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Research Article

Induction of Microspore Embryogenesis of Eggplant (*Solanum melongena* L.) 'Gelatik'

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ABSTRACT

The haploid or double haploid plant of eggplants could be produced from microspore culture (embryogenesis of microspores). In the breeding programs, microspore can be developed into an embryo directly after exposure to stress treatment during cultured. Stress (temperature and starvation medium) is an important factor in the induction of embryogenesis microspore. This study aims to induced embryogenic microspores from eggplant CV. Gelatik. The stage late-uninucleate microspore (*Vacuolate Microspore*/VM) and early binucleate (*Young Bicellular Pollen*/YBP) are the suitable stages to induce multinucleate structure. There are 3 methods used in this research; 1) Determination of the stage development of microspore based on flower buds length and anther length. 2) Induction of embryogenic microspore on the pre-treatment and starvation medium. 3) After giving pre-treatment for 4 days, micropores were transferred to culture medium A2 at 28°C in dark conditions to induce the multicellular structures. This study reported that 50-68.51% of the VM+YBP stage obtained in the range of flower bud lengths of 10-17 mm, and 5.0-6.9 mm, the range of anther length containing VM+YBP of 50-77.48%. The pre-treatment heat shock at 33°C in the medium B for 2 days, produced embryogenic microspores with a high percentage, that is about 50.19%, while microspores at 25°C and 4°C respectively 46.17% and 49.28%. Pre-treatment for 4 days at 4 °C, 25 °C, and 33°C with the percentage of embryogenic microspores apiece 32.87%, 27.45%, and 37.34%. The multicellular (*starlike*) structure begins forming on the fifth day of incubation in culture medium (A2) after pre-treatment in B medium at 33°C.

Keywords: Eggplant, flower bud, microspore, stress treatment, embryogenic microspore

INTRODUCTION

Eggplant (*Solanum melongena* L.) is one of the essential vegetables in tropical and subtropical regions around the world. It is the fifth most economically crucial solanaceous plant after potatoes, tomatoes, pepper, and tobacco (FAO, 2014; Taher *et al.*, 2017). Improvement on eggplant production carried out through biotechnology and hybridization approaches (Kalloo, 1993; Kashyap *et al.*, 2003; Magioli and Mansur, 2005; Bal *et al.*, 2009) such as regeneration of haploid or double haploid plants, through anther or microspore culture (*Microspore Embryogenesis*). Microspore embryogenesis represents a unique system which can produce homozygous lines in a relatively short period and does not require much effort and cost compared to conventional crossing methods which require a lot of crossing over time (Snape, 1989; Maluszynski *et al.*, 2003; Adhikari and Kang, 2017). Usually, microspores will develop into mature pollen (*pollen grain*) and ready to fertilize an egg. Through in vitro culture, the normal development of microspores can be transformed into the development of the sporophytic phase that will form embryos directly. The ability of male gametophytes to change their developmental fate from pollen to embryonic development can occur when exposure to stress treatments during culture. This process referred to as microspore embryogenesis (Soriano *et al.*, 2013). Stress treatment has an essential role in this process, encouraging microspore differentiation and conditioning the androgenic responses (Munoz-Amatriain *et al.*,

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2009).

In microspore embryogenesis, free microspores isolated from anther are cultured in vitro and after pre-treatment stress, microspores will develop sporophytically (Shariatpanahi *et al.*, 2006; Bal *et al.*, 2009). Some stress treatments have been known to induce embryogenic microspores, including cold shock, heat shock, carbon starvation, and nitrogen in growing medium and colchicine treatment (Moraes *et al.*, 2004). During the embryogenesis induction, identification of microspores that have embryogenic abilities and the mechanism of transformation of microspores into embryogenic cells are carried out. Although the initial stage of development of microspores for the induction of embryogenesis differs between species, usually microspores are chosen in the vacuolated stage. Vacuolated microspore stage is the development stage that allows reprogramming of microspores in most species (Olmedilla, 2010). Salas *et al.* (2012) revealed microspores at the stage of young microspore (YM) and mid-microspore (MM) in eggplant did not show a growth response after being transferred to embryogenesis medium. The optimal stage for embryogenesis induction is not the transition stage from vacuolate microspore (VM) to the young bicellular pollen (YBP) stage. When VM and YBP were cultured in a liquid medium, both showed a growth response towards embryogenic microspores. In this study, we determine the stage of microspore development through the selection of flower buds as the first step in microspore culture. To observe the anatomy of the anther, we analyze the correlation between the stages of microspore development and the stage of anther development. Furthermore, induction of embryogenic microspores by pre-treatment of temperature stress (4°C, 25 °C, and 33°C) with a combination of medium starvation (B) for 4 days will follow, the protocol of the microspore culture of eggplant cv. Bambino (Bal *et al.*, 2009).

