	1.0	0.0	0.0	50
8	1.0	0.5	0.0	50
9	1.0	1.0	0.0	50
10	1.0	0.0	1.0	50
11	1.0	0.5	1.0	50
12	1.0	1.0	1.0	50
13	2.0	0.0	0.0	0.0
14	2.0	0.5	0.0	0.0
15	2.0	1.0	0.0	0.0
16	2.0	0.0	1.0	0.0
17	2.0	0.5	1.0	0.0
18	2.0	1.0	1.0	0.0
19	2.0	0.0	0.0	50
20	2.0	0.5	0.0	50
12	2.0	1.0	0.0	50
22	2.0	0.0	1.0	50
23	2.0	0.5	1.0	50
24	2.0	1.0	1.0	50

RESULTS

First experiment:

As shown in Figure 1, most anthers of cucumber were about 4.3 mm in length without anther filament (Figure 1a). Squashed anther samples with the addition of acetocarmine revealed the uni-nucleated pollen stage for these anthers derived from closed male flower buds (Figures 1b). However, a combination of mostly di-nucleated pollen and low frequency of uni-nucleated ones were found in anthers from open flower buds (Figure 1c).

The cultivated anthers start swelling after one week from inoculation, then callus initials appeared and enlarged to maximum size after 45 days in culture. Callus colors ranged from yellow-white to cream, and then turned brownish at the end. No visible shoot or root formation were detected at any treatment. Microscopic enlargement of callus pieces indicated no somatic embryo formation, but only embryo-like structures in rare cases with the "Garawani" variety in MS medium (Figure 2a) or B5 medium (Figure 2b). Differences among cucumber genotypes were significant for their effect on the frequency of anther development into callus (Table 2). Cucumber line 88-83 and Garawani followed by L 82-57 produced more anther-derived callus per jar than the other lines (L 88-93, L 89-08 and WI 1701 G). Percentage callus formation ranged from 60.6% in L 88-83 and Garawani to 43.6% for WI 1701 G.

The overall effect of media formation was significant (Table 2). As tested overall cucumber genotypes and flower bud stages, main effect of medium show positive response to MS basal salts and vitamins than B5 medium. In this respect, the average number of anthers that formed callus per jar (containing 5 anthers) was 2.92 for anthers cultured on MS compared to 2.28 for those cultured on B5. Therefore, 58.4% of anthers on MS developed callus versus only 45.6% on B5 medium.

As indicated in Table (2), anthers isolated from small, closed male flower buds were superior over those taken from larger, open flowers in their development into callus *in vitro*. An average of 3.12 anthers out of 5

cultured per jar (62.4%) had turned into callus with medium friability and creamy color (Figure 2c), as compared to an average of only 2.18 (43.6%) callus/jar if anthers were derived from open flowers (Figure 2d). The aforementioned results were an average over all genotypes and medium types (Table 2).

Regarding the interaction of genotype x media type x flower stage, the most positive combination showing the highest anther development was achieved using anthers from the cucumber genotypes "Garawani", L 82-57, L 88-93 when grown on MS medium utilizing anthers from closed flower. As indicated from the microscopic examination, those anthers will have the uni-nucleated pollen stage (Figure 1b), the most effective for anther culture in this study.

In this experiment, it was observed that not all cultured anthers had the same size, depending on the factors under examination (Table 3). Genotype main effect did not affect the diameter of callus, except with the cucumber L 82-57 which produced larger callus compared to the other genotype-derived anther calluses. However, medium main effect was significant, where anthers cultured on MS medium were larger (mean scale = 2.02) compared to those derived from B5 medium (mean scale = 1.63). Anther from closed male flower buds also produced callus larger than from open male flower buds, as tested over all genotypes and medium types.

The interaction effect of all tested factors was significant for callus diameter. The best combination was the callus derived from cucumber L 89-08 or L 88-93 on MS medium if anthers were isolated from closed male flower buds.

For histological examination, thin callus tissue sections were examined microscopically as shown in Figures (3 a,b,c), all from Garawani cv, indicating calluses grown on B5 medium from open flower (Figure 3a) or closed flower on MS medium (Figures 3b) as well as from close flower on B5 medium (Figure 3c). All photomicrographs indicate no visible bud regeneration or somatic embryo initials. Figure (3a), shown large parenchyma cells in the edge of the callus and smaller meristimatic regions in the middle. Figure (3b), show much organized meristematic tissue with heavy stained region. In Figure (3c), same observation as Figure (3b), but with small bud primordium without vascular connection with the rest of the callus tissue.

