Embryo-gynogenic induction of Indonesia shallot (Allium cepa L. Aggregatum group) cultivars using three different protocol unpollinated flower cultures

E S Lestari¹,², Sulastriningsih³, D C Prayantini ², A Purwantoro¹ and E Sulistyaningih¹,*

¹ Faculty of Agriculture, Gadjah Mada University, Jl Flora, Bulaksumur, Depok, Sleman, Yogyakarta, Indonesia
² PT BISI International, Tbk, Jl. HOS Cokroaminoto 72A, Pare, Kediri, East Java, Indonesia
* E-mail: endangsih@ugm.ac.id

Abstract. Embryo-gynogenic induction was an important step in haploid induction. The research was determined the best method of unpollinated flower culture of Indonesia shallot varieties. Three methods were used including method 1 with two-step cultures using BDS as basic medium supplemented with polyamines 2 mM putrescine on the first step and spermidine 0.1 mM at the second step, method 2 using BDS as basic medium supplemented with 2,4-D 2 mgL⁻¹ and BA 2 mgL⁻¹ on the first step and NAA 1 mgL⁻¹ and 2IP 2 mgL⁻¹ on the second step and method 3 using B5 medium supplemented with 2,4-D 2 mgL⁻¹ and BA 2 mgL⁻¹. Six shallot cultivars used as flower donor were Katumi, Bima Brebes, Tajuk, Trisula, Superphilip, and Bauji. Flower umbel was collected and pretreated using Yoshida liquid medium then placed on incubator at four °C for overnight. The results showed that all flowers cultured could anthesis properly in the first week of cultured. The embryos could emerge from ovaries from week 7th of cultured in all methods. However, the highest percentage of embryos gynogenic developed to seedlings was achieved using method 3 in Superphilip (6.98%) followed by method 2 in Tajuk (4.44%).

Keywords: embryo-gynogenic, haploid induction, unpollinated flower

1. Introduction
Shallot (Allium cepa L. Aggregatum group) is one of Indonesia's valuable and most widely cultivated crops. Most of them were propagated with bulbs and several of them with seeds. Since shallot is a biennial plant, production inbred lines request at least 6-10 years [1]. Inducing haploid plant followed with chromosome doubling can shorten the time required to create inbred lines. Muren [2] reported induction of haploid onion using ovary culture while haploid culture induction using anther culture has been reported [3]. However, there were no embryos respond using anther culture. The successfully haploid induction using gynogenesis method has been reported by several types of research in several genera of Allium such as in Allium cepa L [1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15], Allium cepa L. Aggregatum group [16], Allium ampelorasum L. [17], Allium fistulosum L. [18] and Allium chinense [19]. Several of them used ovule, ovary or flower buds as explants for double haploid induction. Ovary culture and unpollinated flower culture gave similar embryogenic responses, and then unpollinated flower culture was chosen since it was less laborious than ovary culture [1, 20].

Several factors, including genotypes, growing condition of donor plants, and medium composition could be affected the rates of the embryo-gynogenic induction [21]. Gynogenesis induction in shallot
has been reported using one-step culture protocols [16] including B5 [22] medium as a primary medium supplemented with 2,4-D 2 mg L\(^{-1}\) and BA 2 mg L\(^{-1}\). In contrast, research in onion, two-step culture with B5, MS and BDS [23] were used as basic medium supplemented with several plant growth regulators or polyamines [11, 14, 5].

Embryo-gynomonic induction was an important step in haploid induction. Therefore, in this research, the best method of unpollinated Indonesia shallot varieties' flower culture would be determined. Three protocols were used including Protocol 1 [11] which using two-step culture with BDS as essential medium supplemented with polyamines two mM putrescine on the first step and spermidine 0.1 mM at the second step, Protocol 2 [5] which also using BDS as basic medium supplemented with 2,4-D 2 mg L\(^{-1}\) and BA 2 mg L\(^{-1}\) on the first step and NAA 1 mg L\(^{-1}\) and 2iP 2 mg L\(^{-1}\) on the second step and Protocol 3 including B5 Gamborg medium supplemented with 2,4-D 2 mg L\(^{-1}\) and BA 2 mg L\(^{-1}\) [16] (Table 1).

