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ANTHER-DERIVED CALLUS FORMATION IN BITTER MELON (*Momordica charantia* L.) AS INFLUENCED BY MICROSPORE DEVELOPMENT STAGE AND MEDIUM COMPOSITION

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Abstract

Anther culture is one of the powerful techniques to pure line production for the short time. This method can be applied to accelerate the breeding process of F_1 hybrids of bitter melon (*Momordica charantia* L., family *Cucurbitaceae*), which is an important commercial crop in tropical and subtropical countries of South America, Asia and Africa. Although, there are several factors affecting the success of this method, the effective protocol of bitter melon anther culture has not been developed at present. The results of this study showed that the microspore developmental stage has a significant ($p \leq 0.05$) effect on speed and rate of callus formation. It was revealed that the frequency and the rate of callus formation and the morphology of callus substantially depend on the composition and concentration of growth regulators in the nutrient medium. In addition, the dynamics of callus formation in bitter melon anther culture was first studied. The main objective of the work was to study the influence of the microspore developmental stage and the composition of nutrient medium on the ability of callus formation in bitter melon anther culture in vitro. Plants of the F_1 hybrid bitter melon Diago 26 were grown in field in Dailoc district, Quangnam province (Vietnam) in 2018 according to the standard technique for obtaining commercial fruits. The buds harvested at 5-7 am were stored in plastic bags in dark condition at 4 °C for 1 day. Before culturing of anther, flower buds were surfaced sterilized using 70 % ethanol and 5 % sodium hypochlorite (NaOCl). Anther removed from flower buds were inoculated in the induction medium in the horizontal laminar air flow; then incubated at 25±2 °C and a photoperiod of 16 h light/8 h dark for 4 weeks. Three variants of the Murashige-Skoog (MS) nutrient mediums were used, differing in composition and concentration of growth regulators: MK1 with the addition of 1.0 mg/l 2.4-D and 1.5 mg/l BAP; MK2 — 1.5 mg/l 2,4-D and 1.0 mg/l BAP; MK3 — 1.5 mg/l NAA, 1.0 mg/l BAP and 0.5 mg/l kinetin. The completely randomized design with statistical analysis using the software IBM SPSS Statistics Base 22 and Microsoft Excel 2013 were carried out. The cytological analysis result showed that each anther contained microspores with different development stages. Therefore, it is impossible to separate microspores at the unique developmental stage being suitable for anther culture; however, there is always a predominant stage. In this study, buds were divided into two main groups. The first group consisted of buds with the size of 4.0-5.0 mm with early and mid uninucleate microspores. The second group included buds with the size of 5.1-6.5 mm with late uninucleate and binucleate microspore. After inoculation, the beginning of calli formation was observed within the first week for anther of the buds with the size of 4.0-5.0 mm and within the second week for the buds with the size of 5.1-6.5 mm. The highest frequency of callus formation (93.75±2.55 %) was observed on MS medium supplemented with 1.0 mg/l of 2,4-D and 1.5 mg/l of BAP (MK1). Most calli were formed during the second and third week after cultivation. Also, there was the significant difference about the morphology of the calli obtained on three nutrient medium. Calli on medium MK1 were yellow, strongly dense and calli on MS medium with the addition of 1.5 mg/l of 2,4-D and 1,0 mg/l of BAP (MK2) being green, strongly dense. Green-yellow, dense calli were obtained on medium supplemented with 1.5 mg/l NAA and 1.0 mg/l BAP and 0.5 mg/l Kinetin (MK3). However, the effect of

the developmental stage of microspores on the morphology of calli was not revealed. Despite receiving a large number of calli, the formation of embryoids was not observed

Keywords: *Momordica charantia* L., bitter melon, callus, doubled haploid, anther culture, microspore stage

Bitter melon (*Momordica charantia* L.) is an economically important crop distributed widely in tropical and subtropical countries in South America, Asia and Africa [1, 2[. This crop has been cultivated throughout the year in Asia countries [3]. The area of bitter melon is about 340.000 ha, most of the production is concentrated in China and India [4]. The bitter melon fruit is used not only as a kind of vegetable, but also applied in medicine for treatment of various diseases including diabetes, gout, cancer [5, 6]. Chemical constituent analysis proved that *Momordica charantia* L. contains saponins (momordisin, momordin), which have shown promising biological activities including antibacterial, antifungal, antiviral, insecticidal effect [7].