MATERIALS AND METHODS

Plant Material

The seeds of eggplant cv. Gelatik were sown in a plug-tray and transferred to a pot in a greenhouse of Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada. The seedling, one and a half months of age or has 4 leaves transferred to a pot measuring 30x27x17 cm^3 and filled with planting media consisting of soil, manure, and compost. The plants watered daily, and the soil was supplemented with Growmore fertilizer NPK (15:15:15) and for leaves.. The plants were maintained and pruned by bud shoot (*dominancy apical*) to produce many branches. Flower buds then picked as the primary materials of this study.

Determination of Microspore Developmental Stage

The flower buds of various sizes were collected and grouped according to the size of the flower bud length and anther length (shown in Table 1). The buds were measured using Nankai digital calipers 0-150 mm. The length measured from the base of the calyx to the tips. After the measurement, the anther removed from the flower bud. Anther from each flower bud was crushed in medium B to observe the development of microspores by Microscope (Nikon Diaphot 300 Fluorescence; Japan) and images were taken by OptiLab face 2.2 Miconos. Determination of the percentage of each microspore stage in the same anther was counted from 10 randomly selected images from five display fields. The number of each microspore is calculated by dividing the number of microspores at a certain stage by the total number of microspores based on the calculation of 10 images.

Anatomic Preparation of Anthers

An anther of different size flower buds was put in a vial bottle. It is then fixed in FAA fixative solution (formald ehyde: absolute alcohol: glacial acetic acid: aquadest 2:10:1:7) for 24 hours, followed by staining with safranin 1% orange in 70% alcohol for 24 hours, washing and dehydration: 70% alcohol, 80%, 95%, 100% twice, each for 30 minutes, dealcoholization: the ratio of alcohol: xylol 3: 1; 1: 1; 1: 3, and pure xylol twice, each for 30 minutes. The last step was washing by the xylol: paraffin ratio of 1:9 at 57 °C for 24 hours twice. The stage of planting or making a blocking was after incubation for 1 hour in pure paraffin. Paraffin was cut using a Rotary Microtome with a thickness of 15 µm. The last step was washing and staining using safranin 1% in 70% alcohol, and fast green 1%. Washing materials were xylol twice, alcohol: xylol (1: 3, 1: 1 and 3: 1), alcohol 100% twice, 95%, 80%, and 70% each for 3 minutes, Safranin 1% for 1 hour. After safranin, the slides were washed in aquadest, 70% alcohol, fast green staining, 80% alcohol, 95%, 100% twice, alcohol: xylol (3: 1.1: 1, and 1: 3), and lastly with xylol each for a minute. The Slide was mounted by Balsam Canada and covered with glass cover. Anatomy of anther was observed using a Boeco Binocular Microscope (BM-180 SP Germany).

Induction of Microspore Embryogenic with Temperature Stress and Starvation Medium

Anther from flower buds contained high-percentage VM + YBP microspores were used as microspores donors for the induction of embryogenic

microspores by stress treatment. Microspores were cultured in B Medium (Indrianto *et al.*, 2014) containing macronutrients: KCl 1.490 mg/L, $MgSO_4.7H_2O$ 250 mg / L, $CaCl_2.2H_2O$ 110 mg / L, KH_2PO_4 136 mg / L, Mannitol 54.630 mg / L with pH 7. The cultures incubated at 33°C, 25 °C, and 4 °C for 4 days. The observations were presented as a percentage of embryogenic microspores (VM, YBP, and Multinucleates) and non-embryogenic (YM, MM, and lysis microspores).

Development of Embryogenic Microspore

The binucleate and multinucleate microspores from the best pre-treatment combination of temperature stress and starvation medium were transferred to A2 medium (Touraev *et al.*, 1996) containing macronutrients: KNO_3 2.800 mg/L, $(NH_4)_2SO_4$ 460 mg/L, KH_2PO_4 400 mg/L, $CaCl_2.2H_2O$ 166 mg/L, $Mg.SO_4.7H_2O$ 185 mg/L, Iron 1 mL, micronutrients 1 mL, Vitamine (B5) 1 mL, MES (Morfolino Ethanol Sulfonic Acid) 1.950 mg/L, Glutamine 500 mg/L, and Maltose 90.000 mg/L, with pH 6.2. Embryogenic microspores were cultured at 25°C in dark conditions. This development observed for three weeks.