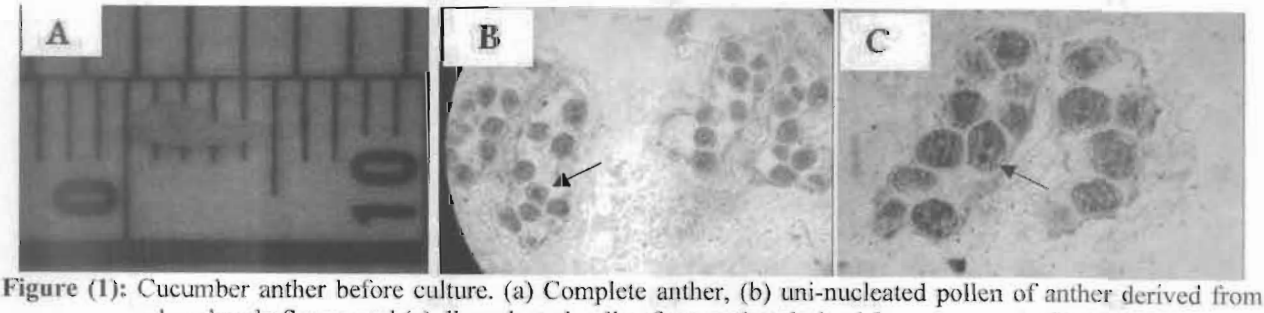

Figure (1): Cucumber anther before culture. (a) Complete anther, (b) uni-nucleated pollen of anther derived from closed male flower and (c) di-nucleated pollen from anther derived from open male flower.

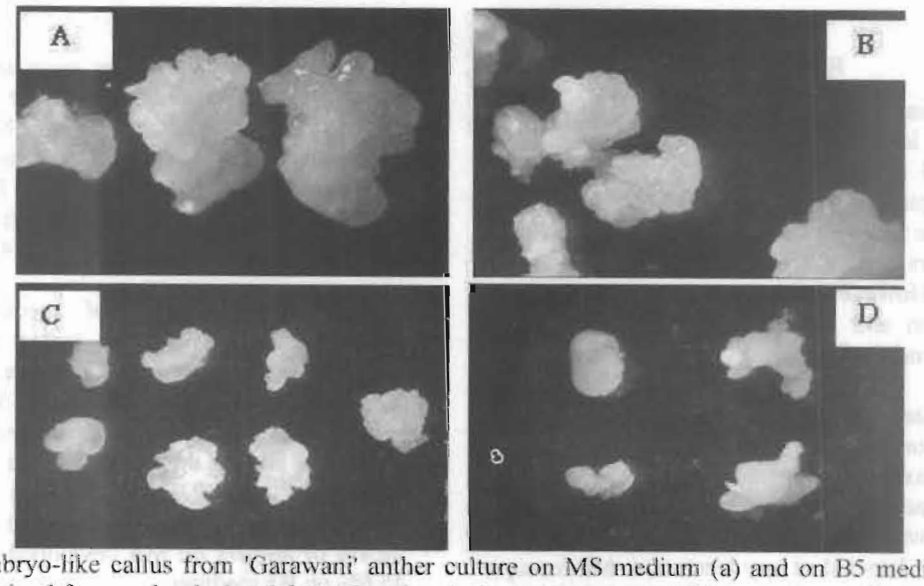

Figure (2): Embryo-like callus from 'Garawani' anther culture on MS medium (a) and on B5 medium (b). Callus derived from anther isolated from closed male flower buds (c) and from open male flower buds (d).

Figure (3): Longitudinal section through callus derived from anther grown on B5 medium from open flower (a), callus section from open male flower on MS medium (b), and callus section from closed flower on B5 medium (c).

Table (2): Effect of cucumber genotypes, medium types, stage of flower development and their interactions on number of anther forming callus and percentage of callus formation for cultured anthers.

Medium	MS				B5				Mean genotype	
Flower stage	Closed		Open		Closed		Open			
Genotype	No/jar	%	No/jar	%	No/jar	%	No/jar	%	No/jar	%
Garawani	3.8^{a}	76	2.6^{bcd}	52	3.3^{abc}	66	2.4^{cde}	48	3.0^{a}	60.6
L 82-57	3.8^{a}	76	2.2^{def}	44	2.8^{a-d}	56	2.1^{def}	42	2.7^{ab}	54.0
L 88-83	3.1^{a-d}	62	2.9^{a-d}	58	3.6^{ab}	72	2.5^{cde}	50	3.0^{a}	60.6
L 88-93	3.8^{a}	76	2.5^{cde}	50	2.0^{def}	40	1.3^{f}	26	2.4^{bc}	48.0
L 89-08	3.8^{a}	76	2.1^{def}	42	2.2^{def}	44	2.0^{def}	40	2.5^{bc}	50.8
WI 1701G	2.9^{a-d}	58	1.5^{ef}	30	2.3^{c-f}	46	2.0^{def}	40	2.2^{c}	43.6
Mean Flower bud	3.53^{a}	70.6	2.30^{c}	46	2.70^{b}	54	2.05^{c}	41		
Mean Medium	2.92^{a} (58.4%)				2.28^{b} (45.6%)					

Values are the means of ten replicates. Values followed by the same letter within a column are not significantly different at the 0.05% level of probability according to Duncan's multiple range test

Table (3): Effect of cucumber genotypes, medium types, stage of flower development and their interactions on callus size scale*.

