DETERMINATION OF SOYBEAN (*Glycine max* L.[Merrill]) MICROSPORES DEVELOPMENT STAGE BASED ON THE LENGTH OF FLOWER BUDS

Sumarmi[1], Budi Setiadi Daryono[2], Diah Rachmawati[3], Ari Indrianto[4]
1. Faculty of Agriculture Slamet Riyadi University
2. Genetics Laboratory, Faculty of Biology, Gadjah Mada University
3. Plant Physiology Laboratory, Faculty of Biology, Gadjah Mada University
4. Biotechnology Laboratory, Faculty of Biology, Gadjah Mada University
e-mail : sumarmi_mp@yahoo.com

ABSTRACT

The number of published reports of flower buds, anthers and on microspore culture of horticultural plants, especially in soybean is very low. The purpose of this research is to determine soybean microspores development stage based on the length of flower buds. Five local cultivars such as Argomulyo, Grobogan,Wilis, Anjasmoro and the black soybean Malika were used. Flower buds were the main material for microspore culture. Anjasmoro cultivar produces the highest number of flower bud. Anjasmoro soybean cultivar at third stage development, 3.1-3.6 mm length, was selected for microspore culture, based on plant height, length of anther, total of microspore per bud. Anjasmoro cultivar has the most late-uninucleate microspore and the total of shed of Anjasmoro microspore in the B medium with 34^{0}C for 4 day is 3820. Results revealed that Anjasmoro soybean cultivars had 354.67±59.67 µm length of anther, 2003±216 microspore per bud and the most mid and late-uninucleat microspore on 2.6-3.6 mm length flower bud. Anjasmoro soybean cultivar can be used for microspore culture.

Key words: microspore, soybean, flower bud

INTRODUCTION

Selection of flower buds is the first step of microspore culture. Within the anther, male sporogenous cells differentiate and undergo meiosis to produce microspores, which give rise to pollen grains. Determination of the proper length of flower buds support the success of microspore culture. The grain legumes are well-known for their recalcitrance to most in vitro approaches, and the development of doubled haploid plants is no exception. To date, this technique is not routinely used in any grain or pasture legume improvement program (Croser et al., 2011). An important issue, however, is to find and choose the suitable conditions at a certain stage in a given species. Microspore culture studies on various types of plants to use the main ingredient of flower buds of different sizes. In *Brassica napus* flower buds used measuring 2-4 mm (Leroux et al., 2009), the length of 'Garbanzo bean' (*Cicer arietinum* L.) buds 2.85 – 3.50 mm (Croser, et al., 2011), on dill (*Anethum graveolens* L.) less than 3 mm (Ferrie et al., 2011) and soybean bud about 3.0 until 3.5 mm (Rodrigues, et al., 2006). Soybean flower buds with 2.5-3.5 mm in size, microspore was uninucleate stage. Soybean cultivars Williams 82 and Asgrow A1929 with bud length of 3.9±0.63 mm had 57% and 42% of the microspores at the uninucleate and binucleate stage respectively and 12% were empty ones (Lauxen et al., 2003).

Microspore androgenesis is the most commonly used method to produce double haploids (Shariatpanahi et al., 2006). The production of double haploid throught androgenesis is a proven method to obtain homozygous individual in a single step. The in vitro induction of androgenesis from unfertilized male gametes is the most widely used method for the generation of haploid populations. Androgenesis can result from the culture of intact anthers or the culture of mechanically isolated microspores (Croser et al., 2011). To achieve best results, microspores in culture need to be relatively pure (Zheng, 2003). Some important factors influencing the efficiency of microspore culture include genotype, donor plant physiology, microspore developmental stage, stress pretreatments and medium composition (Shirdelmoghanloo et al., 2009).

In Indonesia there are 62 cultivars of soybean. Cultivars commonly cultivated in every region is different. In Java, there are 28 cultivars of soybean (Wirawan, 2000). However, the number of published reports on microspore culture of plants, especially Indonesian soybean is very low. Isolated microspore culture could provide better conditions for androgenic plant production in soybean (Rodrigues, et al., 2006). The stage of microspore development at the time of culture initiation markedly affects androgenesis. The period around the first haploid mitosis (late uninucleate or early binucleat microspores) has been shown to be the critical stage for androgenic induction (Cardoso et al., 2004). A triggering factor of stress is necessary to induce embryogenesis in cultured microspores (Shariatpanahi et al., 2006). Therefore, in the present research, we determine of soybean (*Glycine max* L. [Merrill]) microspores development stage based on the length of flower buds five local soybean cultivars for first step in vitro culture.