Statistical Analysis

The quantitative data consists of the number of embryogenic microspores and the increased diameter of microspores at different temperature treatments were analyzed using Microsoft Excel 2019. The data were obtained from three replications and presented as mean ± standard deviation in the tables.

RESULTS AND DISCUSSION

Developmental Stage of Microspore of Eggplant cv. Gelatik

The suitable stage of the flower bud (Figure 1) is a critical determinant of the success of microspores culture. The morphological size of flower buds, the length of flower buds, and the length of anther correlate with the stage of microspore development and the stage of anther development. In some studies, bud length and anther length are also used as benchmarks for the stages of microspore development such as in anther culture of tomato (Segui-Simarro and Nuez, 2005), eggplant (Salas *et al.*, 2012), and correlation with the development of pollen in anther culture of apple (Zhang *et al.*, 2013).

In this study, the microspore development stages were identified based on the length of flower

Table 1. Stage development of microspore in eggplant cv. Gelatik, based on the length of flower bud and length of anther.

Length of a Flower bud (mm)	Stage of Microspore (%±sd)					
	Tetrad/ Meiocyte	Uninucleate			Early Binucleate (Yong bicellular pollen)	Mature Pollen
		Early (Young microspore)	Mid (Mid microspore)	Late (Vacuolate microspore)		
6-7	100±0	-	-	-	-	-
8-9	-	*26.46±4.00*	*40.08±2.37*	33.46±3.88	-	-
10-11	-	10.67±6.52	19.03±6.05	**41.46±4.66**	**17.61±5.62**	11.23±7.02
12-13	-	5.42±4.38	17.42±8.39	**40.22±9.04**	**28.29±4.96**	8.65±4.43
14-15	-	*19.80±5.51*	*23.09±6.41*	**46.63±7.40**	**7.37±3.82**	3.11±1.85
16-17	-	*19.38±3.04*	16.47±4.37	**50.01±7.64**	**6.02±2.22**	8.12±2.52
18-19	-	-	-	11.01±1.17	**21.09±1.26**	67.90±5.08
20-21	-	-	-	-	-	100±0
>22	-	-	-	-	-	100±0
Length of Anther (mm)	Tetrad Meiocyte	Uninucleate			Early Binucleate (Yong bicellular pollen)	Mature Pollen
		Early (Young microspore)	Mid (Mid microspore)	Late (Vacuolate microspore)		
3.0-3.9	100±00	-	-	-	-	-
4.0-4.9	-	*38.93± 3.02*	*27.09±4.14*	**33.98±8.18**	-	-
5.0-5.9	-	16.40±2.04	*26.27±6.17*	**52.84±7.75**	3.11±1.27	1.38±1.08
6.0-6.9	-	-	12.07±4.70	**45.29±6.45**	*32.19±8.42*	10.45±1.23
7.0-7.9	-	-	-	-	*39.33±5.53*	60.67±4.66
8.0-8.9	-	-	-	-	-	100±0
>9.0	-	-	-	-	-	100±0

The result is expressed as a percentage of each stage ± sd
Bold values are flower bud lengths with a high percentage of VM+YBP, whereas *Italicized* values are bud lengths with a high percentage of Young Microspore (YM) and Mid Microspore (MM).

