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Effects of culture conditions on isolated microspore culture of melon (*Cucumis melo* L.)

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Abstract:

Isolated microspore culture is a useful tool to produce pure, fully homozygous parental lines in a short time. This study evaluated factors including the microspore developmental stage, cell culture density, and heat treatment influencing callus formation from melon microspores (*Cucumis melo* L.). The results showed that the obtained number of calli induced was highest (11.00±1.02) when cultivating flower buds with sizes 7.0-7.9 mm that contained microspores at middle-to-late uninucleate stages. The optimal microspore density for culture is 4×10^4 cells. The cultured medium was NLN, containing 130 g/l of sucrose at pH 5.8. Heat treatment at 40°C for 48 hours was best suited for callus induction of all flower bud sizes. The survival rate of microspores after 7 days of culture was lower than before inoculation and was only 75.6%. The development of the microspores and the arising of calli and embryos have been observed and evaluated for morphological cell characteristics. However, in this study, no mature embryo formation or seedling regeneration was observed.

Keywords: **anthers, callus, *Cucumis melo* L., cytology, flower buds, microspore developmental stage.**

Classification numbers: **3.1, 3.4**

1. Introduction

In vitro doubled haploid (DH) plants produced via androgenesis or gynogenesis are useful tools for both basic studies and applied research (e.g., genetic research or crop breeding) [1, 2]. Androgenesis methods are more attractive and have greater potential than gynogenesis ones. Indeed, the number of male haploid cells (microspores/pollens) is much greater than that of female haploid cells (ovules) produced by the same flower. Besides, female haploid cells exist in ovules that are covered by nucellar and tegument tissue layers and these ovules are located inside the ovary while male haploid cells are only included within the anther wall. Therefore, ovary culture requires much more time and advanced technical skills.

The method of androgenic DH plant production based on *in vitro* microspore embryogenesis includes anther culture and isolated microspore culture. Anther culture is widely used to produce DHs, however, there are some limitations involved in anther walls. Anther wall layers have a protective role and provide nutrition for microspores, but they may secrete inhibitors or toxic molecules in the case of injured tissues [3]. Moreover, embryos or calli can develop not only from microspores but also from anther walls when cultured *in vitro* [1, 4, 5]. Isolated microspore culture is an alternative technique used in plant-breeding programs. Over the past few years, studies related to isolated microspore culture have focused on *Brassica* and cereal species [1]. *In vitro* plants can be regenerated directly from microspores, embryos, or indirectly through the arising of calli formed from microspores.

Melon (*Cucumis melo* L.) has economic importance as a fruit crop [6] with more than 1 million ha involved in worldwide production in 2018 (Food and Agriculture Organization Corporate Statistical Database). Currently, most of the widespread melon varieties are hybrids, but up until now the technique of a DH line production in microspore cultures of this crop has not yet been performed. The amount of research related to this technique in melons as well as other species of the cucumber family (cucumbers, watermelons, squash) is still very limited [7]. The first DH melon plant was created in 1987 through the pollination technique of irradiated pollen grains [8]. T. Suprunova, N. Shmykova (2008) [9] obtained callus from cultured cucumber microspores.

The success of the above methods depends on many factors such as genotype, developmental stage of

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microspores, nutrient medium component, the addition of growth regulators, the cell density, heat shock treatment, microspore isolation technique, and other culture conditions [1, 10]. Several studies on crucible plants evaluated the effect of the developmental stage of cultured microspores on embryonic formation, namely, that microspores from the late nucleate to the early binucleate stage were suitable for culture [11-13]. Meanwhile, the development of microspores is associated with the length of flower buds, flower leaves, or their proportions [9, 14]. Other studies focused on assessing the effects of high temperatures on the culture process [15, 16].

In a previous study, we determined the correlation between morphological characteristics, specifically the size of buds and anthers, with the development stages of melon microspore (*Cucumis melo* L.) [17]. Buds 6.0-8.9 mm in length contained mid-to-late vacuolated microspores. The present study aims to investigate microspore embryogenesis under different culture conditions. A few factors include the effects of microspore developmental stages, cell density, and stress treatment on the frequency of microspore embryogenesis in melons.

