

Pollen Viability and Microspore Culture in Three Broccoli Cultivars (*Brassica oleracea* L. var. *italica* Plenck)

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ABSTRACT

Broccoli is a high value vegetable crop in Indonesia, however production is low due to limited number of suitable cultivars, so, breeding hybrid broccoli for warm climate is important. The first step in hybridization is providing homozygote parent plants which can be done efficiently via microspore culture. The objectives of this study were to determine 1) bud size that produce uninucleate microspore stage appropriate for culture; 2) pollen viability, 3) microspore development, in three broccoli cultivars ('BL 10001', 'Royal Green' and 'Green Magic'). Various bud size (1 – 5 cm) was squashed and observed microscopically to determine bud size containing uninucleate microspore. Pollen viability was determined by IKI staining and pollen germination method. Chromosome number was counted on root tips using squash method with aceto-orcein stain. Various heat treatment schemes were conducted to induce microspore development. Result showed uninucleate microspore derived from 2-3 mm and 3-4 mm bud length of 'BL-10001' and 'Royal Green' was responsive for microspore development in culture. Pollen viability varied among cultivars, 78-87% on IKI method and 15-16% on germination test. Microspore culture showed different embryogenesis response; pollen-like structure was produced by 'BL 10001'.

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Introduction

Broccoli (*Brassica oleracea* L. var. *italica* Plenck) is one of important vegetable from Brassicaceae family (Dalmadi, 2010). Broccoli contains high nutritional value and anti-cancer compounds (USDA Nutrient Database, 2011). Broccoli is listed in seven major imported vegetables to Indonesia (Badan Pusat Statistik, 2011). According to the United States Agency for International Development, an increase market broccoli in Indonesia is 15-20% per year (Asril, 2009). However, broccoli production in Indonesia is still limited only at highlands areas. Low broccoli production in Indonesia is caused by non-suitable cultivars, which are not adapted

in Indonesian tropical environments (Jaya, 2009).

Broccoli needed a relatively cool condition for its growth. Most of the broccoli cultivars need temperatures less than 23^oC to encourage vernalization process and allow normal development of flowers and crowns (Farnham and Bjorkman, 2011). When low temperatures are not met, the vegetative phase continues and number of branches increased, and plants produces small size crop (Jaya, 2009).

Indonesia needs to increase broccoli production through plant breeding, producing hybrids which are adapted in high temperatures. Hybrid plants can be produced by crossing two pure/homozygous lines. Pure

line can be obtained through self-pollination followed by selection. Conventional method needs 6-7 years to produce homozygous line (Nurhasanah, 2011). Alternative and fast way to obtain a homozygous line is with microspore culture technique. Homozygous plants produced via microspore culture technique only takes one generation or less than 1 year (Takahira, 2011).

Optimization of microspore culture technique play important role to improve embryogenesis. Several factors affect microspore embryogenesis, including donor plant genotype, donor plant physiology conditions, and developmental stage of microspores, treatment and media composition (Babbar *et al.*, 2004). Palmer *et al.* (2005) revealed that high temperatures can affect microspores embryogenesis. Moreover, Na *et al.* (2011), point out that broccoli microspore culture increased by heat treatment at 32.5⁰C at the beginning of the culture period.

Microspore culture technique has proved efficient in producing double haploid plants in other Brassica species, namely *Brassica napus* (Takahira *et al.*, 2011), *Brassica juncea* (Ali *et al.*, 2008) and *Brassica campestris* (Wang *et al.*, 2009) so that this technique is expected to increase production of broccoli in Indonesia.

The aim of this research was to determine appropriate stage of bud size of each cultivar for microspore culture, to determine pollen viability of each cultivar, to observe microspore development on culture broccoli cultivar.

Material and Methods

Plant materials

Three broccoli cultivars, 'BL 10001', 'Royal Green' and 'Green Magic' were used in this study. Broccoli seed were sown on coco peat media for 2 weeks and then transferred to 30 cm diameter pots containing soil and compost (1:1) in the green house until flowering. Plants were maintained at 27⁰C - 30⁰C temperature.