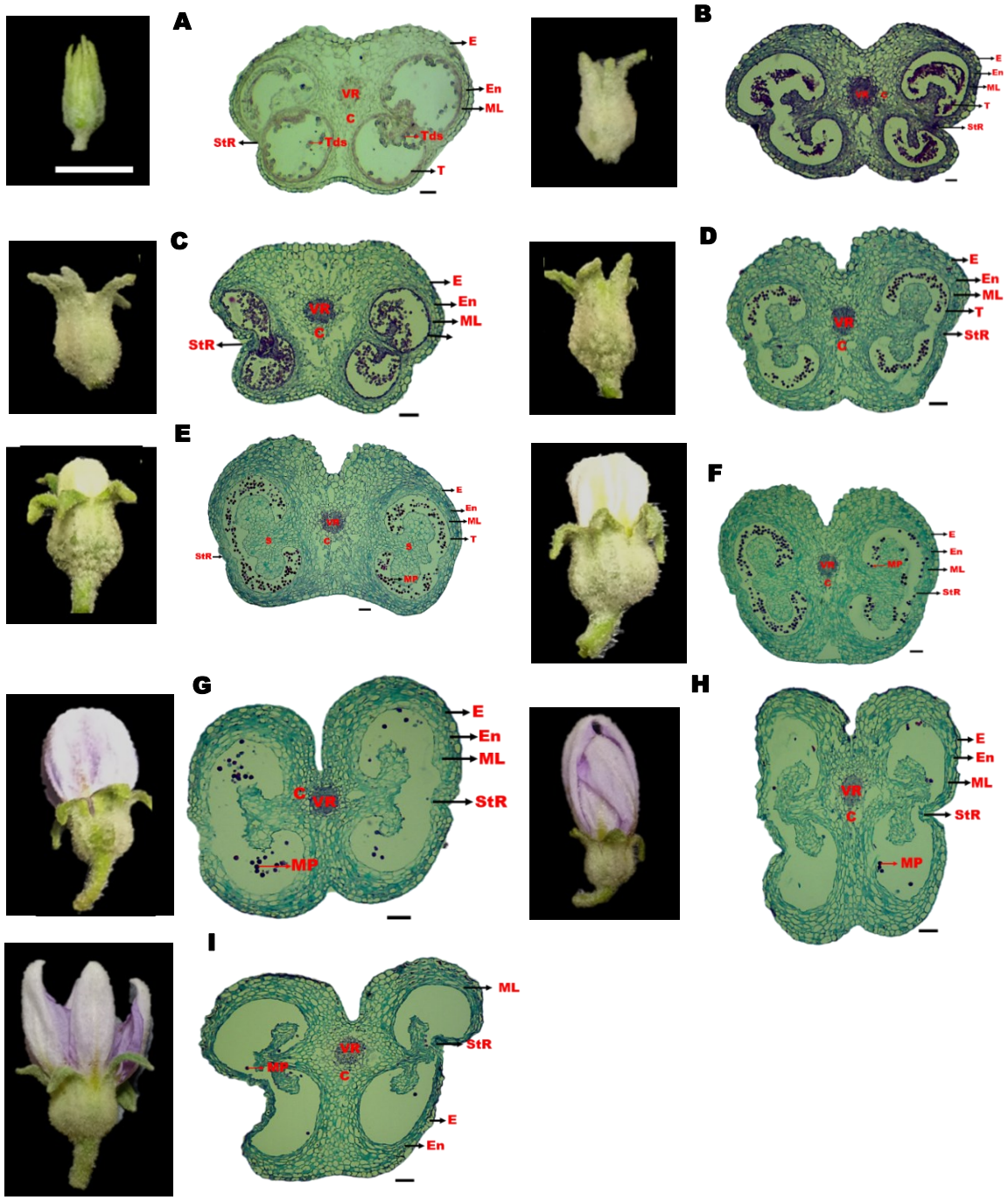

Figure 1. Morphology of the flower bud and transverse section of eggplant cv. Gelatik anther. The associated anther length of the flower bud with the developmental stage of the anther. A=3 mm; B= 4.5 mm; C=5.0 mm; D=5.5 mm; E=6.5 mm; F= 7 mm; G= 7.2 mm; H= 8 mm; I= 8.2 mm (Bar 10 mm). Transverse sections of eggplant anthers. The sample is anther of flower buds (1-9). StR, Stomium region; T, Tapetum; ML, Middle layer; E, Epidermis; En, Endothecium;VR, Vascular region; C, Connective; S, Septume; Tds, Tetrad; MP, Mature pollen (pollen grains). Scale bar =100 μm. (Personal documentation, taken on 19 October 2019, by OptiLab advance 2.2 (Miconos-Indonesia).