Currently, about 80% of bitter melon seeds using in production are hybrids [8]. However, the range of bitter melon hybrids remains limited due to the complicated and prolonged breeding process. Nowadays the obtaining of F_1 hybrids is mainly depends on the pure lines production, while the process of pure lines production is very complicated [9]. By contrast for traditional selection methods, in with self-pollination for 5-7 generations has been carried on to obtain pure lines [10, 11], the technology of double haploids (DH) production can be applied to speed up the breeding process [12, 13]. DH plants have been obtained from more than 200 species. This technology is especially widely applied on cabbage and grain crops [14, 15]. One of the first and most common methods of DH production is anther culture. It is the singular method of DH plants receiving for some crops. The success of this technology depends on many factors, such as genotype, developmental stages of microspores, nutrient medium composition, addition of growth regulators, culture conditions, and the way of plant regeneration from callus [16]. Therefore, the anther culture method needs to be optimized for each crop, even for each genotype, especially in vegetable crops [17].

Researchers, cultivated the bitter melon anthers, received callus, but the plants generation from obtained callus could not be achieved [18-20]. Tang et al. [19] found that callus were formed from the anthers after flower buds pretreating whis low temperature of 4 °C for 48 hours and cultured on Murashige-Skoog medium (MS) with the addition of 3% sucrose, 0.5 mg/l 2.4-D (2.4-dichlorophenoxyacetic acid) and 2.0 mg/l BAP (6-benzylaminopurine). Supplementing vitamin C and $AgNO_3$ resulted in reducing of necrotic callus frequency [19].

There are few published studies that describe callus formation in the bitter melon anther culture, but there is still no clear opinion about the effect of a number of factors.

In the present research, we showed that the size of the flower buds, that is the stage of microspores development, affects significantly ($p \leq 0.05$) the ability of callus formation. We found out, that microspores of different developmental stages can be contained in the same anther. It was revealed that the calluses morphology, the frequency and the time of callus formation in bitter melon anther culture substantially depends on the composition and concentration of growth regulators in the nutrient medium. Besides, the dynamics of callus formation in the process of the bitter melon anthers cultivating was studied for the first time.

The aim of the present research was to study the effects of the microspore developmental stage and the nutrient medium composition on the callus formation in anther culture in vitro.

Techniques. The F_1 hybrid Diago 26 bitter melon was used in this re-

search, seeds producer company is Tropical Development and Investment Company Limited (Công ty trách nhiệm hữu hạn LLC), Vietnam. The plants were grown in 2018 on the farm in Dailoc district, Quangnam province, Vietnam, according to standard technique for commercial fruits obtaining. The flower buds were harvested in the morning at 5-7 am and stored in plastic bags at 4 °C for 1 day.

The developmental stage of microspores was monitored by microscopy using acetocarmine dye [21]. Microspores were isolated from the buds in a drop of glycerin by the clamp on a glass slide. Then they were transferred to a new glass slide containing a drop of 2% acetocarmine solution, covered by a cover glass and observed at magnification ×400 (Axio Imager.M2 microscope, Carl Zeiss, Germany). The developmental stage of microspores was determined by the number of nuclei and their location in the cell [22]. To assess the relationship between the size of the buds and the stages of microspores development, the buds were measured in sizes from 4.0 to 7.0 cm with a distance of 0.1 cm. 450 microspores from three anthers were observed in each group.

Before culturing of anther, flower buds were externally sterilized with 70% ethanol for 30 seconds and sterile distilled water for 15 seconds twice; then buds were immersed into 5% sodium hypochlorite (NaOCl) for 4 minutes and rinsed with sterile distilled water 5 times for 15 seconds. The anthers were taken from buds using tweezers (the filaments were tried to be removed) and placed on the induction medium in plastic Petri dishes of 6 cm in diameter, 8 anthers per dish, in the conditions of horizontal laminar air flow. Anther were incubated at 25±2 °C and a photoperiod of 16 h light/8 h dark for 4 weeks.

In this experiment, three variants of nutrient media based on the Murashige-Skoog (MS) nutrient medium were used. Nutrient media contained 3% sucrose and 0.7% agar (pH 5.8), variants differed in composition and concentration of growth regulators, i.e. 1.0 mg/l 2,4-D and 1.5 mg/l BAP for MK1, 1.5 mg/l 2,4-D and 1.0 mg/l BAP for MK2, 1.5 mg/l NAA, 1.0 mg/l BAP and 0.5 mg/l kinetin for MK3.