MATERIALS AND METHODS

Donor Plant Growth

The cultivation of soybean has been done at the research field, Faculty of Agriculture UGM starting from

May to October 2013. Five soybean cultivars i.e: Argomulyo, Grobogan, Wilis, Anjasmoro and the black soybean Malika were planted. Description of five soybean cultivar guide from Indonesian Legumes and Tuber Crops research Institute (Table 1). Soybean seeds were planted in 10 polibags, with 25 cm of diameters and 35 cm of height. The medium of planting consist of soil, manure and compost. Flower buds was harvested for main material research. Soybean planting has been done once in 3 weeks, to ensure a constant supply of fresh buds for microspores culture.

Table 1. Description of five soybean cultivars

cultivars	100 seeds weigth (gram)	plant height (cm)	age of having flower (days)	age harvest time (days)	harvest (ton)
Argomulyo	15.6-16.0	40-44	38-40	80-82	1.50-2.00
Grobogan	17.8-18.2	50-60	30-32	76-80	2.60-2.70
Wilis	10.0-10.2	47-50	38-40	85-90	1.50-1.60
Anjasmoro	14.8-15.3	64-68	36-39	82-92	2.00-2.25
Black Malika	10.0-10.2	35-50	34-36	85-90	1.60-1.70

Data were taken from Indonesian Legumes and Tuber Crops research Institute, the black soybean Malika cultivar taken from Kastono, D. (2008) with modification.

Flower Buds Size

Forty flower buds were devided to four growth level. First: the youngest buds, 2.0 - 2.5 mm length, all of the part are covered by the petals, it has a sharp tip bud, the tricomas covered the buds. Second: the length of bud is about 2.6 - 3.0 mm. Third: it's length is about 3.1 - 3.6 mm and the inside start to get bigger. Fourth: it's length abaut 3.7 - 4.1 mm (Fig.1). Four groups were sampled from five cultivars. To study microspore developmental stage, anthers were dissected out from ten buds for each size group.

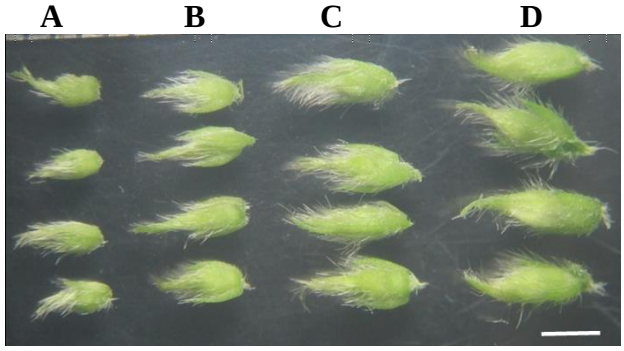

Figure 1. Developmental stadium of soybean Anjasmoro cultivars buds (a) stage 1: 2.0-2.5 mm length (b) stage 2: 2.6-3.0 mm (c) stage 3: 3.1-3.6 mm (d) stage 4: 3.7-4.1 mm, bar = 2 mm

Anther and microspore developmental stage

Soybean flower had ten anthers per bud. The morphology of anthers was desribed using light microscopy. The anthers and microspores was photograph and measured laterally using 'Optilab' software to establish the size range encompassing the mid to late uninucleat stage of development. The amount of microspore per bud was determined using section of bud than 10 anthers were crushed with glassrod to release and appear the microspore. Microspores of five soybean cultivars soybean were analyzed under microscope with 200 magnification. Number of microspores were calculated and classified based on the growth of the microspore according to the shape of cell and the position of the nuclear cell deferentiate them become tetrad, mid-uninucleat, late-uninucleat and binucleat. The development stage of microspore were assessed by Acetocarmine staining and measured diameter of microspore especially Anjasmoro cultivar for each group.