Medium	MS		B5		Mean genotype
Flower stage / Genotype	Closed	Open	Closed	Open	
Garawani	1.60^{de}	1.15^{e}	1.45^{de}	1.90^{b-e}	1.53^{b}
L 82-57	2.50^{abc}	1.50^{de}	2.10^{bcd}	2.15^{bcd}	2.06^{a}
L 88-83	1.90^{b-e}	1.90^{b-e}	1.35^{de}	1.85^{b-e}	1.75^{ab}
L 88-93	2.95^{a}	1.85^{b-e}	1.35^{de}	1.05^{e}	1.80^{ab}
L 89-08	3.05^{a}	1.70^{cde}	1.55^{de}	1.61^{de}	1.99^{a}
WI 1701G	2.65^{ab}	1.15^{e}	2.10^{bcd}	1.35^{de}	1.81^{ab}
Mean flower stage	2.44^{a}	1.54^{b}	1.63^{b}	1.65^{b}	
Mean Medium	1.99^{a}		1.65^{b}		

Values are the means of ten replicates. Values followed by the same letter within a column are not significantly different at the 0.05% level of probability according to Duncan's multiple range test * Scale: 1=small, 2=medium and 3= large callus.

Second experiment:

This experiment was conducted to test the effect of medium growth regulators components and pre-treatment of anther with cold temperature (4 °C) for 48 hrs on callus formation and differentiation from six cucumber genotypes. Twenty four treatment combinations of 2,4-D (1.0 and 2.0 mg/l) + BA (0.0, 0.5 and 1.0 mg/l) +Kin (0.0 and 1.0 mg/l) + glutamine (0.0 and 50 mg/l) were used (Table 1).

When tested over all medium combinations and cold pretreatments, anthers from different cucumber genotypes behave differently under the conditions of this experiment. The genotype L 88-93 and L 82-57 followed by L 88-83 had larger callus than L 89-08, Garawani and WI 1701 G. However, all calluses from the different genotypes were in the range of medium-size callus (scored between 1 and 2) as shown in Table (4).

Regarding the main effect of PGR + glutamine, all media produced callus in varying degree. The most effective medium formula were those of MS amended with 1.0 mg/l 2,4-D + 0.5 mg/l BA + 1.0 mg/l Kin with no glutamine (Medium No. 5). Medium No. 2 (1.0 mg/l 2,4-D + 0.5 mg/l BA) and medium No. 3 (1.0 mg/l 2,4-D plus 1.0 mg/l BA) were similar in their effect. Medium No. 11 (1.0 mg/l 2,4-D + 0.5 mg/l BA + 50 mg/l glutamine) had the least effect on callus development (scale = 1.33).

The most effective anther pre-treatment was the cold treatment of anthers at 4 °C for 48 hrs (scale 2.04) as compared to the non-cold treatment (scale 1.68) as shown in Table (4). The interaction of CV x M was significant, however other interactions were not

significant as indicated in Table (4), indicating different response of cvs. to medium type.

Stereoscopic examination:

Although the previous results of genotype x medium x cold treatment of anther showed some differences in callus size according to the treatment, careful examination of callus under magnification by stereoscope indicated large differences in callus differentiation into organs, or somatic embryo-like structures. As indicated in Figures (4) for the genotype "L 88-83" (No.3), embryogenic-like callus was produced if cold treated anthers were cultured on MS medium amended with 0.5 mg/l 2,4-D, 0.5 mg/l BA (Figure 4a). Shoot primordium appear in a medium containing 1.0 mg/l 2,4-D + 1.0 mg/l BA + 1.0 mg/l Kin + 50 mg/l glutamine, as shown (arrow) in Figure (4b). Shoot bud was also observed in callus from cold-treated anther of the above genotype when cultured on 0.5 mg/l 2,4-D + 0.5 mg/l BA plus 50 mg/l glutamine (Figure 4c) or root formation (Figure 4d) from the same medium. The same differences were also observed in anther culture of the genotype "WI 1701 G" (Figure 4e vs 4f). Very visible shoot bud was observed in this genotype (Figure 4f) without glutamine addition to the medium or anther cooling treatment, indicating difference among genotype in their response to media type.

Histological examination:

A serial thin callus tissue sections (10 µ) representing several anther-derived callus in this experiments was prepared and photographed as show in Figures (5 a,b and c). Callus sections from selected treatments (genotype x PGR-glutamine in combination with cold vs. no cold anther treatments) showed a sequence of anatomically distinguished structures. Some of these structures are meristemoids (Figure 5a) showing apical shoot primordium derived from cold-treated anther culture on MS medium with 0.5 mg/l 2,4-D +0.5 mg/l BA + 50 mg/l glutamine (genotype L88-83). Figure 5 (b and c) represent callus sections from non-cold anther treatment of the same genotype, showing reorganizing vascular tissue towards forming organized structures, surrounded with paranchymatic cells. Density stained tissue are highly active to form organs, but still lacking the triggering mechanism for complete organ formation from anther callus tissues.