The effectiveness of callus formation was assessed by the ratio of the number of formed calluses and cultivated anthers, as well as by the color and density of callus. All the experiments were performed in 4 replications, the number of anthers in each replicate was 24.

The completely randomized design with statistical analysis using the software IBM SPSS Statistics Base 22 (IBM Corporation, USA) was carried out. The mean values of the studied parameters (M), standard errors of the mean (±SEM) and confidence interval were determined at 95% confidence level ($t_{0.05}$ × SEM). The significant difference between the variants was evaluated by Student's t-test. The significant test was set at $p \leq 0.05$. Graphs were built with Microsoft Excel 2013.

Results. The developmental stage of microspores is the key factor determining the success of anther culture technology [23]. Each stage corresponds to a definite range of buds size [24]. Therefore, the relationship between the buds size and the microspore developmental stages was investigated in order to select the suitable buds of the F_1 hybrid Diago 26 bitter melon for following culture.

As shown in figure 1, the single anther contains microspores at different developmental stages, from early uninucleate to binucleate. Therefore, it was impossible to select bitter melon buds with microspores at the definite developmental stage. The ratio of microspores at different developmental stages depended on the bud size. In buds of the same size this ratio was similar. We also observed that it was always possible to detect the predominant developmental stage (Fig. 2).

Cytological analysis of anthers found that the buds with the length of 4.0-4.5 mm contained microspores at four developmental stages, in which, the early uninucleate stage was predominant accounted for 60.19±2.32%, the percentages of microspores at mid uninucleate, late uninucleate and binucleate stages were 24.08±0.98%, 14.99±1.8%, 0.74±0.74%, respectively. For the 4.6-5.0 mm buds, there were also four stages of microspores development with the prevalence of the mid uninucleate stage stood at 71.98±0.42%. Therefore, it could not be separated the anthers with the unique development stage of microspores; however, it was possible to separate the anthers containing microspores at the predominant stage of optimal development. The anthers of the 5.1-6.0 mm long buds were found to contain microspores only at two stages (late uninucleate and binucleate), mostly at binucleate stage (82.64±0.59%). The buds with the length of 6.1-6.5 mm and 6.6-7.0 m had respectively 65.54±4.26%, 88.71±2.54% of binucleate microspores.

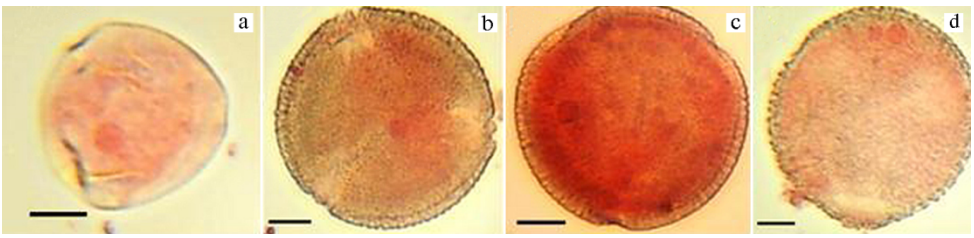

Fig. 1. The stages of microspores development of bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26: a — early uninucleate, b — mid uninucleate, c — late uninucleate, d — uninucleate (Axio Imager.M2 microscope, Carl Zeiss, Germany; magnification ×400, scale bars = 10 μm).

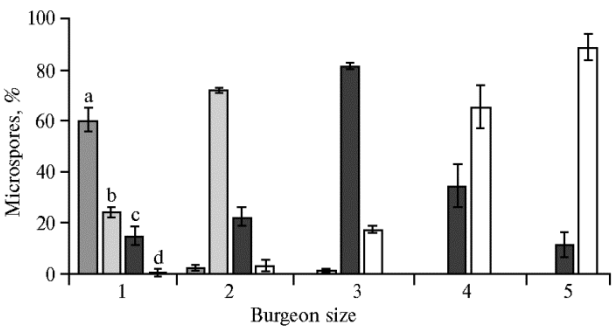

Fig. 2. The percentages of bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26 microspores at early uninucleate (a)**, mid uninucleate** (b)**, late uninucleate** (c) **and binucleate development stages in the buds of different sizes:** 1 — 4.0-4.5 mm, 2 — 4.6-5.0 mm, 3 — 5.1-6.0 mm, 5 — 6.6-7.0 mm.