Bud stage as microspore donor

Broccoli flower buds, with approximate size of 1-5 mm were collected and brought to

lab under cool condition. Anthers were taken and placed on glass objects and given 1 drop of distilled water, tap gently until microspore released and then observed under a light microscope. Broccoli buds size that have microspore in uninucleate stage were used as microspore donor of microspore culture.

Pollen Viability

Anthers from 20 broccoli flowers of each cultivar were collected at anthesis. Anthers were placed in a 50 ml beaker glass and 2 ml aquadest was added. Pollen was released by squeezing with glass rod and filtered with 100 µm filter. Pollen viability was tested using two methods, IKI staining and pollen germination test (Sato *et al.*, 1998). For IKI staining method, one drop of pollen solution was placed on an object glass and given a drop of IKI solution, covered with a cover glass and observed under a light microscope. The percentages of cells absorbing IKI color were calculated as viable pollen. On pollen germination test, pollen was grown on 5 ml of liquid Kwack's medium and incubated 16 hours at 20⁰C in dark. Pollen viability/germination was observed at 3 microscope field-of-view and 3 replicates. Percent of pollen viability of the two techniques was calculated according to Dafni *et al.* (2000).

Microspore culture

Microspore culture was undertaken by three methods: I. Cousin *et al.* (2009), II. Yuan *et al.* (2011) with modification, and III. Winarto and Silva (2011) with modification. On the method I (Cousin *et al.*, 2009), a total of 80 buds of each broccoli cultivar were sterilized with 50 ml, 5% sodium hypochlorite for 15 minutes, rinsed with distilled water on a 100 µm filter. Buds were then transferred to beaker glass containing 20 ml of ½ B5 medium, and microspore were released using glass rod. Microspores were then transferred to 50 ml tubes with ½ B5 medium, centrifuged at 1200 rpm, 5 minutes. Microspores were cultured in petri dish containing NLN-13 medium + 1% activated charcoal + 0.5% colchicine. Petri dishes were

sealed with parafilm and wrapped in aluminum foil and incubated at 32.5°C for 3 days for heat treatment to induce microspore growth. After 3 days, NLN-13 solution was added, and petri dish were covered with aluminum foil again and placed at 25°C for 2 weeks. Subsequently, cultures were transferred to the shaker 60 rpm for 1 week, and exposed to light to produce green embryos.

On the second method II (Yuan *et al.*, 2011), firstly buds were given 4°C pre-treatment in several days period (P1-P5: 0, 1, 2 and 3 days). Subsequently, 80 buds of each broccoli cultivar were surface sterilized with 70% alcohol for 30 seconds followed by 7% sodium hypochlorite for 25 minute and rinsed with sterile distilled water. Buds were squashed gently using glass rod and 5 ml of B5 medium was added. Microspore suspension was filtered using a 45 µm filter and filtrate was centrifuged three times at 100 g for 3 min. Microspore were cultured in petri dish containing 1/2 NLN-13 medium + 1% activated charcoal, and exposed to 32.5°C heat treatment (H1-H3: 1, 2, and 3 days), before transferred to 25°C culture environment. Pre-treatment and heat treatment combination are presented on Table 1.

Table 1. Treatment combinations according to Yuan *et al.* (2011)

Treatment	H1	H2	H3
P0	P0H1	P0H2	P0H3
P1	P1H1	P1H2	P1H3
P2	P2H1	P2H2	P2H3
P3	P3H1	P3H2	P3H3

*P0=without 4°C pre-treatment, P1= 4°C pre-treatment for 1 day, P2= 4°C pre-treatment for 2 days, P3= 4°C pre-treatment for 3 days.

*H1= 32.5°C heat treatment for 1 day, H2= 32.5°C heat treatment for 2 days, H3= 32.5°C heat treatment for 3 days.

On the method III, 80 uninucleate stage buds of each cultivar were sterilized using 2% sodium hypochlorite + 2 drops of Tween for 10 minutes and then rinsed with distilled water. Buds was crushed with glass rod in NLN-13 media. Microspore suspension was filtered using a 45 µm filter then centrifuged using a rate of 100 g at 4°C and repeated 3

times. Microspores were cultured on four types of media (M1-M4, Tabel 2), full/half-strength NLN-13 media added by 0 and 1% activated charcoal with the density of 5 x 10⁴ ml /petri dish. Cultures were exposed to various heat treatments (Table 2) before incubated in 25°C culture condition.