buds and the length of anther of eggplant cv. Gelatik. Table 1 shows that the VM + YBP stage of 50-68.51% is in the range of flower bud length of 10 -17 mm, and 5.0-6.9 mm range of anther length containing VM + YBP of 50-77.48%. The highest percentage of YM + MM was observed in the range of flower bud length and anther length of 8.0-9.0 mm with a percentage of about 66.54% and 4.0-4.9 mm at 66.02%. The length of anthers was suitable for cultured in the starvation medium at 5.0 – 5.9 mm, not for all measures. In this study, the same flower bud length has a different anther length. Therefore the percentage of mature pollen in the range of flower bud length 14-15 mm is lower than 16-17 mm or 12-13 mm. The YM and MM after pre-treatment at 33^{o}C, 25^{o}C, and 4^{o}C, then subcultured into A2 medium did not show a growth response, even some microspores experienced shrinkage. Salas *et al.* (2012) also stated that in the microspore culture of eggplant cv. Bandera. In the study of

morphological markers for the development of microspores in maize, Moraes *et al.* (2008) stated that anther length was the most reflected cytological stage of microsporogenesis. In wheat, it is also found that the length of the anther is considered an appropriate morphological marker for the assessment of the specific development of microspores (Immonean and Antila, 1998). However, it is essential to recognize that the measurements obtained for these parameters in each microspore developmental stage varied according to the genotype and the cultivation place.

Developmental Stage of Eggplant Anther

Stamens consist of two different structures, namely anther and filament. The length of the anther indicates the stage of anther development and the stage of microspores in the anther locus. Microspore development stages were identified based on cellular characteristics such as cell shape, cell number, nucleus type, and position in the cell, and state of the chromosomes (Adhikari and Kang, 2017). According to Browne *et al.* (2018) the length of the wheat anther is an accurate parameter measure to determine the stage of anther development and can be applied among wheat cultivars. Each stamen contains filaments and anthers with four lobes connected to the filament by connective tissues (Zhang and Wilson, 2009). In this study, the stage of anther development of wheat (Browne *et al.*, 2018), used as a reference for determining the developmental stage of the anther in eggplant cv. Gelatik.

In this research, the observation of anther development is starting from stage 8 (Figure 1A), namely the *Programmed Cell Death* (PCD) tapetum stage and the last tetrad stage (before the microspore tetrad is released as young microspore). Stage 9 anther (Figure 1B), which is the stage in which tetrad microspores are released as young microspores. Stage 10 (Figures 1C, 1D, 1E) is the vacuolated microspore stage. Stage 11 (Figure 1F), the anther contains the early binucleate microspores. Stage 12 (Figure 1G), the tapetum portion is completely degraded. Stage 13 (Figure 1H), the septum between the upper and lower locus begins to degrade. Stage 14 (Figure 1H), initial dehiscence, and stomium are degraded. The last stage of anther development is stage 15 (Figure 1I), where pollen released from the anther locus.

Induction of Embryogenic Microspore

The range of flower bud lengths 10-11, 12-13, 14-15 and 16-17 mm each contains VM + YBP 59.07%, 68.51%, 54%, and 56.03. The size of the flower bud used 12-13 mm for embryogenic microspores. Another main factor besides the phase of flower buds is the stress treatment because this is the main factor to determine the success of microspore embryogenesis induction. Microspores were cultured in 2 ml of liquid B medium for 4 days and incubated at three different temperatures (4°C, 25°C, and 33°C). The parameters observed were the percentage of embryogenic microspores (late uninucleate, binucleate and multinucleate microspores), and non-embryogenic microspores including young microspores (YM), mid- uninucleate (MM) and lysis or shrinking microspores. Microspores at this stage, if exposed to stress treatments (such as heat shock, cold shock, and starvation medium) will develop into an embryo. A high percentage of embryogenic microspores is also a determinant success of the stress treatment used. The results showed (Table 2) that, embryogenic microspores were observed during pre-treatment at 33 °C (heat shock), 25°C and 4°C (cold shock) for 2 days of culture and liquid B medium, respectively 50.19%, 46.17%, and 49.28%, after 4 days of incubation obtained embryogenic microspores of 32.87%, 27.45% and 37.34% at each incubation temperature. After 4 days of incubation, several microspores were lysis and shrinking. Incubation microspore of eggplant cv. Gelatik at 33°C for 2 days, enough to induce embryogenic microspore. This is different from broccoli when the embryo production is significantly increased in almost all genotypes of broccoli, which is incubated in a medium of ½ NLN-13 at 32°C for 1 day, compare to if it is incubated at a standard temperature of 30°C for 2 days. Bal *et al.* (2009) revealed, the combined effects of heat pre-treatment

Table 2. Percentage of embryogenic microspore and non-embryogenic.

