Shallot haploid breeding for sustainable production - callus induction from shallot anther tissues

J Irawan\textsuperscript{1,2}, Sudarsono\textsuperscript{3}, A Maharijaya\textsuperscript{3} and D Dinarti\textsuperscript{*}

\textsuperscript{1} Plant Breeding and Biotechnology Graduate Program, IPB Graduate School, IPB University, Bogor 16680, Indonesia
\textsuperscript{2} Department of Agrotechnology, Faculty of Agriculture, Teuku Umar University, Meulaboh 23615, Indonesia
\textsuperscript{3} Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Bogor 16680, Indonesia

\*Email: dinydinarti@gmail.com

Abstract. Shallot is an essential vegetable crop in Indonesia, and its cultivation requires labor and capital intensive. Availability of high yielding and disease resistant shallot varieties will ensure its sustainable production. Haploid breeding should be beneficial for supporting the shallot breeding program. This study aims to identify suitable media for inducing callus growth and subsequent shoots or somatic embryo (SE) regeneration from anther tissues. In this study, successful callus induction was obtained from shallot anther but not for shoots or SE regeneration. The three different flower-sized responses were also investigated, and large flowers (5mm x 3mm) with an average anther length of 1.44 ± 0.04 mm were the best for callus induction. The microscopic observation indicated that large and medium flowers contain most bicellular pollen developmental stages, while small flowers are mostly tetrad stage. It also showed that the bicellular stage was more responsive to callus induction than the tetrad one. Moreover, the C1 medium consisted of MS basal medium supplemented with 3 mg L\textsuperscript{-1} BAP, 3 mg L\textsuperscript{-1} NAA, and 30 g/L sucrose was the best medium for inducing callus from anther explant.

1. Introduction
Anther culture is one method for obtaining haploid plants by growing anther plant in an \textit{in-vitro} medium. This method was one of several methods to accelerate the process of breeding plants that have been applied to cereal, horticultural, and also on some woody plants \cite{1}\cite{2}\cite{3}\cite{4}. The breeders will rapidly obtain homozygous strains with certain superior qualities that are beneficial for the development of plant species compared to the conventional methods \cite{5}\cite{6}\cite{7}\cite{8}\cite{9}\cite{10}. In conventional breeding, to obtain a homozygous pure strain is needed several times planting season to produce F5 with 93.75 and 96.88% homozygous ratio or until F7 to get 99.22% homozygous ratio, and the utilization of haploid culture can reduce the time in producing a homozygous plant \cite{8}.

The application of anther culture in allium has not given quite a meaningful result. The only gynogenesis has succeeded in producing haploid allium plant and most effective \cite{11}. Based on that fact, it is important to develop research that starts from the study of donor plants, anther phase in the uninucleated cell, and the development of microspores in shallot. Therefore, this research should be
done due to the regeneration of anther on in-vitro condition strongly influenced by plant genotypes, microspores density, and stadia development of microspores [12][13].

Development of anther culture could be induced by the indirect embryogenesis method, where anther was induced to produce callus first. The growth of callus In-vitro was influenced by several important factors such as the type of exportation, sugar (sucrose, maltose, fructose), amino acids such as arginine, asparagine, glycine, and plant hormones [14][15].

The medium formulation and in-vitro conditions are important for regenerating anther culture in this study. The composition of micro-macro elements, growth regulator, incubation room condition (light, temperature, humidity) is very important in regenerating the anther of a plant in vitro.

2. Materials and method

2.1. Preparation of donor plants

The donor plants were planted in the experimental field of IPB University Pasir Sarongge, the regency of Cianjur West Java. In vitro culture was conducted in Tissue Culture Laboratory 3 Department of Agronomy and Horticulture Faculty of IPB University.

Sixty shallot bulbs cv. Bima Brebes were planted in planting boxes (95 x 180 cm) shaded with UV-resistant plastic. The medium consisted of soil, charcoal husk, and chicken manure (4:2:1). The medium's mixtures were incubated for three days before the shallot bulbs were planted [16]. The bulbs were planted by burying 3/4 of them into the soil with the bud eye's position upwards with 15 x 20cm of plant spacing. NPK Fertilizer(16:16:16) was applied with 600kg/ha or 102.6 g/plot [17]. Plants were treated intensively, including watering and weed control. Control of pest manually, the pesticide used when the pest attack can not be done manually [18].