Table 2. Treatment combination according to Winarto and Silva (2011) with modification.

Treatment	H1	H2	H3	H4	H5	H6
M1	M1H1	M1H2	M1H3	M1H4	M1H5	M1H6
M2	M2H1	M2H2	M2H3	M2H4	M2H5	M2H6
M3	M3H1	M3H2	M3H3	M3H4	M3H5	M3H6
M4	M4H1	M4H2	M4H3	M4H4	M4H5	M4H6

*H1=heat treatment 30°C for 1 day, H2=heat treatment 30°C for 2 days, H3=heat treatment 30°C for 3 days, H4=heat treatment 31°C for 1 day, H5=heat treatment 31°C for 2 days, H6=heat treatment 31°C for 3 days. M1=NLN medium with 1% activated charcoal, M2=NLN medium without activated charcoal, M3=½ NLN medium with 1% activated charcoal, M4=½ NLN medium without activated charcoal.

In all three methods, the microspore was cultured in the density of 5 x 10⁴ ml / petri dish. Microspore density was calculated using haemocytometer.

Variable observed and data analysis

Variable observed were 1) size of bud of each cultivar suitable for donor plants for microspore culture, 2) percentage of viable pollen of each cultivar, 3) microspore developmental stage in culture condition and 4) chromosome number of each cultivar. Data were presented descriptively on tables and pictures.

Results and Discussion

Stage of bud growth and developmental stage of microspores

Bud growth of 'BL 10001' and corresponding microspore stage were obtained (Figure 1 and 2). At 1 mm bud size the stage of microspores were pollen mother cell, dyad and tetrad. At 2 – 3 mm bud size contained uninucleate stage. Mature pollen was observed at 4 mm, 5 mm bud size and fully bloom flower (Figure 2).

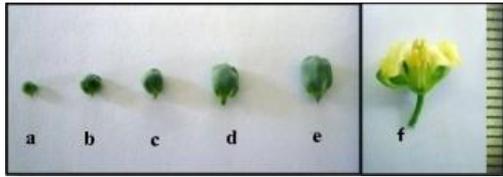


Figure 1. Stage of Bud Growth on 'BL 10001' (Bud size: a. 1 mm; b. 2 mm; c. 3 mm; d. 4 mm; e. 5 mm and f. flower)

Microspore development on 'Royal Green' begins with pollen mother cell and dyad on 1 mm bud size, while tetrad stage on 2 mm. Uninucleate stage was observed at 3 mm - 4 mm bud size, while at 5 mm, pollen grain has formed (Figure 3, 4). On 'Green Magic', pollen mother cell was observed at 1 mm bud size. At 2 mm, dyad and tetrad were observed, while at 3-4 mm and 5 mm, mature pollen was observed (Figure 5, 6).

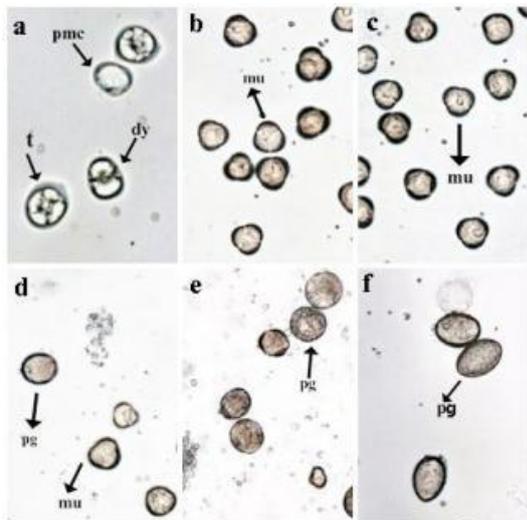


Figure 2. Developmental stage of 'BL 10001' microspore (400x magnification)(Bud size: a. 1 mm, b. 2 mm, c. 3 mm, d. 4 mm, e. 5 mm and f. flower, pmc: pollen mother cell, dy: Dyad, t: tetrad, mu: uninucleate microspores, pg: pollen grain)