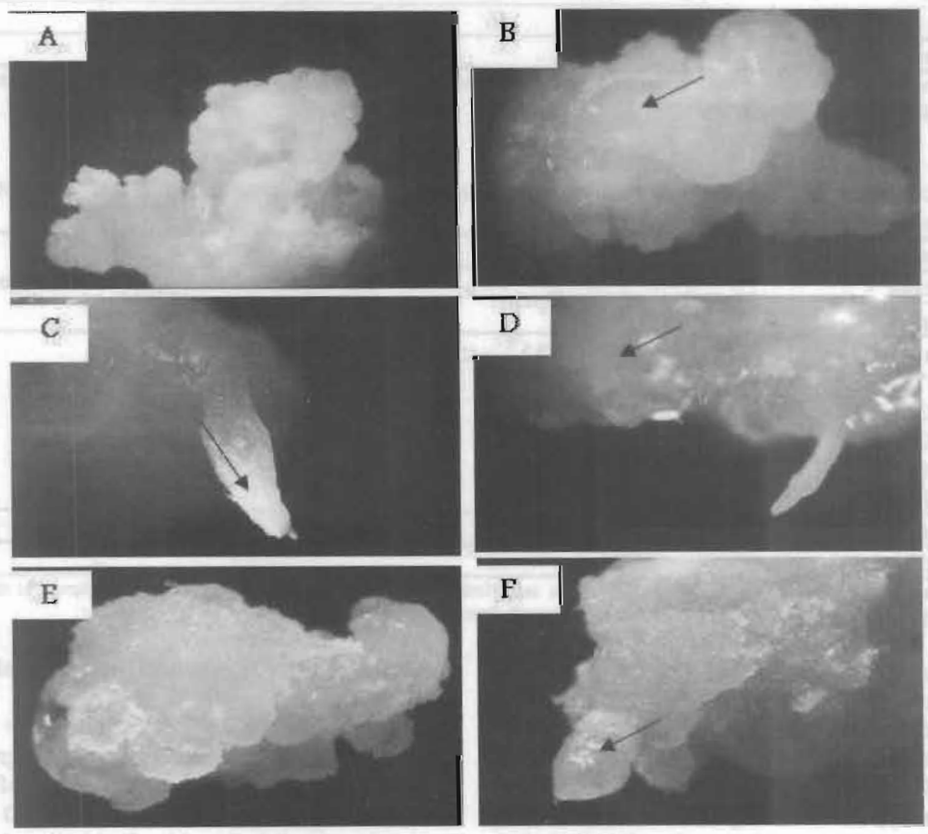

Figure (4): Anther callus from cucumber "L 88-83" showing (a) callus from cold-treatment anther on MS medium + 0.5 mg/l 2,4-D + 0.5 mg/l BA, (b) callus showing shoot primordium on MS + 1.0 mg/l 2,4-D +1.5 mg/l BA+ 1.0 mg/l kin + 50 mg/l glutamine, (c) shoot bud in callus from cold-treated anther on MS+ 0.5 mg/l 2,4-D + 0.5 mg/l BA + 50 mg/l glutamine and root formation from the same medium(d). In addition, callus from anther of "WI 1701 G" treated with cold (e) or visible shoot bud without glutamine-amended medium (f).

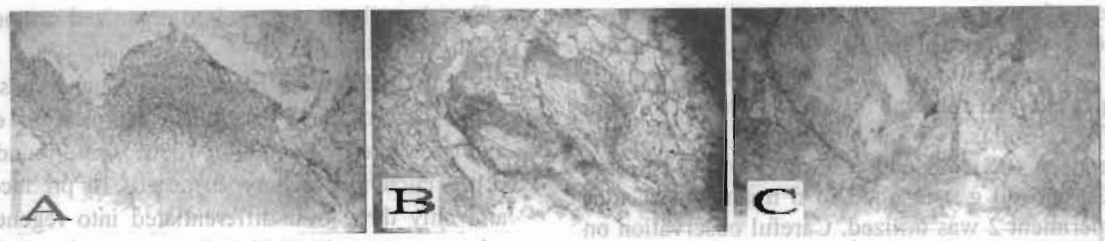

Figure (5): Histology of callus derived from anther culture showing meristemoid (a) from cold-treated anther on MS + 0.5 mg/l 2,4-D + 0.5 mg/l BA + 50 mg/l glutamine, and from non-cold treated anther callus (b) and (c)

Table (4): Effect of cucumber genotype (CV), medium PGR components (M) and cold pre-treatment (T) of anther on callus growth scale[z].