Inflorescence/umbel presence at 30 days after planting, umbels with 2-weeks-old (Fig 1) harvested and flower buds were grouped into three sizes: small (3mm x 2mm), medium (4.8 mm x 2.9 mm), and large (5 mm x 3mm).

![Figure 1](image)

Figure 1. (a) umbel with two weeks old (b) shallot flower buds with different sizes (small, medium, large).

2.2. Calculation of the number and development of microspores

Calculation of the number of microspores was done for several sizes of the anther. The Microspores were collected from two weeks old umbels (Fig. 1b). The length of the anther was measured using the ImageJ software. Calculation of the number of microspores was done manually using a hand counter. Anther from different flower sizes of shallot was placed on the object-glass and then cut into two parts using a scalpel. The anther's surface was pressed slowly using the surgical blade's blunt side until the liquid containing microspores came out. To the microspores were added 1% I2KI solution, and they were incubated for two minutes. The object-glass was observed under a binocular microscope at ten times magnification. The number of microspores was calculated using the hand counter, and the calculation was conducted three times. The microsphere development was observed either as the uninucleate (early, middle, late) or the binucleate stages.
2.3. Invitro Medium Preparation
Invitro Medium was prepared into two types: the callus induction medium and the callus regeneration medium.

2.3.1. Callus Induction Medium. Callus induction used four media types, including (1) MS medium [19] supplemented with 3 mg L\(^{-1}\) BAP, 3 mg L\(^{-1}\) NAA 30g/L sucrose (C1 medium), (2) N6 medium [20] supplemented with 65 g/L sucrose, 2mg NAA, 0.5 mg L\(^{-1}\) Kinetin, 10\(^{-3}\) M putrescine [21] (C2 medium), (3) BDS medium (Dunstan and Short) supplemented with 1 mg L\(^{-1}\) of kinetin, 1 mg L\(^{-1}\) of NAA, 30g/L sucrose (C3 medium), and (4) B5 medium supplemented with 1-2 mg L\(^{-1}\) 2,4 D, 1-2 mg L\(^{-1}\) BA, 75g/L sucrose [22, 23] (C4 medium). Phytagel at 2 g of L\(^{-1}\) was added to the media to solidify the medium.

2.3.2. Regeneration Medium. The Medium used to stimulate callus regeneration include (1) MS Medium [19] supplemented with 30g/L sucrose, 0.5 mg L\(^{-1}\) NAA, 2 mg L\(^{-1}\) 6-Benzylaminopurine (BAP), 2 mg L\(^{-1}\) kinetin, 20g/L sucrose [25] (R2 medium), and (3) MS medium [19] supplemented with 1 mg L\(^{-1}\) BA and 0.2 mg L\(^{-1}\) NAA [3] (R3 medium). Phytagel at 2 g of L\(^{-1}\) was added to the media to solidify the medium.

2.4. Induction of callus on various basic media in vitro
This experiment was designed as a Completely Randomized Design with two factors. The first factor was the flower bud sizes (small, medium, and large), while the second factor was medium composition (C1, C2, C3, and C4 media). A total of 12 treatment combinations and each treatment was repeated five times (total 60 experimental units). Each unit of trial consists of 60 shallot anther. Planting was done in the petri dish. The observation variable of this experiment is the percentage of the callus formed.

2.5. Callus Regeneration
Callus with size ± 4 – 6 mm, obtained from the previous callus induction, was transferred to the callus regeneration medium. The regeneration medium consisted of three types (R1, R2 and R3). This experiment used the Completely Randomized Design with the medium as a single factor, and each treatment was repeated ten times. The average regeneration was observed every 45 days after callus transplant into the regeneration medium.

2.6. Data Analysis
All observation data evaluated using analysis of variance (ANOVA) in the R program. Significant differences between means were compared using Duncan Multiple Range Test (DMRT) at P<0.05.