2. Methods

2.1. Growing donor plants
Six shallot cultivars Katumi, Bima Brebes, Tajuk, Trisula, Superphilip, and Bauji, were used as donor plants. Donor plants were planted in the Pujon farm (1100 asl) PT BISI International Tbk, Batu Malang from January to May 2018. Bulbs were used as a source for donor plants. Donor plants were planted in a field with space 15 cm x 15 cm each plant and irrigated with a drip irrigation system.

2.2. Flower bud harvested
Flower buds were harvested when 20-30% flower buds opened or 3-5 days before anthesis. Flowers with stalk (inflorescences) were placed on beaker consist of Yoshida liquid medium [24] covered with plastic wrapping then incubated in the dark at 4 \(^\circ\)C for overnight. Flower buds were selected with the size approximately 3-4 mm in diameter.

2.3. Unpollinated flower buds cultured
The selected flower buds were sterilized using 70% ethanol for 1 minute, followed by 40% commercial bleach (5.25%) for 15 minutes, then rinsed three times with sterile water. Buds were dried on the filter paper then flower stalk was cut off. Buds were placed on three induction medium (Table 1.) based on [11], [4] and [16]. The medium composition of each protocol was shown in Table 1. Protocols based on [16] was one step culture in which there was no transferred to another medium during the culture until embryo emerge and break the ovum wall. Whereas protocols based on [11] and [4] were two-step culture while flower buds were transferred from first medium (R1) during two week of culture to the second medium (R2) until embryos were emerged. Among three medium were solidified with gelrite 2 g L\(^{-1}\). Flower buds culture were placed on 90 mm x 15 mm Petri dishes consisted of 25 mL of medium then sealed with plastic wrapping. Culture condition was set at 25 ± 1 \(^\circ\)C under Philip fluorescent lamps with 16 h photoperiodism. Exceeds gynomonic embryo transferred to the first regeneration medium (R1) consist of B5 supplemented with 1 mg L\(^{-1}\) IBA, sucrose 30 g L\(^{-1}\) and gelrite 2 g L\(^{-1}\). Regenerants then were transferred to the second regeneration medium consist of B5 medium supplemented with 1 mg L\(^{-1}\) IBA, sucrose 30 g L\(^{-1}\), activated charcoal 10 g L\(^{-1}\) and gelrite 2 g L\(^{-1}\). All emerged embryos were counted for number of embryos and and calculated the percentage of embryos from all flowers cultured in induction media.

| Composition          | Protocol 1 [11] | Protocol 2 [4] | Protocol 3 [16] |
|----------------------|----------------|----------------|-----------------|
|                      | R I  | R II | R I  | R II | B5  |
| Basic media          | BDS  | BDS  | BDS  | BDS  | B5  |
| Macronutrient (mg/L) |      |      |      |      |
| KNO\(_3\)            | 2530 | 2530 | 2530 | 2530 | 2500|
| NH\(_4\)NO\(_3\)     | 320  | 320  | 320  | 320  | 134 |
| CaCl\(_2\)2H\(_2\)O  | 150  | 150  | 150  | 150  | 150 |
| MgSO\(_4\)7H\(_2\)O  | 247  | 247  | 247  | 247  | 250 |
| Compound          | R1 | R2 | R3 | R4 |
|-------------------|----|----|----|----|
| NH₄H₂PO₄          | 230| 230| 230| 230|
| Na₂HPO₄·H₂O       |    |    |    |    |
| Na₂HPO₄·2H₂O      | 152| 152| 152| 152|
| KH₂PO₄            |    |    |    |    |
| (NH₄)₂SO₄         | 134| 134| 134| 134|