Genotype	Garawani		L 82-57		L 88-83		L 88-93		L 89-08		WI 1701 G		Ave T x M		Ave
Medium[x]	C[y]	NC	C	NC	C	NC	C	NC	C	NC	C	NC	C	NC	M
M1	1.33	1.33	1.33	1.33	2.00	2.33	2.33	2.66	1.00	1.33	1.00	1.00	1.50	1.66	1.58^{fg}
M2	2.33	2.33	2.33	3.00	2.00	1.33	2.33	2.66	2.00	1.66	2.66	3.00	2.28	2.33	2.31^{ab}
M3	2.33	1.33	2.66	3.00	3.00	2.33	3.00	2.33	2.33	1.66	2.00	2.00	2.55	2.11	2.33^{ab}
M4	2.33	2.33	3.00	1.66	2.33	2.33	2.00	1.33	2.00	3.00	2.33	2.00	2.33	2.11	2.22^{abc}
M5	2.66	3.00	2.33	3.00	2.66	3.00	2.33	2.33	2.33	3.00	2.00	1.00	2.39	2.55	2.47^{a}
M6	2.00	1.33	2.33	1.33	2.33	1.67	2.33	2.33	2.66	1.66	1.66	1.33	2.22	1.61	1.92^{def}
M7	2.00	1.0	2.33	1.00	2.66	2.66	2.33	2.00	2.00	2.33	2.33	2.33	2.27	1.88	2.08^{bcd}
M8	1.33	1.0	2.33	2.33	1.66	1.66	2.33	2.00	1.66	1.66	2.33	2.00	1.94	1.77	1.86^{def}
M9	2.00	1.0	2.66	1.33	2.00	2.33	2.33	1.66	2.66	2.33	1.66	1.66	2.22	1.72	1.97^{cde}
M10	1.66	1.33	2.00	1.00	1.33	1.33	2.33	2.33	2.33	1.33	1.66	1.33	1.88	1.44	1.67^{ef}
M11	1.67	1.33	1.66	1.33	1.00	1.00	1.66	1.00	1.66	1.00	1.33	1.00	1.50	1.11	1.31^{g}
M12	1.00	1.00	2.33	1.00	2.33	2.00	2.00	1.33	1.66	1.66	2.00	1.66	1.88	1.44	1.67^{ef}
M13	1.66	1.00	1.66	1.33	2.66	1.33	2.00	1.66	1.33	1.00	2.00	1.66	1.89	1.33	1.61^{fg}
M14	1.33	1.00	1.66	1.00	2.33	1.66	2.66	2.66	2.00	1.33	2.33	1.33	2.05	1.50	1.78^{def}
M15	2.00	1.33	2.00	2.00	2.00	1.33	1.66	1.33	1.66	1.00	1.66	1.66	1.83	1.44	1.64^{ef}
M16	1.33	2.00	2.00	1.33	2.00	1.33	2.33	2.00	2.33	2.00	2.00	1.66	2.00	1.72	1.86^{def}
M17	1.33	1.00	2.00	1.33	2.00	1.33	2.33	1.33	2.00	1.66	1.66	1.66	1.89	1.39	1.64^{ef}
M18	2.33	2.00	2.00	1.66	1.33	1.00	1.66	1.33	2.33	2.00	2.00	2.00	1.94	1.66	1.81^{def}
M19	2.33	1.33	2.00	2.00	2.66	2.00	2.00	1.33	1.33	1.00	2.00	1.66	2.05	1.55	1.81^{def}
M20	2.00	2.00	2.00	1.33	2.33	2.00	2.33	1.33	2.00	2.00	1.33	1.33	2.00	1.66	1.83^{def}
M21	2.00	1.33	2.33	1.66	2.00	2.33	2.33	2.33	2.00	1.33	2.33	1.66	2.17	1.77	1.97^{cde}
M22	2.33	1.66	2.00	2.00	2.00	1.33	3.00	1.00	2.00	1.33	1.33	1.66	2.11	1.50	1.81^{def}
M23	1.66	1.00	2.33	2.00	1.66	1.00	1.66	1.66	1.66	1.00	2.00	1.66	1.83	1.38	1.61^{fg}
M24	2.33	2.66	2.66	2.33	1.66	1.66	2.33	2.33	2.00	2.00	2.66	1.66	2.27	2.11	2.19^{ab}
T x CV	1.89^{cde}	1.52^{f}	2.17^{ab}	1.72^{ef}	2.08^{abc}	1.76^{de}	2.23^{a}	1.85^{de}	1.94^{bcd}	1.67^{ef}	1.93^{cd}	1.67^{ef}	2.04^{a}	1.70^{b}	
CV	1.71 ^{b}		1.93 ^{ab}		1.92 ^{ab}		2.04 ^{a}		1.82 ^{b}		1.799 ^{b}				

Significance

S.O.V	SS	df	MS	F	p
CV	10.315	5	2.063	5.451	0.000065***
M	65.218	23	2.836	7.492	0.000000***
T	25.352	1	25.352	66.985	0.000000***
CV*M	89.796	115	0.781	2.063	0.000000***
CV*T	0.856	5	0.171	0.453	0.811476 NS
M*T	10.093	23	0.439	1.159	0.276064 NS
CV*M*T	38.366	115	0.334	0.881	0.796215 NS

Values are the means of ten replicates. Values followed by the same letter within a column are not significantly different at the 0.05% level of probability according to Duncan's multiple range test. NS and ***, Non-significant and significant at 0.1% level, respectively.
(x) Media components from 1-24 are shown in Table (1) (y) C = Cold treatment of anther (4 °C for 48 hr), NC = No Cold,
(z) Callus scale: 1= small, 2= medium, 3= large callus

Third experiment:

As outlined in previous reports, ABA and / or high sucrose concentrations may enhance somatic embryogenesis and subsequent germination of shoot or buds from callus. A combination of 3 ABA concentrations (0.1, 0.2 and 0.3 mg/l) with 2 sucrose levels (30 and 90 g/l) resulted in 6 treatments. Callus from experiment 2 was utilized. Careful observation on cultures during the *in vitro* growth period up to 2 months did not show visible green shoot regeneration from any treatment combination. Only some treatment showed roots derived from callus at the bottom of the culture jars as shown in Figure (6 a&b). These two pictures show hairy roots on the surface of callus grown on MS + 0.2 mg/l ABA + 30 g/l sucrose (Figure 6 a), while Figure (6 b) show very visible root from another callus on the same medium component.