Fisrt step in vitro microspore culture

Eighty soybean buds containing anther with microspore at late uninucleat stage, were washed with liquid detergent for thirty minutes then rinsing water three times, after that 70% alcohol for 2 minutes. Soybean buds were opened with two spluit, take the anther, place them in a petridish. All of the anther were put in the laminar air flow cabinet to be sterilized using Hg Cl_2 1% for 10 min, rinse three times with sterilized water, followed by 96% alcohol for one minutes. All of the anther were crushed with glassrod to release and appear the microspore, then put on 1 ml of medium B (Kyo and Harada, 1986). Starvation medium, the B medium consist of: 1.49 g/l KCl, 0,25 g/l Mg SO_4.7 H_2O, 0.11 g/l $CaCl_2$.2H_2O, 54,70 g/l manitol, and 0.14 g/l KH_2 PO_4 of pH 7,0. The mix of microspores and 2 ml B medium in 2 microtubes were sentrifuged 1000 rpm for 10 min. Then, threw the B medium, and changed it with the 2 ml B medium till clearly and keep them in the incubator 34^0C for 4 days. The shed microspore were counted at the fourth day.

Statistical analysis

Data length of soybean's anther, total of microspore, diameter of Anjasmoro soybean's microspore and number of shedding microspore in the B medium were analyzed by ANOVA, and means were compared using Duncan's multiple range test at significant level 5% ($P < 0.05$).

RESULTS

The five soybean cultivars have excellent characteristic. The three of them are categorized as the big seeds: Argomulyo, Grobogan and Anjasmoro. The Wilis

and black Malika cultivar had small seeds and short stem plants. The data in Table 1 describes the variety of five soybean cultivars. Flower buds were the main material for microspore culture, so choosing cultivars based on amount buds produced per plant. Generally, flower buds were proceeds both up and down the main stem and spreads along the branches. Anjasmoro cultivar which had the highest number of nodes (11-16) produces the highest number of flower bud too.

Mean length of anthers in five cultivars various between 278.00±17.51 µm and 354.67±59.67 µm. The longest anther is Anjasmoro, althought Argomulyo and Grobogan are big too. Anthers of the apple-shaped were notably shorter and smaller in size, than those of the suboblate group (Fig.2).

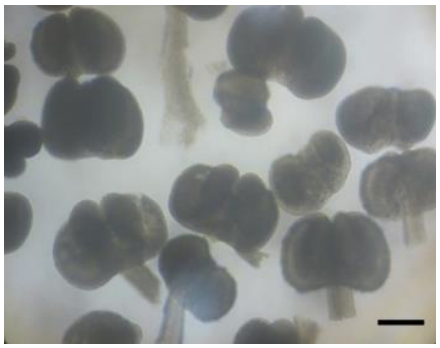

Figure 2. Various anthers soybean of Grobogan cultivar shape, bar = 100 µm

Anthers contained a number of microspore and pollen grain. Anjasmoro cultivar is the largest anther had the big number microspore per bud (Table 2). Stage 1 (2.0-2.5 mm), the youngest buds consist tetrad microspore more than another stadium. Tetrads are from polen mother cell, had four nucleus with a thin cell wall. The biggest number of tetrad find on Anjasmoro and the less is Wilis cultivar. The proportion of late-uninucleat were find on stage 3 (3.1-3.6 mm) of Anjasmoro. Stage 4, more than 3.7 mm length, buds developed to be a mature flower bud, so microspore will be changed to binucleat pollen grain. Although our sample size were small, we have determine that comparing cultivars, buds of the same size necessarilly do not have microspores at the same stage (Fig. 3).