The obtained in the present study results were different from previously published by other researches. As Tang et al. [18] reported, the buds size corresponds to a specific microspores developmental stage: 3.0-4.0 mm length bud contains early uninucleate microspores, 3.0-4.0 mm buds contain late uninucleate microspores, binucleate stage being observed at bigger buds (above 5.0 mm). This difference could be explained by a genotype specific relationship between the bud size and the developmental stages of anther. The same conclusions were also obtained for other crops [25]. Therefore, in order to achieve the best result of anther culture, it was necessary to determine the relationship between the bud size and the microspore development stages corresponding to each genotype.

To study effects of the developmental stages of microspores on the efficiency of callus formation, flower buds of two size ranges were cultivated in vitro: 1) 4.0-5.0 mm buds that contained early uninucleate and mid uninucleate microspores; 2) 5.1-6.5 mm buds with uninucleate and binucleate microspores. Two anther groups were cultured at three nutrient media, MK1, MK2 and MK3, with different concentrations and compositions of growth regulators. The result of the research revealed that the supplement of growth regulators and the

bud size had significant effects ($p \leq 0.05$) on the efficiency of callus formation in bitter melon anther culture; there was no effect of interaction of these factors. This finding is consistent with previous researches on the bitter melon as well as on other crops [18, 26, 27]. Using the buds containing early uninucleate and late uninucleate microspores induced the high frequency of callus formation. The average frequencies of callus formation were 79.17±4.57%, 68.23±4.00% when 4.0-5.0 mm of and 5.1-6.5 mm buds were used.

The results of the table show that callus formation was the most effective on MK1 medium with the 4.0-5.0 mm long buds. The frequency of callus formation was 93.75±2.55%. This is higher than the best result of Tang et al. [19] who reached 80.55% in culturing anthers on MS medium supplemented with 0.5 mg/l 2,4-D and 2.0 mg/l BAP. There was no significant difference between MK1 medium and MK3 medium. Culturing on MK2 medium resulted in the lowest frequency of callus formation (51.56±3.93%). Furthermore, there was no interaction effect between medium composition and bud size on the frequency of callus formation.

Effects of bud size and medium composition on callus formation in anther culture of bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26 (*M*±SEM)

Medium	Plant growth regulators, mg/l				Callus formation		Callus morphology
	2,4-D	BAP	K	NAA	percentage, %	starting time	
4.0-5.0 mm buds							
MK1	1.0	1.5	–	–	93.75±2.55[a]	week 1	Yellow, strongly dense
MK2	1.5	1.0	–	–	60.94±5.33[be]	week 1	Green, strongly dense
MK3	-	0.5	1.0	1.5	82.81±2.99[af]	week 1	Green-yellow, dense
5.1-6.5 mm buds							
MK1	1.0	1.5	–	–	73.44±2.99[cbf]	week 2	Yellow, strongly dense
MK2	1.5	1.0	–	–	51.56±3.93[de]	week 2	Green, strongly dense
MK3	–	0.5	1.0	1.5	79.69±2.99[af]	week 2	Green-yellow, dense

N o t e. For description of the media composition, see the *Techniques* section. 2,4-D is 2,4-dichlorophenoxyacetic acid, BAP is 6-benzylaminopurine, K is kinetin, NAA is 1-naphthaleneacetic acid. Different alphabets indicate statistically significant differences at $p \leq 0.05$.

Therefore, anthers of the F_1 hybrids Diago 26 bitter melon are able to form callus when containing microspores at various developmental stages. The early uninucleate and mid uninucleate stages are the most suitable, though late uninucleate and early binucleate stages are the best for *Brassicaceae* DH technology [28] and mid uninucleate and late uninucleate stages are the most suitable for cucumber [29, 30]. Tang et al. [18] proved that culturing of bitter melon anthers containing late uninucleate microspores resulted in higher efficiency of callus formation comparing to late uninucleate.

The date of start of callus formation varied and depended on the predominant stage of microspore development. For small size buds, calli formation could be observed within the first week of culture. Meanwhile, calli formation was found on the second week for larger buds. So, there sizes of calluses can be different on the same medium. It is very important to determine the time of callus formation for further sub-culturing or plant regeneration.

It was also found, that the rate of callus formation varies due to culture period. On MK1 medium, there was a difference between the buds of different sizes, and the largest number of callus was formed within the second and the third week (Fig. 3). On MK2 and MK3 medium, the highest callus formation frequency was within the second week. The callus formation was asynchronous. So, there were different sizes of calluses even at one Petri dish, in one bud passage.