2. Materials and methods

2.1. Materials

The melon called F_1 hybrid Kim Hong Ngoc produced by Chia Tai Seed Company in Thailand was used for this study. Flower buds of all lengths ranging from 6.0 to 8.9 mm at 1 mm intervals were collected at 5:30-6:30 am and stored for 24 hours at 4°C in the dark. At least 10 buds were collected for each of the intervals.

2.2. Method

Assessing the viability and developmental stage of the microspores before culture: The analysis of the viability and developmental stage of the microspores was performed as described previously [17]. Briefly, the microspores were incubated with 2% acetocarmine for 30 min and observed under the Zeiss Axio Lab1 optical microscope (Suzhou Co., Ltd). The survival rate of the microspores was assessed by three different observation fields with 100 cells (a) per field. The percentage of living cells was calculated by:

$$\text{Percentage of living cells (\%)} = \frac{\text{live cells}}{\text{a}} \text{ x } 100.$$

Staining with 4, 6-diamidino-2-phenylindole (DAPI) fluorescent dye was used to identify the cell developmental stage. After staining, cells were observed through a Zeiss Axio Lab1 fluorescent microscope (Suzhou Co., Ltd).

Isolated microspore culture procedure: The protocol of isolated microscope culture was performed as described previously [18]. Briefly, the collected healthy flower buds were classified into three different size groups before cold pre-treatment. The bud surfaces were sterilized in 2.5% NaClO for 10 min and were rinsed in cold sterile distilled water three times for 1, 4, and 10 min, respectively. The buds were transferred to a mortar with 2 ml of sterile NLN liquid medium (pH 5.8) with 13% sucrose (NLN-13) that was slightly squeezed to free the microspores. After that, the suspensions were filtered in a centrifuge tube through two layers of nested sterile filters and the volume of each tube (10 ml) was adjusted before centrifugation at 4°C at 800 rpm for 5 min before resuspension (repeated twice). After the final washing, the pellet was suspended in 1 ml cold MS liquid medium (pH 5.8) with 13% sucrose (MS-13) and 20 µl of suspension was used to count cell density by a Fuchs-Rosenthal counting chamber and photographed under the Zeiss Axio Lab1 microscope (Suzhou Co., Ltd) at 10X magnification. The Fuchs-Rosenthal chamber has 16 squares, each with an area of 1.0×1.0 mm^2, and the depth of the counting chamber is 0.2 mm. Microspores were counted in 5 sub-cells, then multiplied by 16,000 to give a sub-microspore density of over 1 ml. The plating density for culture was adjusted by MS-13, and 3 ml microspore suspension was poured into sterile plastic Petri dishes (50×12 mm) wrapped with Parafilm membranes. The cultured plates were incubated in the dark at different temperatures for heat shock and then at 25°C continuously.

Effect of flower bud sizes and cell densities on the induction of embryogenesis from microspores: In the previous study, a correlation of bud and anther morphology with developmental stages of microspores in melon (*Cucumis melo* L.) was conducted [17]. In fact, 56.8% of microspores from 6.0-8.9 mm long buds were in the late uninucleate stage. In this study, three different groups of bud sizes i.e., 6.0-6.9, 7.0-7.9, and 8.0-8.9 mm were tested and different microspore densities were also tested: 4×10^4, 6×10^4, 8×10^4, and 16×10^4 cells/ml. Both factors were combined as a treatment. The cultured plates in this experiment were incubated at 37°C for 48 hours and then transferred to 25°C. All samples were kept under dark conditions.

Effect of temperature on the induction of embryogenesis from microspores: The appropriate density of microspores from the previous experiment was used in this experiment. Four different temperatures (33, 35, 37, and 40°C) over three periods (24, 48, and 72 hours) in three flower bud sizes (6.0-6.9 mm, 7.0-7.9 mm, and 8.0-8.9 mm) were studied.

After heat shock treatment, the culture samples were kept at 25°C in dark conditions, periodically tested, and evaluated for callus arising by direct observation under a stereoscopic microscope.