3. Results and discussion
Length of anther and number of microspores have a higher value in line with flower bud size (table 1). The different size of anther also shows the difference in the development of microspore (Fig. 2). Anther with medium and large flowers contain a majority of bicellular/binucleate symmetrical microspores, while the anther in small-sized flower bud mostly at the tetrad phase. The development of the microspores in the anther species of allium has been reported [26] and showed that the development of anther in allium species begins with the early prophase stage, late prophase I, prophase II, and pollen grain (bicellular pollen grain and pollen grain).
Table 1. Anther length and the number of microspores.

| Flower Size | Anther Length (mm) | Number of Microspore |
|-------------|--------------------|----------------------|
| Small       | 1.13±0.04<sup>c</sup> | 1301.33±110.35<sup>c</sup> |
| Medium      | 1.26±0.01<sup>b</sup> | 3730.67±63.88<sup>b</sup> |
| Large       | 1.44±0.04<sup>a</sup> | 4163.00±69.28<sup>a</sup> |

<sup>a,b,c</sup> Value followed by the same letter in the same column are not significantly different based on DMRT (<i>p = 0.05</i>)

Figure 2. Anther and microspores of shallots were observed using a microscope. (a) anther from a small flower bud as well as microspores tetrad phase. (b) anther from the medium flower bud and microspores bicellular phase. (c) anther of a large flower bud and microspores bicellular phase.

Anthers gave different responses (color and shape) when grown in different media (figure 3). Anther from a large flower bud can form a higher callus than anther from medium and small-sized flower buds (Table 2). Large-sized of flower bud has more microspore than medium and small flower bud sizes (Table 1)

Figure 3. Callus which formed from anther showed the differences in shape and color of callus

The development of microspores <i>in vitro</i> conditions was strongly affected by anther phase [27]. The size of flower buds greatly affected the development of microspores to form calli. The bicellular/binucleate phase was the best in androgenesis formation [8]. Microspores will respond to
forming embryos before the microspore and pollen stage [29], forming embryos formed from immature pollen when the balance of hormones in the medium will stimulate embryos to form organs [28]. On the other hand, some plants have good results in forming callus through anther at the uninucleate phase as in the kenaf plant [30], late uninucleate-early binucleate phase on Brassica [31].

Table 2. Percentages of callus, callus form and callus color.

| Medium | Flower size | Anther | Callus | Callus Form | Warna Kalus |
|--------|-------------|--------|--------|-------------|-------------|
|        |             | 180    | 0.56^b | Friable     | White       |
| C1     | Small       | 180    | 1.67^{ab} | Friable     | White       |
|        | Large       | 180    | 4.44^a  | Friable     | White       |
|        | Medium      | 180    | 0.56^b  | Friable     | White       |
|        | Large       | 180    | 1.11^{ab} | Compact     | White       |
|        |             | 180    | 2.78^{ab} | Friable     | White       |
| C3     | Small       | 180    | 0.00^b  | -           | -           |
|        | Medium      | 180    | 0.00^b  | -           | -           |
|        | Large       | 180    | 1.67^{ab} | Compact     | White       |
|        |             | 180    | 0.00^b  | -           | -           |
| C4     | Small       | 180    | 0.56^b  | Friable     | White       |
|        | Medium      | 180    | 0.56^b  | Friable     | White       |
|        | Large       | 180    | 1.11^{ab} | Friable     | White       |

\(^{a,b,c}\) Value followed by the same letter in the same column are not significantly different based on DMRT (p = 0.05)

Table 3. Callus regeneration in the regeneration medium

| Medium | Average of callus regeneration | Bentuk Kalus | Warna Kalus |
|--------|---------------------------------|--------------|-------------|
|        | 45 days                         | 90 days      |             |
| R1     | 1.86                            | 2            | Friable     | White-green |
| R2     | 2                               | 2.71         | Friable     | White-green |
| R3     | 1.86                            | 2.43         | Compact     | White-yellow|

Figure 4. Callus in regeneration medium showed different shapes and colors.
(a) callus in R1 medium (b) callus in R2 medium (c) callus in R3 medium.

Murashige and Skoog medium (C1 medium) supplemented with 3 mg L\(^{-1}\) BAP, 3 mg L\(^{-1}\) NAA 30g/L sucrose was the best medium for inducing callus anther on shallot. For the regeneration medium, the whole media has not significantly influenced regenerating callus (Table 3). However, the regeneration of callus showed the different shapes and colors (figure 4), the regeneration of the callus was very poor; all callus did not survive in the regeneration medium after 115 days. Basic media formulations are important and major to induction callus form, ratio of nitrate-ammonium ions (NO3- -
NH4 + 66:34) on MS medium and optimal composition of hormones spur a callus formation. The ratio of ions nitrate-ammonium was an important factor in selecting an in-vitro medium because it will greatly impact the explants' growth [32]. On the other side, the hormone substance added to the media was crucial in regenerating callus growth [4]. Plant hormones like auxin and cytokinin were the most common hormones used to induce indirect regeneration [22]. More factors cause the callus can not undergo regeneration to embryo somatic, such as in-vitro medium, wounding on explant, and hormone concentration [35]. The current issue showed that molecular mechanism plays a role in embryo development but is poorly understood [36], the transcription factor of BABY BOOM, Leafy Cotyledon (LEC) and AGAMOUS expressed during somatic embryogenesis [37]. Optimization on that factors and more information about molecular mechanisms will give the best result of shallot anther culture.