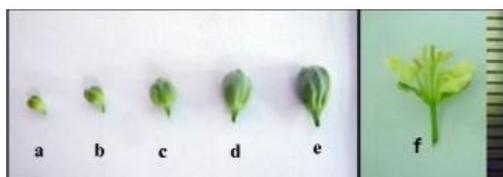


Figure 3. Stage of bud growth on 'Royal Green' (Bud size: a. 1 mm; b. 2 mm; c. 3 mm; d. 4 mm; e. 5 mm and f. flower)

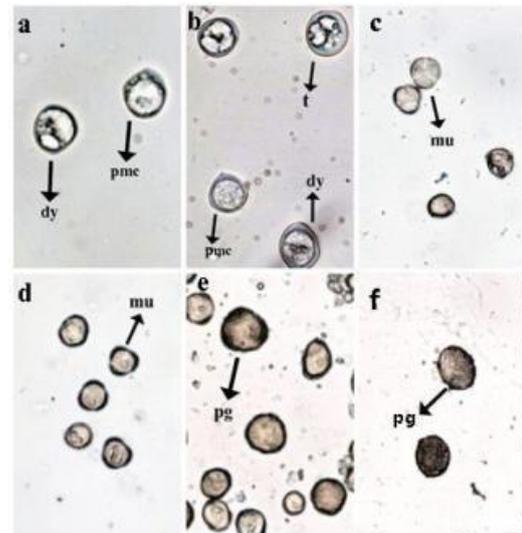


Figure 4. Developmental stage of 'Royal Green' microspore (400x magnification)(Bud size: a. 1 mm, b. 2 mm, c. 3 mm, d. 4 mm, e. 5 mm and f. flower, pmc: pollen mother cell, dy: Dyad, t: tetrad, mu: uninucleate microspores, pg: pollen grain)

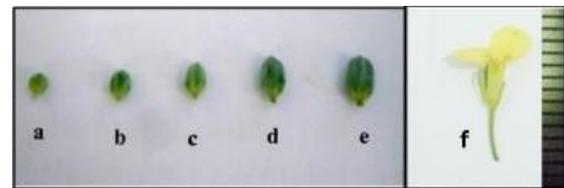


Figure 5. Stage of bud growth on 'Green Magic' (Bud size: a. 1 mm; b. 2 mm; c. 3 mm; d. 4 mm; e. 5 mm and f. flower).

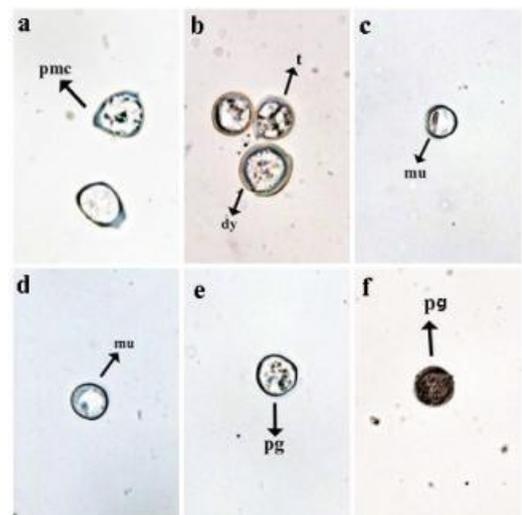


Figure 6. Developmental stage of 'Green Magic' microspore (400x magnification)(Bud size: a. 1 mm, b. 2 mm, c. 3 mm, d. 4 mm, e. 5 mm and f. flower, pmc: pollen mother cell, dy: Dyad, t: tetrad, mu: uninucleate microspores, pg: pollen grain)

Uninucleate stage of microspore was obtained on 2-3 mm bud size of 'BL 10001' and 3-4 mm bud size of 'Royal Green'

Pollen viability

Pollen viability varied among cultivars (Table 3). 'Royal Green' has the highest pollen viability (87.09%), followed by 'BL 10001' (77.8%) using IKI method. Indicator of viable pollen on IKI (Iodine Potassium Iodide) staining method was revealed by pollen color changes (Figure 7). Viable pollen absorbed IKI stain so that pollen become turn black/dark, while non-viable pollen did not absorb color so that pollen as lighter color (Roubik, 1995).