Histological observation: For observation of early development in culture, during the first 7 days, 50-100 µl of sample from a dish was collected, stained with 2% Acetocarmine, and observed under a Zeiss Axio Lab1 fluorescent microscope (Suzhou Co., Ltd). The survival rate of the cultured microspores was determined at two different times (T_1: at the time of culture; T_2: after one week of culture).

Periodic observations were carried out under a Zeiss Stemi 2000-C stereomicroscope (Suzhou Co., Ltd) to document each alteration during sporogenesis at intervals of 2 days until embryos or calli formed.

Experimental design and statistical analysis: Each experiment was conducted using a completely randomised design with three replications and 5 Petri dishes per replicate. The number of calli or embryos per dish was recorded over 30 days. The data was processed using IBM SPSS Statistic 23 (IBM Corporation, USA) with average M and standard error (±SEM) with a confidence interval of 95% ($t_{0.05}$×SEM) as outputs. Statistically significant differences were determined by the student's t-test value.

3. Results and discussion

3.1. Viability of microspores

For microspore culture, it is necessary to evaluate the number of cells for culture. For melons, the total number of microspores per flower bud is about 180,000 to 510,000 cells with an average number of 336,204±118,695 cells/bud. The highest number of cells was found in the 7.0-7.9 mm-long buds, followed by 6.0-6.9-mm buds and the 8.0-8.9-mm ones (Table 1). The number of cells tended to decrease as the length of the flower bud increased. The petals at bud sizes of 8.0-8.9 mm grew outside sepal and thus were affected by environmental factors such as light intensity or insect activity. This trend has also been recorded in *Raphanus sativus* L. cvs. Taebaek and Chungwoon (Monsanto Co., Seoul, Korea). Indeed, M-sized (4.0±0.5 mm) flower buds had the highest number of microspores, followed by the S- and L-sized flower buds (2.0±0.5 mm and 6.0±0.5 mm, respectively) [5]. In rapeseed (*Brassica napus* L.), a flower bud could contain more than 100,000 microspores, and about 50-70% of that number can be easily separated and served for the culture process [18].

Cell viability is one of the important factors in embryo or callus production. The viability rate of microspores after cold pre-treatment (T_1) in all three groups reached about 80%. In particular, the highest was recorded at 6.0-6.9 mm (Table 1). According to the study of D. Yi, et al. (2019) [19], cold pre-treatment under dark conditions at 4°C for 24 hours gave a 20% tree regeneration efficiency. This incurred rate was higher than at 4°C for 12 hours and 4°C for 48 hours (14.17 and 9.17%).

Table 1. Survival rate of microspores of different sizes after cold pre-treatment (T_1).

No.	Flower bud size (mm)	Number of microspores (cells/bud)	Viability rate (%)
1	6.0-6.9	436,500±105,831	88.78±3.30[a]
2	7.0-7.9	442,500±28,722	86.80±1.47[a]
3	8.0-8.9	251,500±86,094	79.06±3.18[a]

Note: Different letters on the same column indicate a statistically significant discrepancy in the sample average with p<0.05.

Microspores were assessed for their ability at three different times (T_0: before cold pre-treatment; T_1: at the time of culturing; T_2: after a week of culture). Table 2 shows that the survival rate of microspores reached over 80% at the culture time. The proportion of cells living before culture reaches 86.5% and decreases slightly after cold pre-processing. After a 7-days culture period, the survival rate of cells is about 75.6%.

Table 2. Survival rate of microspores at different times.

No.	Time	Proportion of living cells
1	T_0	86.50±2,45[a]
2	T_1	83.45±2,09[a]
3	T_2	75.64±1,99[b]

Note: Different letters on the same column indicate a statistically significant discrepancy in the sample average with p<0.05.

The results in this study were higher than in the study of tomatoes [20]. At the time of separation from the anarchy, the viability of microspores reached nearly 90%; however, after six days, this rate dropped to about 50%. This difference may be due to differences in genotype and culture conditions.