Length of a flower bud (mm)	Temperature stress (°C)	Time of Incubation (days)	Percentage of microspore±sd	
			Embryogenic	Non-embryogenic
12-13	33	2	**50.19±2.45**	49.81±3.53
		4	32.87±2.76	67.13±5.49
	25	2	**46.17±2.16**	53.83±3.02
		4	27.45±4.04	72.55±4.94
	4	2	**49.28±1.58**	50.72±4.14
		4	37.34±2.86	62.66±1.87

Table 3. The increase of diameter late uninucleate and binucleate microspores.

Temperature stress (°C)	Time of Incubation (day)	Average of microspore diameter (µm) ±sd	
		Late uninucleate	Binucleate
33	0	**17,240±1,072**	**20,030±0.874**
	2	19,121±1,276	23,090±1,381
	4	**19,999±1,270**	**23,775±1,629**
25	0	**17,534±1,115**	**20,985±1,760**
	2	18,787±0,963	21,852±1,022
	4	**19,134±1,263**	**23,270±1,661**
4	0	**17,469±0,954**	**20,237±0,818**
	2	17,378±0,874	20,336±1,296
	4	**17,890±0,909**	**21,362±1,595**

and starvation were the same as Miyoshi's (1996) study, which cultured eggplant microspores in mannitol starvation medium and incubated at 35°C for 3 days.

The incubation for 48h at 32°C obtained unoptimized results for embryo production. That research reported that broccoli is sensitive to high temperatures (Carlos and Dias, 2001). In eggplant cv. Bambino, microspores treated with cold shock rapidly will lose viability after being subcultured into an AT3 medium (Bal *et al.*, 2009). The same problem was also found in this study, the pre-treatment incubation of microspores at 4°C for 4 days after subculture to A2 medium were found many microspores underwent lysis. Microspores that underwent nucleus symmetrical division and form multinucleate structures were obtained at low frequency after the microspores were transferred from heat shock and starvation to the AT3 medium containing 0.25 M maltose. The frequency of multinucleate structures were 17.3% and 19.4% after one week of incubated in an AT3 medium (Bal *et al.*, 2009).

Table 3. showed, the late uninucleate and early binucleate microspores were cultured in liquid B medium and incubated at 33°C for 4 days. There is an increase in the diameter size of microspores, respectively about 2.759 µm and 3.745 µm. Late uninucleate and binucleate culture of the microspores at 25 °C for 4 days was 1.6 µm and 2.285 µm respectively, whereas microspores were at 4 °C for 4 days each at 0.421 µm and 1.125 µm. Microspores incubated at 4°C developed very slowly. The increase in the diameter size of the microspore of both the late uninucleate and binucleate is unlike if the microspores incubated at temperatures 25°C and 33°C. According to Bal *et al.* (2009), heat shock was successfully used as a stress factor for microspore embryogenesis in eggplants. However, starvation pre-treatment, combined with heat shock, namely the microspore embryogenesis protocol in tobacco, is more effective. Stress treatments such as heat shock also provide optimal results for the induction of embryogenic microspores in eggplant cv. Gelatik. In addition to temperature stress, incubation time is also important in the induction of embryogenic microspores in eggplant.

Development of Microspore Embryogenic in Culture Medium

Embryogenic microspores have been treated with heat and cold shock temperature stress for 4 days, then subcultured into embryogenesis medium A2 to release microspores from stress to develop microspores towards embryo formation. Embryogenic microspores from B medium at 33°C (Figure 2) after one week of incubation in a new medium (medium A2), swoll very quickly, while in embryogenic microspores from 25°C to 25°C, many microspores were lysed. Thus microspores from 4°C temperature were more lysed with very slow development and the number of embryogenic and *starlike* microspores was less than the others.

Embryogenic microspores formed after stress treatment were characterized by several physiological and morphological changes (Figure 2). In tobacco and wheat, embryogenic microspores form a nucleus in the middle surrounded by structures like vacuoles. Microspores with cytoplasm form *starlike* structures, commonly found in microspores of wheat, barley, and tobacco. Swollen in *Brassica* is marked as embryogenic microspores (Bal *et al.*, 2012). In this study, several embryogenic microspores were observed, swollen, increased diameter of the microspores, the cell nucleus continuously divided to form multinucleate structures. The induction of microspores embryogenesis in eggplants in A2 medium (Figure 2), after 3 weeks of incubation produced no globular embryo, only microspore enlargement or bulging into swollen microspore, and