In a medium amended with 0.3 mg/l ABA and 30 g/l sucrose, shoot bud was visible under stereoscope (Figure 6c). Increasing sucrose to 90 g/l resulted in larger shoot initial (Figure 6d). Embryogenic callus with side shoot bud was formed in medium with 0.1 mg/l ABA + 90 g/l sucrose (Figure 6e) or MS + 0.2 mg/l ABA + 90 g/l sucrose (Figure 6f).

These observations revealed the importance of sub-culturing the anther callus previously formed in 2,4-D-amended medium to ABA or high sucrose level.

Clear evidence of the above mentioned result was shown by histological studies. Histological analysis of callus sub-cultured into MS medium with ABA and high sucrose demonstrated visible shoot bud meristem ready to initiate shoot from callus, but still under microscopic level (Figure 7a&b). Callus cells and tissue became active in systematic tissue organization into meristemoids, but lacking the somatic embryo initials, since the connection of meristemoid regions with the inside callus tissue via vascular elements is apparent (Figure 7 c).

DISCUSSION

The present study demonstrated the importance of several factors for the anther culture success in cucumber, including genotypes, basal medium components, plant growth regulators in the culture medium, in addition to the stage of anther development and thermal pre-conditioning anther treatment.

Studies with cucumber showed different response of six genotypes to the *in vitro* anther development, depending on the interaction between genotype and medium, stage of male flower, and thermal pre-cooling treatment. Anthers from the cucumber "line 88-83" and the local cv. "Garawani" showed higher frequency of callus formation (60.6%) than the other tested genotypes. However, the development of sporophyte (microspore-derived plantlet) was not clearly visible under any of the tested factors. It is well recognized in plant tissue culture studies that the genetic background of mother plant influences the *in vitro* developmental pattern, and the different explants from the same plant affect the culture success into organized or unorganized (callus) structures.

The differential responses of genotypes through callus growth have been also reported in cucumber anther culture by Song *et al.* (2007), who found that, out of 20 cucumber genotypes tested, 16 produced callus and only three cvs. differentiated into regenerants. In other report, no haploid embryos were detected from 10 tested cucumber genotypes (Superunova and Shnykova, 2008). Difference among five cucumber cvs were also reported by Mohamed *et al.* (2005) using somatic tissues. Our results are in harmony with those found by other researchers in anther culture of cucurbits (Badawi *et al.*, 2005; Kurtar *et al.*, 1999; Kurtar *et al.*, 2002). The difference in genotype response is unknown, and there is evidence suggested that a few genes may have an effect on the regeneration capacity of plant tissue (Reisch and Bingham, 1980).

The present investigation indicated that medium type affected anther development and MS was better than B5 medium formula in cucumber, especially for callus growth and frequency of callus formation (58.4% in MS vs. 45.6% in B5). This finding may be due to the difference in nitrogen source between the two media (all N from nitrate in B5 vs. NO_3 and NH_4 in MS) or vitamine concentration (much higher in B5 than MS). This was in agreement with other reports on cucumber anther culture that used MS medium (Song *et al.*, 2007). In our results with cucumber, a significant genotype x medium type interaction was detected in callus formation, indicating that some genotype had preference to specific medium than the other. Similarly, Kumar *et al.* (2003) used B5 medium for callus induction and embryogenesis. Therefore, both media could be used, depending on the genotype.

Plant growth regulator type and concentrations affected *in vitro* anther development. MS medium amended with 1.0 mg/l 2,4-D + 0.5 mg/l BA + 1.0 mg/l Kin was the most effective in callus growth (out of 24 combinations with Glutamine), while the addition of glutamine to this medium increased the induction of shoot bud initials or embryogenic callus formation. The amino acid glutamine was also effective in anther culture of sweet melon and snake melon in the production of embryo-like structures from anther-derived callus (El-Magawry, 2010). It is known that 2,4-D is a strong auxin for callus growth, while both BA and Kin with their effect in increasing cell division rate, will contribute in adjusting the auxin/cytokinin balance for the required culture development. Glutamine may increase N from organic source in the medium, which might influenced the observed callus differentiation pattern. The utilization of the aforementioned PGR is in agreement with the reports of other researchers on cucurbit anther culture (Eun and Bak, 1974; Kumar *et al.*, 2003 and 2004; Song *et al.*, 2007 and Metwaly *et al.*, 1998). When several amino acids were tested by Kumar and Murthy (2004) glutamine was effective in anther differentiation.