Table 2 . Length of soybean's anther and total of microspore

cultivars	Lenght of anther (µm)			total of microspores per bud (mean±sd)
	minimum	maximum	mean±sd	
Argomulyo	294.0	363.7	326.03±20.81[b]	1644±173[bc]
Grobogan	294.6	412.6	344.56±35.19[b]	941±142 [a]
Wilis	262.8	310.6	284.98±18.16[a]	1839±156 [c]
Anjasmoro	261.0	432.5	354.67±59.67[b]	2003±216 [c]
Black Malika	248.9	300.9	278.00±17.51[a]	1277±147[ab]

Mean values followed by different upper-case letters different significantly by Duncan's multiple range test at P ≤0.05. Sd = standard deviation

Microspore tetrad stage (a) are discovered in buds 2.0-2.5 mm soybean cultivar like Anjasmoro, Argomulyo, Grobogan, Black Malika, and Wilis. Mid uninucleat microspore (b) with centrally nucleus, late uninucleat microspore with nucleus close to the polen wall (c). Binucleat stage (d) have two nucleus, generative and vegetative nucleus (Fig 4). Soybean flower buds with 2.5-3.5 mm in size presented 57.15% stage was uninucleate microspore (Lauxen et al., 2003, Cardoso et al., 2004).

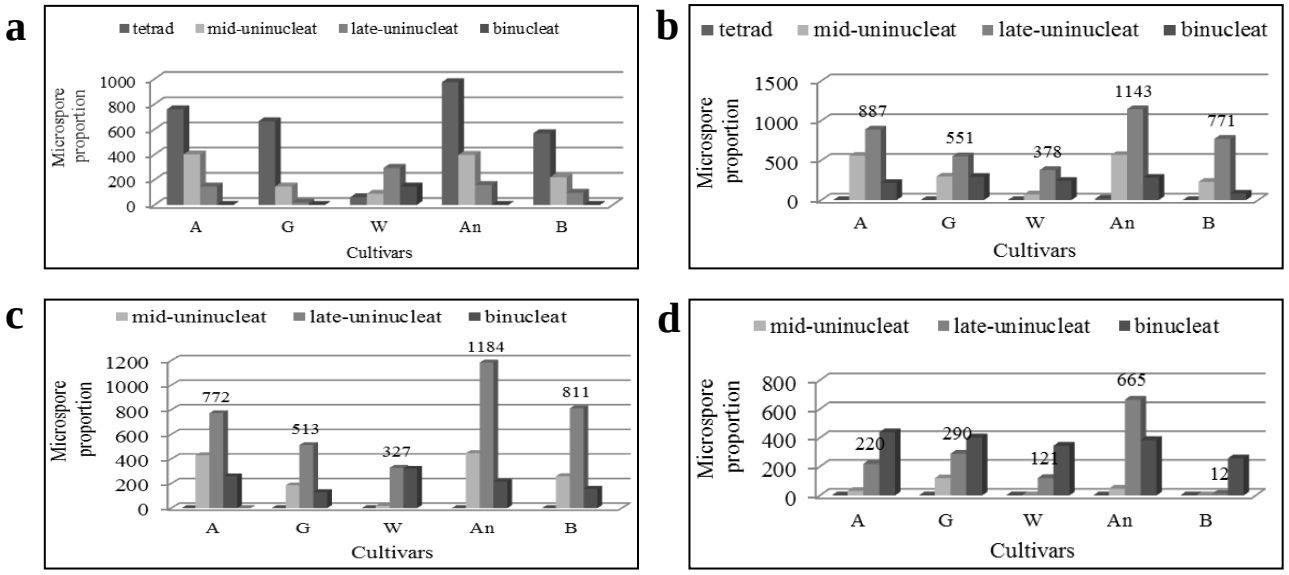

Fig.3. Diagram of the soybean microspore growth based on 4 stadium (tetrad, mid-uninucleat, late-uninucleat and binucleat) on 5 cultivar: A: Argomulyo, G: Grobogan, W:Wilis, An: Anjasmoro, B: Black Malika, shows the level of flower buds growth depend on the length: stage 1(a) 2.0-2.5 mm length, stage 2 (b) 2.6-3.0 mm, stage 3 (c) 3.1-3.6 mm, and stage 4 (d) 3.7-4.1 mm