3.2. Anatomical observations of the microspore developmental stages and embryo or callus formation

Morphological changes in the microspores and the formation of the embryo or callus during cultivation have been monitored and periodically observed under fluorescent and stereoscopic microscopes. At the time of culture, most microspores were at the middle-to-late uninucleate stages (Fig. 1A1-A2). After 5 days of culture, microspores began to break out of the microspore wall and divide to form a

primordium (Fig. 1A3-A8 and Fig. 2A1, arrow marks) and form disorganized cell structures (astrocyte figure, Fig. 2A1). After germination, that germ continued to divide (Fig. 1) and bind together (Fig. 2A2). Similar observations have been reported for beetroot trees. The spores after division have different tendencies, for example, some stop growing after a few divisions, some form callus-like structures, and only a few form embryos [21].

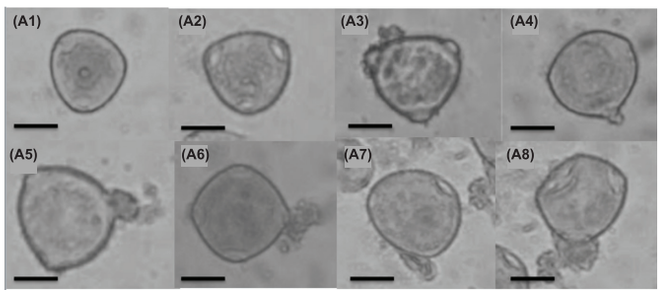

Fig. 1. The development of microspores during culture. (A1) Middle uninucleate stage; **(A2)** Late uninucleate stages; **(A3-A8)** Forming a primordium from microspores. Ratio bar = 20 µm.

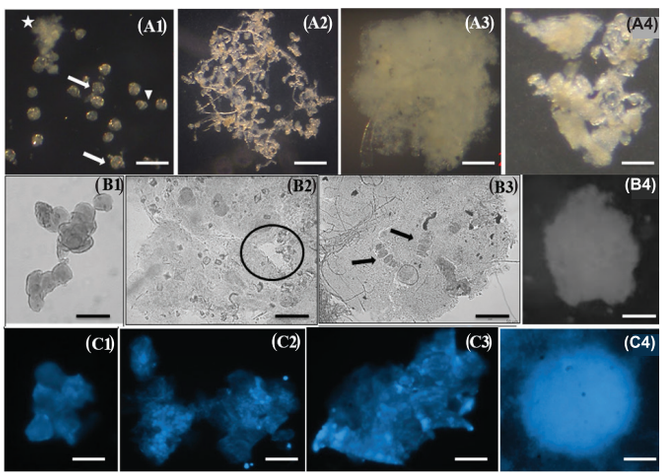

Fig. 2. Callus growth of melon microspores *in vitro*. (A1-A4) Callus formation process (ratio bar = 0.1 mm); **(B1-B4)** Histological observation of callus (scale bar = 50 µm); **(C1-C4)** Histological observation of callus. Scale bar = 50 µm.

Different forms of cell induction involved in embryonic or callus formation were all observed in a cultured environment. After stress treatment, cell observations showed that some cells continue to grow in a gamenial pathway, becoming mature pollen (triangular symbol, Fig. 2A1). In addition, a certain number of microspores did not show any morphological change from the initial implantation and some cells died or stopped growing. This is similarly indicated in the other studies [21, 22].

After 15 days, callus-like structures began to appear in two different forms. Fig. 2A3 shows the form of block callus, which is tightly bound, white, and amorphic. Meanwhile, Fig. 2A4 represents the cluster callus structure, which is discrete, yellowish-white, and amorphic. Histological observations show that inside the cluster callus is the presence of cells that are continuing to divide (arrow mark Fig. 2B3) and form a space between parts in a discrete callus (circled, Fig. 2B2).

After eight weeks, arising calli and the continuation of the growth phase is no different from the original and embryo-like structures can be observed on the surface of some discrete calli (Fig. 2). Embryonic structures at the late spherical and heart-shaped stages were given in which a protective layer was distinguished. However, under these conditions, further development of these structures into mature embryos or germinated seedlings were not observed.