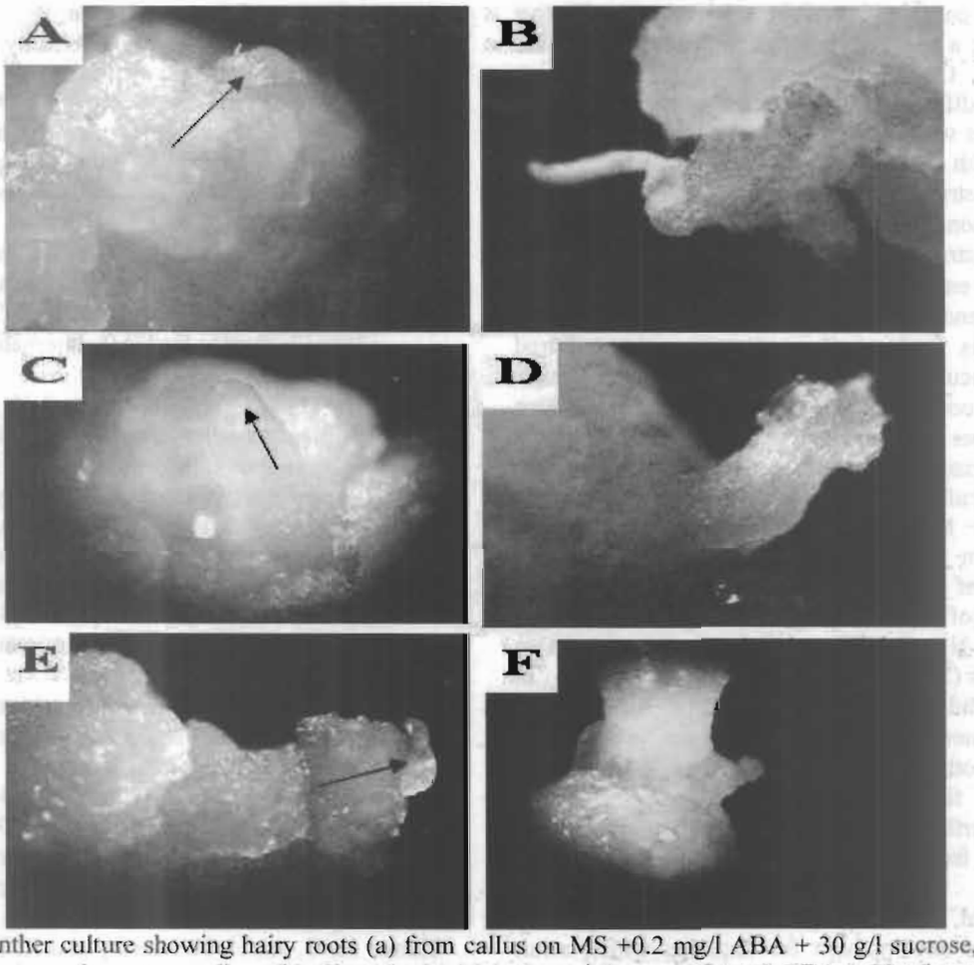

Figure (6): Anther culture showing hairy roots (a) from callus on MS +0.2 mg/l ABA + 30 g/l sucrose, and visible roots on the same medium (b). Shoot bud initials from callus on 0.3 mg/l ABA + 30 g/l sucrose (c) and on 0.3 mg/l ABA +90 g/l sucrose (d) with larger shoot initial. Embryogenic callus with side shoot bud on MS + 0.1 mg/l ABA + 90 g/l sucrose (e) and on MS +0.2 mg/l ABA +90 g//l sucrose (f).

Figure (7): Histology of callus derived from anther culture on ABA–amended MS medium with high sucrose showing clear shoot bud meristem (a and b) and vascular elements supporting meristemoid (c).

The cold treatment of anthers before culture was significantly effective in callus development in all anther culture examinations in these studies, perhaps through affecting the division of the generative cell after the pollen grains have been discharged from the anther (Bhojwani and Bhatngar, 1978). The positive effect of cold treatment on anther culture was also reported to increase number of microspores to develop sporophytes instead of gametophytes (Kumar *et al.*, 2003 and Song *et al.*, 2007).

Anthers from closed male flower differentiated better than from open flowers, perhaps because in closed buds, microspores are in the uni-nucleate stage, as indicated by our microscopic examination. In most reports of anther culture, this stage is usually used to produce haploid clones, symmetrical nuclei division, and globular embryo (Sharp *et al.*, 1972; Yinnan *et al.*, 1999; Bal and Abak, 2005). It could be speculated that the responsive stage for microspore embryogenesis is the uni-nucleate stage, and that the culture of young anthers (from closed flower buds) provides suitable environment for the microspore, i.e. acts as a nurse culture and helps them reaching the uni-nucleate stage. In cucumber and squash, flower buds were harvested when the microspores were at mid to late uni-nucleate stage (Metwally *et al.*, 1998; Kumar *et al.*, 2003 & 2004; Kumar and Murthy, 2004; Badawi *et al.*, 2005 and Song *et al.*, 2007).

Although different factors in these studies were examined, no combination of these factors was effective for producing a regenerating complete haploid plantlet from callus. Only callus, embryogenic callus, or shoot/root differentiations were visible under microscope or with the histological analysis. This is in agreement with other reports in cucurbit anther culture which demonstrated low frequency of obtaining haploid plants. This conclusion is in contrast with other reports which demonstrated the possibility of obtaining somatic embryos and embryo germination in cucumber. Kumar *et al.* (2003) and Kumar and Murthy (2004) reported on embryogenesis and plant regeneration in cultured anthers of cucumber (cvs. Calypso and Green Long). Their reports contained only microphotographs showing the phenotypes of obtained structures, however, the histological examination was made only for anthers at the time of culture showing uni-nucleate microspores (Kumar and Murthy, 2004). Song *et al.* (2007) presented, in addition to microphotographs of phenotypes of produced structures, the cytological examination of 20 randomly selected R0 plants of cucumber cv. Ningjia which revealed that there were no haploid plants (2n = 2x = 7), 18 plants were diploid (2n = 2x = 14) and one was tetraploid (2n = 4x = 28). In another regenerant, chromosome counts showed the presence of both haploid (8.3%) and diploid cells in the same tendril tip, suggesting that it was mixoploid. Therefore, further studies are needed to obtain high regeneration frequency from the cultured anthers in cucumber.