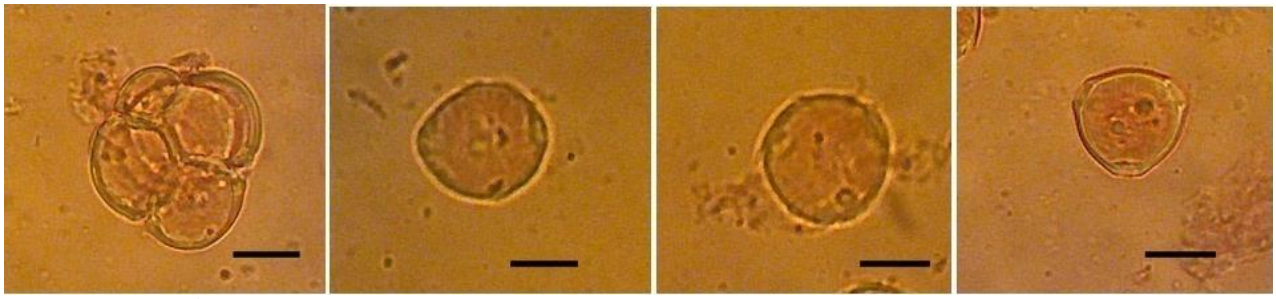

Fig.4. Microspores of Anjasmoro soybean stained with Acetocarmine (a) tetrad (b) mid-uninucleat (c) late-uninucleat (d) binucleat, bars = 10 µm

The result of soybean microspore diameter measurement was between 17.8 and 23.8 µm (Table 3). Indonesian soybean microspore smaller than Brazilian soybean. The microspores of the soybean cultivars tested in Brazil study presented an average diameter of 25 µm (Rodrigues, et al., 2006). Microspore on stage 1 and 2 bigger than stage 3 and 4. The cytoplasm binucleat pollen consist of starch grain. The measurement of binucleat microspore and pollen grains shown be smaller than mid and late-uninucleat microspore.

Table 3 . The diameter of Anjasmoro soybean's microspore

stage	length of soybean flower buds	Diameter of microspore (µm)		
		minimum	maximum	mean±sd
1	2.0-2.5 mm	19.3	22.7	21.47±1.06 a
2	2.6-3.0 mm	20.4	23.8	21.64±1.06 a
3	3.1-3.6 mm	17.8	21.2	19.38±1.35 b
4	3.7-4.1 mm	18.2	20.8	19.29±0.98 b

Mean values followed by different letters different significantly by Duncan's multiple range test at $P \leq 0.05$. Sd = standard deviation

Treatment in starvation (B medium) and height temperature (34^0C) for four days was first step for in vitro micropore culture. A number of shed microspores rise from stomium. Respons of five soybean cultivar are different. The result shown that black soybean Malika had 1729 ± 292 shedding microspore and the most, Anjasmoro had 3820 ± 516 (Table 4).

Table 4 . Number of shedding microspore in the B medium

cultivars	Number of shedding microspore in B medium with 34^0C for 4 day
Argomulyo	3527 ± 396^b
Grobogan	2149 ± 61^a
Wilis	3658 ± 652^b
Anjasmoro	3820 ± 516^b
Hitam Malika	1729 ± 292^a

Mean values followed by different upper-case letters different significantly by Duncan's multiple range test at $P \leq 0.05$. Sd = standard deviation

DISCUSSION

Stamens are the male reproductive organs of flowering plants. They consist of an anther, the site of pollen development, and in most species a stalk-like filament, which transmits water and nutrients to the anther and positions it to aid pollen dispersal. Within the anther, male sporogenous cells differentiate and undergo meiosis to produce microspores, which give rise to pollen grains, whereas other cell types contribute to pollen maturation, protection, or release.

The condition of donor plant affects both anther and microspore culture. The growth of flower buds were observed to select soybean cultivars responsive. Healtly donor plants are important for success to minimize contamination (Zheng, 2003). The number of flower buds depends on cultivars plant. Grobogan soybean cultivars produce flowers fastest, but quickly develops to the fruit. This condition was difficult for micropsore culture progress.

The microspore build a cell wall, therefore its lost effect totipotent cell. This rapid process makes it difficult for microspore culture. Willis and Black Malika cultivars have small grain. Soybean seed small produce small flower buds, anther and microspore size are small too. Table 1 shown that Argomulyo soybean looks like Anjasmoro cultivars, but Anjasmoro cultivar is the highest. Tall plants with many branches have a lot of flowers. The main ingredient of microspore culture are buds flower. Anjasmoro cultivars produce a lot of flowers. Buds of Anjasmoro soybean cultivars at third stage development are selected for microspore culture according length of anther and total microspore per bud.