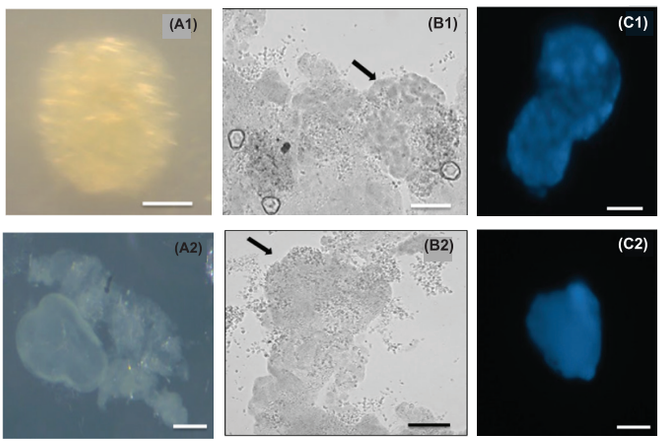

Fig. 3. The development of embryos in the culture environment of melon microspores. (A1) Spherical embryo; **(A2)** Heart-shaped embryo, scale bar = 0.2 mm; **(B1)** Spherical workpiece (arrow mark); **(B2)** Heart-shaped embryo (arrow mark), scale bar = 100 µm; **(C1)** Spherical embryo; **(C2)** Heart-shaped embryo (even arrow), scale bar = 50 µm.

3.3. Influences of microspore density and flower bud size on callus induction

Microspore density and bud size significantly affected the success of isolated microspore cultures [4]. In this study, a significant number of calli were observed at 4×10^4 cells/ml in three size groups (Table 3). In particular, the highest number of calli was recorded in flower buds of 7.0-7.9 mm (11.00±1.02 callus/petri dish).

In studies of *Brassica napus* L., the culture density for embryonic growth was 4×10^4 (cells/ml) in flower buds of 3.3 to 3.4 mm [23] while other studies showed that the highest embryo induction was given in 2.5-3.0 mm buds containing middle nucleate microspores in the range of 7×10^4 to 10×10^4 (cell/ml) [24]. In the study on *Brassica oleracea* cabbage, the cell density of 15×10^4 (cells/ml) in flower buds was 4.5 to 4.6 mm long for the highest embryonic arising rate of 15.2±2.17 [25].

Table 3. Interactive effect of microspore density and flower bud size on callus induction.

No.	Cell density (cell/ml)	Number of calluses at different flower bud sizes (callus/petri dish)		
		6.0-6.9	*7.0-7.9*	*8.0-8.9*
1	4×10^4	4.33 ± 1.06^{a}	11.00 ± 1.02^{a}	1.33 ± 0.51^{a}
2	6×10^4	2.58 ± 0.89^{f}	5.00 ± 1.13^{b}	0.67 ± 0.26^{a}
3	8×10^4	0.5 ± 0.29^{b}	2.92 ± 1.05^{b}	0.5 ± 0.23^{a}
4	16×10^4	0.83 ± 0.41^{b}	4.08 ± 0.89^{b}	0.33 ± 0.14^{a}

Note: Different letters on the same column indicate a statistically significant discrepancy in the sample average with $p<0.05$.

The establishment of optimal culture density is the initial step toward an efficient protocol. In previous studies, a lower density than the optimal value affected embryo production [25]. In addition, high density increases the single effect between cells that release nicotinamide and purine metabolising agents, which have a strong effect on the early stages of embryogenesis. However, when plating density is higher than the optimal concentration, autoxidation or inhibitory compounds can affect the reduction of embryos or calli [26, 27] and increase abnormal morphology [28].

Effect of heat shock and flower bud size on callus induction: Heat shock is an important factor in isolated microspore culture. Without heat stress, microspores form pollen through gametogenesis. The appropriate temperature will stimulate and transfer from the gametophytic to the sporophytic pathway [2].

Temperature stress during culture depends on the ecological type of the mother plant. For example, cucumbers from cold regions like Jinglv No. 4 cucumbers in the northern region of China have a good responses to cold pre-treatment while varieties from more temperate regions such as Erzaozi in southern China have a better responses to high-temperature conditions. For melons, the optimum development temperature is between 18 and 28°C and can withstand up to 40°C for several hours per day [26]. In this study, high-temperature experiments over three different periods were conducted and the results are shown in Table 4.

Table 4. The interaction effect of flower bud heat shock and size on callus induction.