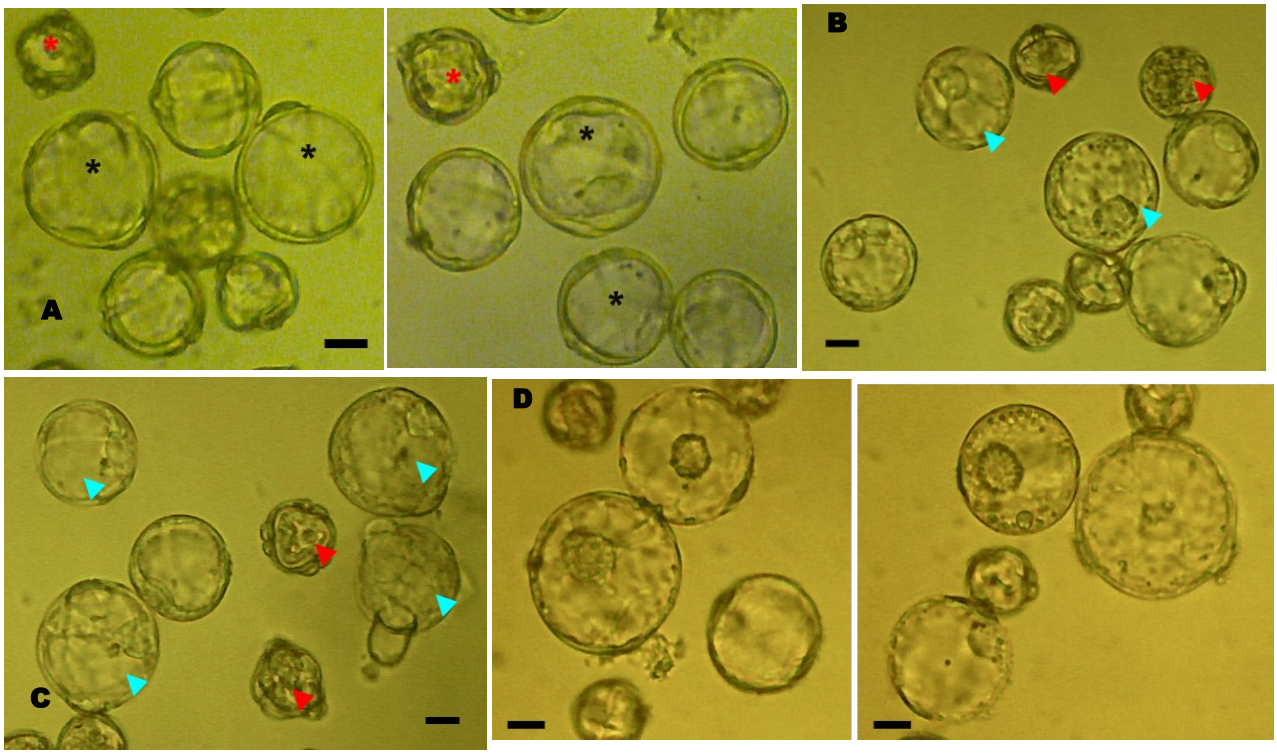

Figure 2. Development of embryogenic microspores in A2 medium at 25°C for 3 weeks. A) Embryogenic microspores from B medium at 33 oC for 4 days Incubation, B,C) Microspores resemble star-like structures (blue arrows: star-like structures, red arrows: microscopic lysis (1 week incubation) D) Star-like microspores, with the diameter of the microspores increases and swollen occurs (3 weeks incubation). Scale = 10 μm.

only formed a *star-like* structure. This shows that the A2 medium is less suitable for eggplant embryogenesis. According to Sumarmi *et al.* (2014) A2 medium is more suitable for the microspore culture of monocot plants. Medium A2 succeeded in spurring the growth of embryogenic microspores, resulting in symmetrical division and multinucleate structures formed in the culture of palm microspores (Indrianto *et al.*, 2014). Besides the A2 medium, some embryogenesis medium often used for induction of embryogenesis is AT3 medium in the culture of eggplant cv. Bambino (Bal *et al.*, 2009), and B5 medium with 9% maltose in wheat microspore culture (Zheng, 2003).

CONCLUSION

The incubation of microspores at 33°C in the medium for 4 days is effective to induce embryogenic microspores in eggplants. In addition, the early binucleate stage is preferably chosen for the induction of embryogenic microspores.

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