4. Conclusion
The size of flower buds indicated the microspore phase, and the development of callus from anther shallot depends on the microspore and in-vitro basal medium. Callus from anther cannot achieve regeneration to somatic embryos or shoots. Further research is required to obtain the best regeneration medium capable of inducing somatic embryos.

Acknowledgment
All authors are grateful to the Minister of Education and Culture for providing a research grant for this study through Beasiswa Pendidikan Pascasarjana Dalam Negeri (BPPDN).

References
[1] Otani M, Wakita Y, Shimada T. 2005. Doubled haploid plant production of transgenic rice (Oryza sativa L.) using anther culture. Plant Biotech. 22(2) 141–143.
[2] Asanaviciute R, 2008. Androgenesis in anther culture of Lithuanian spring barley (Hordeum vulgare L.) and potato (Solanum tuberosum L.) cultivars. Turk J, Biol. 32 155-160.
[3] Li Y, Li H, Chen Z, Ji LX, Ye MX, Wang J, Wang L, An XM. 2013. Haploid plants from anther cultures of poplar (Populus x Beijjingensis). Plant Cell Tiss Organ Cult. 114 39-48.
[4] Bhatia R, Dey SS, Sood S, Sharma K, Sharma VK, Kumar R, Parkash C. 2016. Optimizing protocol for efficient microspore embryogenesis and doubled haploid development in different maturity groups of cauliflower (B. Oleracea var. botrytis L.) in India. Euphytica. 212 (3) 439-454.
[5] Afza R, Shen M, Zapata FJ, Xie J, Fundi HK, Lee KS, Mucino B, Kodym A. 2000. Effect of spikelet position on rice anther culture efficiency Plant Science. 153 155-159.
[6] Aulinger IE, Peter SO, Schmid JE, Stamp P. 2003. Rapid attainment of a doubled haploid line from transgenic maize (Zea mays L.) plants by means of anther culture. In vitro Cell Dev Plant. 39 165-170.
[7] Hassawi DS, Qrunfleh I, Dradkah N. 2005. Production of doubled haploids from some Jordanian wheat cultivars via anther culture technique. J Food Agric Environ. 3 161-164.
[8] Neto RF, Garbuglio DD, Borem A. 2014. Double haploid Biotechnology Applied to Plant Breeding. Elsevier inc. Chapter 9.
[9] Dwivedi SL, Britt AB, Tripathi L, Sharma S, Upadhayaya HD, Ortiz R. 2015. Haploid: constraints and opportunities in plant breeding. Biotechnology advances. Elsevier Published.
[10] Ren J, Wu P, Trampe B, Tian X, Luberstedt T, Chen S. 2017. Novel technologies in double haploid line development. Plant Bio J. 15 1361-1370.
[11] Fayos O, Valles MP, Claver AG, Mallor C, Castillo AM. 2015. Double haploid production from Spanish onion (Allium cepa L.) germplasm: embryogenesis induction, plant regeneration and chromosome doubling. Front Plant Sci. 6 384.
[12] Prem D, Gupta K, Sarkar G, Agnihotri A. 2008. Activated charcoal induced high-frequency microspore embryogenesis and efficient doubled haploid production in Brassica juncea. Plant Cell Tiss Organ Cult 93 269–282.
[13] Wan GL, Naeem MS, Geng XX, Xu L, Li B, Jilani G, Zhou WJ. 2011. Optimization of microspore embryogenesis and plant regeneration protocols for Brassica napus. Int J Agric Biol. 13 83–88.

[14] Purnamaningsih R. 2002. Regenerasi Tanaman Melalui Embriogenesis dan Beberapa Gen yang Mengendalikannya. Buletin AgroBio 5(2) 51-58.

[15] Saepudin A, Khumaida N, Sopandie D, Ardie S. 2016. Induksi dan Proliferasi Embriogenesis Somatik In Vitro pada Lima Genotipe Kedelai. J Agron Indonesia. 44(3) 261-270.