In general, cucumbers seem to be recalcitrant in anther culture development for haploids which await further investigations regarding medium component or environmental factors. Pollen culture might be the choice instead of whole anther culture to avoid many obstacles related to this technique.

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دراسات على بعض العوامل المؤثرة على زراعة المتك فى ستة تراكيب وراثية من الخيار

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أجريت ثلاث تجارب على كفاءه وفاعليه زراعه المتك في الخيار. الأولى لدراسة تأثير التراكيب الوراثية ونوع البيئة المستخدمه ومرحلة تطور الزهرة المذكرة المأخوذة منها المتك المستخدم. وجدت اختلافات معنوية بين التراكيب الوراثية المستخدمه في نسبه تطور المتك إلى كالوس ولكن لم يتأثر حجم الكالوس الناتج باختلاف التراكيب الوراثية. و قد أثرت نوع البيئات معنوياً على نسبة تطور المتك إلى كالوس وكذلك على معدل نمو هذا الكالوس. ولكن لم يتأثر حجم الكالوس الناتج باختلاف التراكيب الوراثية المستخدمه و كانت المتك المعزولة من البراعم الزهرية المقفلة اكبر معنويا في نسبه تكوين الكالوس بالمقارنة بتلك المعزولة من الأزهار المتفتحة. بالنسبة للتفاعل بين العوامل الثلاثة (التراكيب الوراثية ×البيئة×مرحله تطور الزهرة) كان أفضلها تأثيرا في نمو الكالوس زراعه المتك من البراعم الزهرية المقفلة للأصناف جروانى و L82-5٧ أو L88-83 على بيئة MS الصلبة والمضاف إليها منظمات النمو 2,4-D (٢مجم/لتر) + بنزيل أدنين (١ مجم/لتر) وقد أوضحت الدراسة التشريحية لأنسجه الكالوس المتكون في المعاملات السابقه عدم وجود تكشفات برعمية.

أجريت التجربة الثانية لاختبار بيئات مكونة من الحمض الامينى جلوتامين وأنواع من منظمات النمو بتركيزات مختلفة مع معاملتين حراريتين للمتك قبل زراعتها من ستة تراكيب وراثية للخيار. أوضحت النتائج أن بيئة MS المحتوية على ١مجم/لتر 2,4-D + 0.5مجم/لتر بنزيل أدنين + ١مجم/لتر كينيين كانت الأكثر تأثيرا على نمو الكالوس. أنتجت المتك المعامله بالتبريد اكبر نمو للكالوس بالمقارنة بغير المعامله. عند اختبار الكالوس المتكون تحت المجهر لوحظ تركيب شبيه بالأجنة الجسمية في الكالوس الناتج من المتك المبردة للصنف L88-83 على بيئة MS المحتويه على ٠,٥ مجم/ لتر 2,4-D + ٠,٥ مجم/ لتر بنزيل أدنين ، بينما ظهرت مبادئ تفريع خضري من المتك المنزرعة على بيئة MS + ١مجم/ لتر 2,4-D + ١مجم/ لتر بنزيل أدنين + ١مجم/ لتر كينتين + ٥٠ مجم/ لتر جلوتامين. لوحظ مبادئ جذور وتبرعم خضري في الكالوس الناتج من زراعه المتك الغير مبردة للصنف WI 1701G في بيئة لا تحتوى على الجلوتامين مما يوضح وجود اختلافات بين أصناف الخيار في استجابة المتك المنزرعة منها لمحتوى البيئة ومعاملات التبريد. وقد تأكدت النتائج السابقة بالدراسة التشريحية.

فى التجربة الثالثه تم إعادة زراعه الكالوس الناتج من التجربة الثانية على بيئة MS المضاف إليها ثلاث تركيزات من هرمون ABA مع تركيزين من السكروز بهدف اختبار تأثير هذه البيئات على تكشف الكالوس إلى نبتيات. أوضحت الدراسة الميكروسكوبية للتكوينات الناتجة من إعادة زراعه الكالوس في بيئة MS + ٠,٣ مجم/ لتر ABA + ٩٠ جرام/ لتر سكروز ظهور تبرعم خضري - بينما ظهرت شعيرات جذرية على الكالوس في البيئة المحتوية على ٠,٢ مجم/ لتر ABA + ٣% سكروز.أتضح تكوين كالوس شبه جنيني مع تفريع خضري جانبي على بيئة MS المحتوية على ٠,١ مجم/ لتر ABA+ ٩٠ جرام/لتر سكروز. قدمت الدراسة التشريحية دليلاً واضحاً على تكوين مبادئ تفريعات خضرية على البيئات السابقة.