In five cultivars soybean anther shape is highly diverse. Black Malika soybean had the smallest anther (Table 2). Size of anthers is influenced by flower size. The level of pollen production is directly related to anther size and inversely related to pollen grain size (Nagy-Déri, 2011). The five cultivars showed that microspores from different anthers of the same flower are not at the same developmental stage

Soybean have papilionaceus flower consists of nine fused stamens with a single separate posterior stamen. Soybean flower bud stage 3 has the highest proportion of the number of late uninucleat microspore. In some types of crops such as brassica, tobacco, rice and wheat, flower buds with the above conditions are best for as microspores cultured (Wang et al., 2011). The third stage development of soybean flower buds (3.1-3.6 mm) contain mostly late-uninucleate microspores. Maize anthers are staged by length are early uni-nucleate at stage 3.0 mm length, middle uni-nucleate at stage 3.5 mm length, late uni-nucleate at stage 3.8 mm length and pollen mitosis on 4.0 mm length

(Wang et al., 2011), in Anjasmoro cultivars containing mostly late-uninucleate microspore. In soybean, the association between bud size and microspore stage should be re-checked under each different situation, such as cultivar, donor plant age, donor plant growth environments, etc. (Lauxen et al., 2003).

Stage of development plays an important role in the induction of microspore embryogenesis. Stage of microspore development in the anther begins with tetrad. The tetrad stage proceeds rapidly, and the infratectal columellae are the first exine elements to form (Taylor and Osborn, 2006). As the growth of flower buds, tetrad microspores well release by called, their microspore to develop into early uninucleate microspore, mid uninucleate and late uninucleate stage. Early uninucleate stage with the location of the cell nucleus in the middle, to the middle uninucleate stage (mid uninucleat) with the location of the cell nucleus shifted from the middle and late uninucleate stage the cell nucleus is in the periphere close to the pollen wall. There is a correlation between the length of the flower bud with stage of microspore development. To confirm our observations of optimum bud length range, counts were undertaken of microspores at each developmental stage for these three most responsive cultivars (Croser et al., 2011). Total microspores per anther in 5 cultivars of wheat (*Triticum aestivum* L.) ranged from 1230 to 1940 microspores (Touraev et al., 1996). Total microspore per bud in 5 cultivars of soybean ranged from 941-2003. Moreover, the number of pollen grains per anther also varied significantly and ranged from 4720 to 9840 among the walnut (*Juglans regia* L.) cultivars (Mer, 2010).

For many species, the most amenable stage is either the uni-nucleate stage of the microspore or, at or just after the first pollen mitosis, which is the early bi-nucleate stage. At this time the transcriptional status of the microspore may still be proliferative and not yet fully differentiated (Indrianto et al., 2001). The number of shed microspores from anther after stress in medium B at a temperature of 34^0C for 4 days for 5 soybean cultivars give in Table 4. Malika black soybean cultivars microspores most difficult out of stomium anther. Malika black cultivars stomium are not easily broken even though exposed to high temperature stress. Usually microspores from anther will shedding if it is in minimal medium or B medium. Grobogan cultivars are nearly the same as the Malika black soybean. In three soybean cultivars squandered another easy out microspores after stress treatment. Stomium anther cultivars Argomulyo, Wilis and Anjasmoro easy open for anther cell walls thin. Among the five soybean cultivars studied, the most widely Anjasmoro shed microspores after stress treatment. This supports the determination of the most responsive cultivar that will be used for microspore culture. Anjasmoro soybean cultivar can be used microspore culture to develop homozygus pure lines for crop-breeding programs.

ACKNOWLEDGEMENTS

Author expressed thanks to the Directorate General of Higher Education, Ministry of Education and Culture Republic Indonesia for giving BPPS scholarship to persue the Doctoral program. This research was financially supported by Doctoral Disertasion Research Program DIKTI No.16/S9/AD/2014.