Pollen germination revealed much lower percentage, i.e. 16% on 'Royal Green' and 15% on 'BL 10001'. Pollen viability using pollen germination method on Liquid Kwack media identified by emergence of pollen tube after incubation for 16 hours (Figure 8).

Table 3. Pollen viability based on IKI staining and pollen germination method

No	Cultivar	IKI staining		pollen germination	
		total pollen	viable pollen (%)	total pollen	germinated /viable pollen (%)
1	BL 10001	135	77.8	127	14.96
2	Royal Green	155	87.09	258	15.89
3	Green Magic	3	*	5	*

*Indicates that 'Green Magic' does not qualify for counting pollen viability as total count below 100.

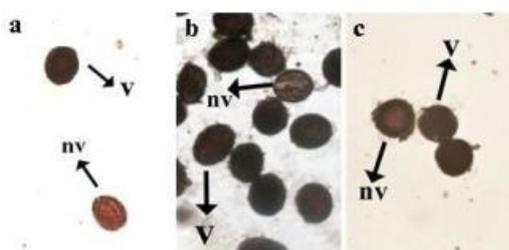


Figure 7. Pollen viability test using IKI staining technique, 400x magnification. (Mature pollen at a. 'BL 10001', b. 'Royal Green', c. 'Green Magic', nv: non viable; v: viable).

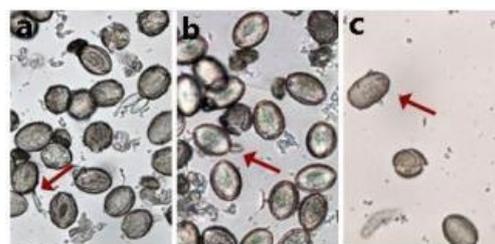


Fig 8. Pollen viability using germination test, 400x magnification (Pollen at a. 'BL 10001', b. 'Royal Green', c. 'Green Magic', arrows indicate germinated pollen).

Pollen viability test is an important factor for successful microspore culture. Pollen viability test is used to determine the percentage of cells that have the potential for microspore culture so that it can respond embryogenesis (Mariani and Bots, 2005).

Variation in pollen viability occurred between cultivars and between techniques. Using IKI staining techniques, pollen viability was above 50% on 'BL 10001' and 'Royal Green', which mean both cultivars have good pollen viability according to Dafni *et al.* (2000). Therefore, both cultivars can be a good donor plants for microspore culture.

'Green Magic' produced only three pollen per anther. 'Green Magic' may have Genetic Male Sterility (GMS) mechanism. Suwarno (2008) reported that GMS plants occur due to the genes that inhibit the normal development of anther or pollen. Expression of specific genes can be complete so there is no longer pollen in the anther. Gene expression may also be partly so that viable pollen is formed in small quantities such as at 'Green Magic'.

The standard technique of testing pollen viability has not been established so that more than one technique should be done to confirm pollen viability (Warid and Palupi, 2009). In this study, pollen germination technique using Kwack liquid medium was employed following study on *Brassica campestris* (Sato *et al.*, 1998). 'BL 10001' has 15% pollen viability, while 'Royal Green' has 16%. The low pollen viability on this technique was due to pollen unable to form germination tube during incubation. It may be due to environmental factors such as temperature, pollen density, medium used and incubation time (Mariani and Bots, 2005). Differences

on pollen viability percentage on different technique occurred frequently so that more than one technique should be employed to obtained better picture of the pollen viability.

Microspore culture

Microspore culture using first method did not lead to embryogenesis. There was no development on culture of 'BL 10001' and 'Royal Green'. Cell lysis occurred at 30 days after culture (Figure 9.i and 9.ii). Microspore on 'Green Magic' developed into multicellular stage at 25°C after 7 days incubation (Figure 9. iii). However, that microspore was deteriorating at 30 days.

Microspore culture employing second method (Yuan *et al.*, 2011), was responded only by 'Royal Green', resulted in callus formation on 4°C pre-treatment for 3 days followed by 32.5°C heat treatment for 3 days (Figure 10). No development of microspore was observed on all other treatment combinations.