There was also a significant difference in calli morphology depending on concentration and composition of growth regulators in nutrient medium after 4

weeks of culturing. Calluses on MK1 medium were yellow, strongly dense, calluses on MK2 medium were green, strongly dense and green-yellow, dense calluses were obtained on MK3 (Fig. 4). By contrast, effect of development stages of microspores on callus morphology was not observed.

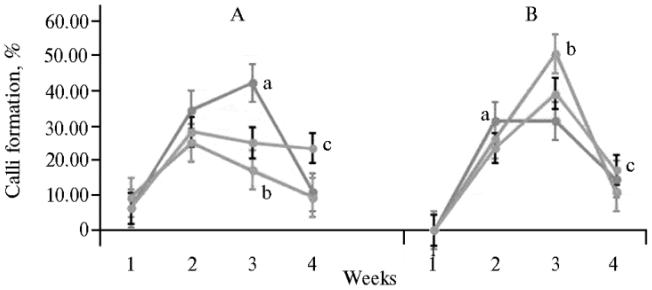

Fig. 3. The rate of callus formation in bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26 derived from microspores of 4.0-5.0 mm (A) **and 5.1-6.5 mm** (B) **buds on MK1 medium** (a)**, MK2 medium** (b) **and MK3 medium** (c) **depending on culturing time** (for description of media composition, see the *Techniques* section).

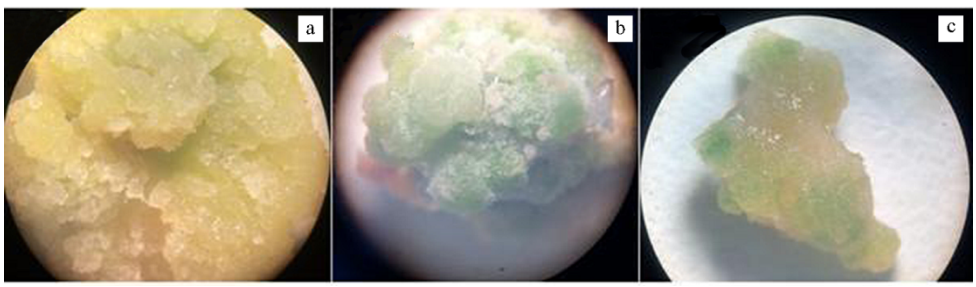

Fig. 4. Callus morphology of bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26 on different media after 4-week culturing: MK1 (a), MK2 (b), MK3 (c) (Axio Imager.M2 microscope, Carl Zeiss, Germany; magnification ×40; for description of media composition, see the *Techniques* section).

The time when callus formation began and growth rate of calli have not been mentioned in previous studies which have just focused on general calli formation time. However, the period of callus formation able to plant regeneration can vary depending on genotype and medium composition. For example, cucumber calli can form yellow embryoids after 6 weeks of culturing [12, 31], and this period can take 4 months for banana [32].

Although the high frequency of callus formation was obtained on all media, the formation of embryoids was not observed. Culturing for more than 4 weeks caused the calli to turn brown. This finding was consistent with the results of Tang et al. [20]. It is possible that the momordica callus cells accumulate secondary metabolites which prevent embryoid formation and organogenesis. By comparing concentration ratio of endogenous growth regulators in callus cells (gibberellic acid — GA3, zeatin, indole-3-acetic acid — IAA, abscisic acid — ABA), Tang et al. [20] found that stem tissues had higher concentration of Zeatin than callus formed from anthers. Besides, the regeneration occurred only in calli formed from stem tissues. In addition, callus able to plant regeneration had the lower ratios of IAA/zeatin and GA3/zeatin in [20].

Thus, the results of this study proved that the concentration of growth regulators significantly affects the rate, frequency of callus formation, and the callus morphology in anther culture of bitter melon (*Momordica charantia*) F_1 hybrid Diago 26. It has been established that one anther contains microspores at different developmental stages. The significant difference in the callusogenesis ability was revealed between the anthers containing early uninucleate, mid uninucleate microspores and those containing late uninucleate and binucleate microspores. For the highest frequency of callus formation (93.75%), anthers of flower buds of 4.0-5.0 mm length should be cultured on MS medium with 3%

sucrose and 0.7% agar supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid and 1.5 mg/l 6-benzylaminopurine.

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