REFERENCE

Cardoso, M.B., Kaltchuk-Santos, E., Munstock, E.C. and M.H. Bodanese-Zanettini. 2004. Initial segmentation patterns of microspores and pollen viability in soybean cultured anthers: indication of chromosome doubling. *Brazilian Archives of Biology and Technology*. 47 (5): 703-712.

Croser, J.S., Lulsdorf, M.M., Grewal, R.K., Kayley M. Usher, Kadambot H. and Siddique M.,. 2011. Isolated microspores culture of chickpea (*Cicer arietinum* L): induction of androgenesis and cytological analysis of early haploid divisions. *In Vitro Cell. Dev. Biol Plant*. 47:357–368

Ferrie, A.M.R., Bethune, T.D. and Mykytyshyn, M.. 2011. Microspore embryogenesis in Apiaceae. *Plant Cell Tiss Organ Cult* 104: 399-406

Indrianto, A., Barinova, I., Touraev, A., and E. Herberle-Bors. 2001. Tracking individual wheat microspores in vitro: identification of embryogenic microspores and body axis formation in the embryo. *Planta* 212:163-174

Kastono, D. 2008 Malika jejak sinergi pada sebutir kedele. Unilever Peduli Foundation, Universitas Gadjah Mada, dan Yayasan Kehati. ISBN: 978-979-18211-0-0.

Kyo, M. and Harada, H. 1986, Studies on conditions for cell division and embryogenesis in isolated pollen culture of *Nicotiana rustica*. *Plant Physiol*., 79, 90-94

Lauxen, M.S., Kaltchuk-Santos, E., Hu, C., Callegari-Jacques, S.M. and Bodanese-Zanettini, M.H. 2003. Association between floral bud size and developmental stage in soybean microspores. *Brazil. Arch. Biol. Technol.* 46(4):515-520.

Leroux, B, Carmoy, N., Giraudet, D., Potin, P., Larher, F., and Bodin, M. 2009. Inhibition of ethylene biosynthesis enhances embryogenesis of cultured microspores of *Brassica napus*. *Plant Biotechnol Rep.*3:347–351

Mer, C. 2010. Anther and pollen morphology and anatomy in walnut (*Juglans regia* L.). *Hortscience* 45(5):757–760.

Nagy-Déri, H. 2011. Morphological investigations on anthers and pollen grains of some quince cultivars. *Acta Biologica Szegediensis* 55(2):231-235

Rodrigues, L R, Camargo-Forte, D. D. and Zanettini, M.D.B. 2006. Isolation and culture of soybean (*Glycine max* L. Merril) microspore and pollen

grains. *Brazillian Achieve of Biology and Technology*. .49(4): 537-545

Shariatpanahi, M.E., Bal, U., Heberle-Bors, E., and Touraev, A. 2006. Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. Review. *Physiologia Plantarum* 127: 519-534.

Shirdelmoghanloo, H., Ahmad Moieni, A., and Mousavi, A. 2009. Effects of embryo induction media and pretreatments in isolated microspore culture of hexaploid wheat (*Triticum aestivum* L. cv. Falat). *African Journal of Biotechnology*. Vol. 8 (22), pp. 6134-6140

Taylor, M.L., and J.M. Osborn. 2006. Pollen ontogeny in Brasenia (Cabombaceae, Nymphaeales). *American Journal of Botany* 93: 344-356.

Touraev, A.,Indrianto, S., Wratschko, I., Vincente, O. and Heberle-Bors, E. 1996, Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperature. *Sex Plant Reprod* 9:209-215

Wang, D., Skibbe, DS., Walbot V. 2011. Maize csmd1 exhibits pre-meiotic somatic and post-meiotic microspore and somatic defects but sustains anther growth. *Sex Plant Reprod* 24:297–306

Wirawan,S.R.S. 2000. Studi Taksonomi kultivar Kedelai (*Glycine max* L. Merr) Di Pulau Jawa[Thesis]. Program Pasca Sarjana, Fakultas Biologi Universitas Gadjah Mada, Yogyakarta

Zheng, M.Y. 2003. Microspore in wheat (*Triticum aestivum*)- double haploid production via induce embryogenesis. Review of Plant Biotechnology and Appied Genetics. *Plant Cell, Tissue and Organ Culture*. 73:213-230.