On the third method (Winarto and Silva, 2011), some (16.2%) microspores of 'BL 10001' developed into pollen-like structure when employing 31°C heat treatment for 3 days, without activated charcoal (Figure 11). One day 31°C heat treatment induced cell division on microspore, while 2 days 31°C heat treatment causing star-like structure formation. Other treatment combinations did not induce response to the microspore.

Development of pollen-like structure has decreased by 7.4% after culture were transferred to 25°C incubation for 1 days, further decrease to 3.2% after 3 days incubation and no pollen-like structure were observed after 7 days incubation at 25°C due to cell lysis.

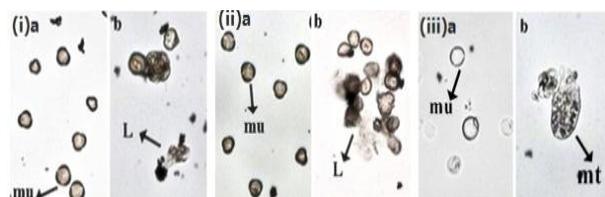


Figure 9. Development of microspore culture, 400x magnification (microspore culture at i. 'BL 10001'; ii. 'Royal Green'; iii. 'Green Magic' (a: fresh culture; b:

30 days of culture; mu: uninucleate microspores; L: lysis cell; mt; multicellular)

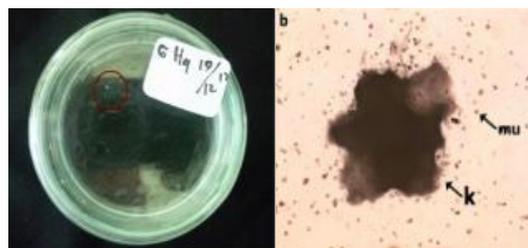


Figure 10. Callus structure of 'Royal Green' (a. on a petri dish (red circle), b. callus at 100x magnification)

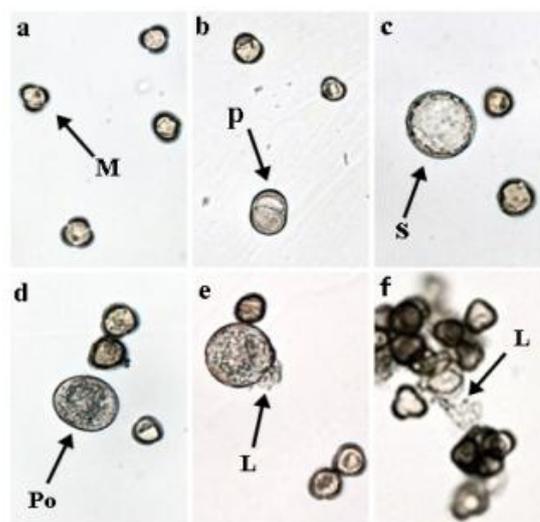


Figure 11. Development of microspore culture on 'BL 10001' (400x magnification) (a. fresh culture, b. 31°C heat treatment for 1 day; c. 31°C heat treatment for 2 day; d. 31°C heat treatment for 3 day; e. 1 day incubation at 25°C incubation after 31°C heat treatment, f. 7 day incubation at 25°C after 31°C heat treatment, M: uninucleate microspore; P: cell division; S: star-like structure; Po: pollen-like structure; L: lysis cell)

Bud stage and microspore development

The latest technology in the production of homozygous lines of broccoli is microspore culture techniques. The success of microspore culture technique was affected by several factors, i.e. plant genotype, physiological status of donor plant, stage of microspore development, treatment and media composition (Babbar *et al.*, 2004).

One important factor on development of microspore culture is the bud stage as donor plant. There is no standard bud size for

optimum microspore culture in each cultivar and therefore need to be determined at the beginning of microspore culture (Abraha *et al.*, 2008). Winarto and Silva (2011) reported that difference in size of the buds indicates differences in developmental stage of microspores. Babbar *et al.* (2004) revealed that Brassica microspore culture responsive to the varying culture conditions only in the early uninucleate to early binucleate stages. This study found that uninucleate stages on 'BL 10001' is on buds size 2-3 mm, while 'Royal Green' and 'Green Magic' is in 3 - 4 mm bud size. Therefore, this study uses only uninucleate stage as donor plants for microspore culture.