No.	Temperature and time	Number of calli of different flower buds per a petri dish		
		6.0-6.9	*7.0-7.9*	*8.0-8.9*
1	33°C in 24 h	0.6 ± 0.16^{a}	3.3 ± 0.47^{a}	3.5 ± 0.72^{ab}
2	33°C in 48 h	1.8 ± 0.33^{abc}	7.0 ± 0.67^{abc}	3.8 ± 0.49^{abc}
3	33°C in 72 h	1.3 ± 0.42^{ab}	4.1 ± 0.46^{f}	2.2 ± 0.36^{ab}
4	35°C in 24 h	1.3 ± 0.34^{ab}	4.1 ± 0.72^{f}	1.6 ± 0.45^{a}
5	35°C in 48 h	2.9 ± 0.28^{abc}	7.7 ± 0.75^{bc}	4.0 ± 0.42^{abc}
6	35°C in 72 h	4.3 ± 0.40^{cd}	6.5 ± 0.64^{abc}	4.7 ± 0.50^{bc}
7	37°C in 24 h	3.3 ± 0.50^{bc}	5.2 ± 0.42^{ab}	3.5 ± 0.4^{ab}
8	37°C in 48 h	7.0 ± 0.86^{e}	9.4 ± 0.85^{c}	6.1 ± 0.66^{cd}
9	37°C in 72 h	3.3 ± 0.42^{bc}	6.4 ± 0.54^{abc}	4.2 ± 0.36^{bc}
10	40°C in 24 h	6.5 ± 0.86^{de}	8.1 ± 1.13^{bc}	6.2 ± 0.73^{cd}
11	40°C in 48 h	8.5 ± 0.95^{e}	17.9 ± 1.89^{d}	8.9 ± 0.53^{e}
12	40°C in 72 h	7.1 ± 0.48^{e}	9.3 ± 0.79^{c}	7.6 ± 0.60^{de}

Note: Different letters on the same column indicate a statistically significant discrepancy in the sample average with $p<0.05$.

In general, the temperature has a significant effect on callus induction. In this study, at lower stress temperatures for short times (i.e., 33°C for 24 hours), the lowest number of calli was generated (0.6±0.16). In contrast, heat shock treatment at 40°C for 48 hours was the most suitable treatment for callus production in all flower bud sizes. Specifically, 17.9±1.89 calli/petri dish were formed in buds 7.0-7.9 mm-long and 8.9±0.53 calli/petri dish in 8.0-8.9 mm-long buds. However, at the same temperature, the heat shock period lasting 72 hours affected the number of calli in all three flower bud size groups.

Treatment at high temperatures from 32 to 35°C is commonly used to induce the embryogenesis of microspores in different plant species [29]. For the cucurbits, the microspore culture method is less studied but mainly focuses on the anther culture method. Kumar et al. showed that the best response of cucumber microspores in terms of androgenic capability was obtained by incubating anther at 32°C for 1 day [30]. This is in agreement with the observation of H. Song, et al. (2007) [31] who mentioned that heat treatment at 33°C was optimal for embryo induction from microspores in cucumbers. Meanwhile, heat shock at 35°C allowed the formation of haploid plants in squash [32]. However, in watermelon and squash, haploid plants could be obtained without high-temperature treatments [33, 34].

4. Conclusions

From this study, the conditions of isolated microspore culture of melon (*Cucumis melo* L.) were established. Three different groups of bud size, i.e., 6.0-6.9, 7.0-7.9, and 8.0-8.9 mm, which contained the most microspores in the late uninucleate stage of development, were studied. The highest number of calli was observed when 7.0-7.9-mm buds were cultured on MS-13 at pH 5.8. The suitable density for high callus growth is 4×10^4. Heat treatment at 40°C for 48 hours was best suited for callus arising at all flower bud sizes. The cell viability after 7 days reached 75.6%. The development of microspores as well as the formation of calli and embryos were observed and evaluated for cell characteristics and morphology. This study provides the first result of microspores isolated from melon (*Cucumis melo* L.).

CRediT author statement

Minh Ly Nguyen: Conceptualization, Methodology, Investigation, Writing - Reviewing and Editing; Ton Nu Bao Tien Huyen: Data curation, Writing - Original draft preparation.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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