[16] Suhesti K. 2017. Kajian fotoperiodisme dan vernalisasi untuk induksi pembungaan bawang merah (Allium cepa L. Agregatum group) di dataran rendah. [Tesis]. Bogor (ID): Institut Pertanian Bogor.

[17] Rosliani R, Palupi ER, Hilman Y. 2013. Pengaruh benzil amino purin dan boron terhadap pembungaan, viabilitas serbuk sari, produksi, dan mutu benih bawang merah di dataran rendah. J Hort. 23(4) 339-349.

[18] Dinas Pertanian Yogyakarta. 2012. Standard Operating Procedure Bawang merah gunung kidul.

[19] Murashige T, Skoog F. 1962. A revised medium for rapid growth on bioassays with tobacco tissue culture. Physiol Plant 15 473-497.

[20] Chu CC. 1978. The N6 medium and its applications to anther culture of cereal crops. Proc. Symp. Plant Tissue Culture. Science Press. Peking.

[21] Dewi IS, Syaffi M, Purwoko BS, Suwarno WB. 2017. Efficient indica rice anther culture derived from three-way crosses. Sabrao Journal of breeding and genetics. 49 (4) 336-345.

[22] Muren RC. 1989. Haploid plant induction from unpollinated ovaries in onion. Hort Science. 24 833-834.

[23] Geoffriau E, Kahane R, Rancillac M. 1997. Variation of gynogenesis ability in onion (Allium cepa L.). Euphytica. 94 37-44.

[24] Murashige T, Skoog F. 1962. A revised medium for rapid growth on bioassays with tobacco tissue culture. Physiol Plant 15 473-497.

[25] Bhatia R, Dey SS, Sood S, Sharma K, Parkash C, Kumar R. 2017. Efficient microspore embryogenesis in cauliflower (Brassica oleracea var. botrytis L.) for development of plants with different ploidy levels and their use in breeding programs. Scientia Horticulturae. 216 83-92.

[26] Dorota T, Kamil D, Krystyna W. 2017. Cytological and biophysical comparative analysis of cell structures at the microsporogenesis stage in sterile and fertile Allium species. Planta. 245 137-150.

[27] Marta CW, Sandra MR, Joyce AB, Wyne JC. 1991. Effect of microspore stage and media on anther culture of peanut (Arachis hypogaea L.). Plant Cell Tiss Organ Cult. 24 25-28.

[28] Cardoso MB, Santos EK, De-Mundstock ES, Bodanese-Zanettini MH. 2004. Initial Segmentation Patterns of Microspores and Pollen Viability in Soybean Cultured Anthers: Indication of Chromosome Doubling. Brazilian Archives Of Biology And Technology An International Journal. 47 703-712.

[29] Zaki MAM, Dickson HG. 1990. Structural changes during the first division of embryos resulting from anther and free microspore culture in Brassica napus. Protoplasma. 156(3): 149-162.

[30] Neto RF, Garbuglio DD, Borem A. 2014. Double haploid Biotechnology Applied to Plant Breeding. Elsevier inc. Chapter 9.

[31] Ferrie AMR, Caswell KL. 2010. Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tissue Organ Culture. 104(3): 301-309.

[32] Edwin FG, Michael AH, Geert-Jan DK. 2008. Plant propagation by tissue culture 3rd edition. Springer. The Netherlands.

[33] Bhaskaran S, Smith RH. 1990. Regeneration in serial tissue culture: a review. Crop Sci. 30 1328-1337.
[34] Ikeuchi M, Sugimoto K, Iwase A. 2013. Plant callus: Mechanism of induction and repression. *The Plant Cell*. 25 3159-3173.

[35] Hugo AMH, Maharshi LR, Randy NAM, Yary LJG, Analesa S, Johny AM, Clelia D, Victor MLV. 2019. Signaling overview of plant somatic embryogenesis. *Front Plant Sci*. 10 1-15.

[36] Cetz-Chel JE, Loyola-Vargas VM in 2016. Transcriptome profile of somatic embryogenesis. In: Loyola-Vargas VM, Ochoa-Alejo N. (eds). Somatic embryogenesis: Fundamental aspects and applications, Chapter 4. Springer pp 39-52.

[37] Stella AGDS, Candice NH, Buell CR, Shawn MK, Heidi FK. 2014. Whole transcriptome profiling of maize during early somatic embryogenesis reveals altered expression of stress factors and embryogenesis-related genes. *PloS one*. 9 1-17.