Microspore culture protocol has been effectively employed on rapeseed or canola. Embryogenesis response occurs after 14 days in culture of microspore (Cousin *et al.*, 2009). However, in this study the method did not successfully induce embryogenesis of microspore isolated from three broccoli cultivars trialed. Isolated microspores in 'BL 10001' and 'Royal Green' did not show any development after 30 days in culture, while 'Green Magic' developed abnormal multicellular microspores but finally deceased after 30 days. Palmer *et al.* (1996) point out that each genotype has a different response to stress treatment. Stress response in the treatment of certain genotypes may trigger embryogenesis; while for other genotype can make cell death because microspores cannot survive in culture conditions (Wahidah, 2010).

Yuan *et al.* (2010) uses a variety of stress treatments to stimulate embryogenesis. Combination of 4⁰C pre-treatment for donor plant before culture and 32.5⁰C heat treatment during culture has stimulated callus development in 'Royal Green'. It is in accordance with Segui-Simaro and Nuez (2008) reported that stress treatment can stimulate microspore embryogenesis and may cause microspores develop into callus.

The best stress treatment of method III that can stimulate embryogenesis in microspore culture is heat treatment 31⁰C in 'BL10001', resulting in star - like structure, then

developed into pollen - like structure, however it ended in the death of the cell. Microspores uninucleate can develop into embryogenic if key embryonic regulators BABY BOOM transcription factors are activated (Maraschin *et al.*, 2005) as shown in 'BL 10001' using Winarto and Silva (2011) methods. Heat treatment of 31⁰C was also successfully employed to induce embryogenesis on cabbage microspore culture (Winarto and Silva, 2011).

The regeneration of microspores into embryos in microspore culture can be stimulated by several stress treatments. Stress treatment can be heat, cold, carbon starvation, chemicals such as colchicines, and gamma irradiation (Yuan *et al.*, 2011). Without stress treatment, microspores developed following the normal path, gametophytic form into pollen, while stress treatment can make microspores develop into sporophytic path to form embryo (Ferrie and Caswell, 2010).

Environmental factors such as light intensity, photoperiod and temperature affect the physiological condition of donor plant and highly influence microspore culture (Swanson, 1990). Donor plants should be grown in a constant temperature of 18⁰C with 16 hours lighting photoperiod. Setting environmental conditions are intended to obtain a healthy donor plants (Taji *et al.*, 2002). Donor plants with 9-10 leaves were then moved to 10⁰C temperature for 9-11 days to induce flowering. Temperature decrease in the donor plant is needed to extend flowering time and to induce embryogenesis during culture (Winarto and Silva, 2011). In this study, donor plants were grown in the green house with temperature of 27⁰C - 30⁰C. Lower temperature is required to induce embryogenesis in microspore culture (Ferrie and Caswell, 2010).

There is no universal protocol in microspore culture techniques to produce embryos in all species. Differences occur depending on the species and genotypes in response embryogenesis (Ferrie and Caswell, 2010). The success of microspore culture in Brassica genotypes only occurred in a limited number of each species. Microspore culture

response can vary depending on the genotypes. Certain genotypes may respond embryogenesis in microspores culture but some genotypes are not able to respond embryogenesis (Ferrie and Caswell, 2010). To date, only a small number of embryos have been produced from Broccoli microspore culture (Na *et al.*, 2011). Microspore culture condition needs to be optimized using varied treatment such as heat, cold or starvation using PEG. Also, further research should be done with more genotypes to be able to select genotypes that response to microspore culture and produce homozygous lines.

Conclusions

The responsive stage of developmental microspore for culture is uninucleate stage derived from 2-3 mm size bud of 'BL 10001' and 3-4 mm size bud of 'Royal Green' and 'Green Magic' respectively. Pollen viability varied among cultivars, ranging from 78-87% on IKI method and 15-16% on germination test. Microspore culture showed different embryogenesis response, in which pollen-like structure was only produced in 'BL 10001' using Winarto and Silva (2011) technique. Chromosome number was $2n=18$ on 'BL 10001', $2n=17$ on 'Royal Green' and $2n=14$ on 'Green Magic'.

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