**Mikronutrien (mg/L)**

| Compound          | R1 | R2 | R3 | R4 |
|-------------------|----|----|----|----|
| KI                | 0.75| 0.75| 0.75| 0.75|
| H₃BO₃            | 3   | 3   | 3   | 3   |
| MnSO₄·H₂O        | 10  | 10  | 10  | 10  |
| MNSO₄·4H₂O       |    |    |    | 10  |
| ZnSO₄·7H₂O       | 2   | 2   | 2   | 2   |
| Na₂MoO₄·2H₂O     | 0.25| 0.25| 0.25| 0.25|
| CuSO₄·2H₂O      | 0.025| 0.025| 0.025| 0.025|
| CoCl₂·6H₂O      | 0.025| 0.025| 0.025| 0.025|
| FeSO₄·7H₂O      | 27.8| 27.8| 27.8| 27.8|
| Na₂-EDTA      | 37.3| 37.3| 37.3| 37.3|

**Vitamins (mgL⁻¹)**

| Vitamin           | R1 | R2 | R3 | R4 |
|-------------------|----|----|----|----|
| Myo-inositol      | 100| 100| 100| 100|
| Nicotinic acid    | 0.5| 0.5| 0.5| 0.5|
| Pyridoxine-HCl    | 0.5| 0.5| 0.5| 0.5|
| Thiamine HCl      | 0.1| 0.1| 0.1| 0.1|
| Glycine           | 2   | 2   | 2   | 2   |

**Polyamines**

| Polyamine       | R1 | R2 | R3 | R4 |
|-----------------|----|----|----|----|
| Putrecine (mM)  | 2  |    |    |    |
| Spermine (mM)   |    | 0.1|    |    |
| Sucrose gL⁻¹     | 100| 100|    |    |

**R1 : Flower buds culture in the first media for two week on Protocol 1 and 2.**

**R2 : Flower buds culture transferred to the second media after two week in R2 on protocol 1 and 2.**

### 3. Results and discussion

Flowers could anthesis properly in the first week of cultured. Flower petals opened spontaneously within the first week of culture in all protocols. The size of flower buds in protocol 2 and 3 grew 2-3 times bigger than their original size. In contrast, explants in protocol 1 showed a smaller size comparing with protocol 2 and 3.

After seven weeks of culture, ovaries colour in protocols 2 and 3 turned into green, and some of them formed callus at the base of flower buds but there were no embryoids responses observed after seven weeks in culture. While in protocol 1, ovaries showed yellowish colour, no callus response at the base flower and several embryos from Katumi genotype started to emerge from ovaries wall. The embryoids emerged continuously after seven weeks of culture in protocol 1. In protocol 2 and 3 several embryos were emerged from ovarium wall at 80 days of culture. The first embryoid emerged from Philippine using protocol 3. Several embryos emerged directly through septum in the ovarium wall. Most of them coming out with structure like seed coat with black colour. Emerging embryos in the Protocols 3 were shown in Figure 1.
Figure 1. (a) Flower buds culture after 80 days using Protocol 3. (b), (c) Embryo emerged from the ovarium using Protocol 3.

The number of percentage of embryos responses among three protocols in six genotype of shallot were showed in the Table 2.

Table 2. Gynogenic embryo of six shallot cultivars.

| Genotype     | The number of flower buds | Number of embryos | % embryos |
|--------------|---------------------------|-------------------|-----------|
|              | Protocol 1 | Protocol 2 | Protocol 3 | Protocol 1 | Protocol 2 | Protocol 3 | Protocol 1 | Protocol 2 | Protocol 3 |
| Katumi       | 4150     | 367     | 3745     | 37       | 0       | 49       | 0.89      | 0       | 1.31 |
| Bima brebes  | 702      | 297     | 355      | 4        | 0       | 4        | 0.57      | 0       | 1.13 |
| Tajuk        | 422      | 383     | 68       | 0        | 17      | 0        | 0         | 4.44    | 0    |
| Trisula      | 514      | 508     | 227      | 0        | 7       | 2        | 0         | 1.38    | 0.88 |
| Superphilip  | 300      | 337     | 43       | 0        | 3       | 3        | 0         | 0.89    | 6.98 |
| Bauji        | 950      | 623     | 366      | 0        | 2       | 1        | 0         | 0.32    | 0.27 |

Protocol 1 employed two different media, first media consisted of BDS supplemented with putrescine 2 mM then were subcultured to the second medium containing BDS medium supplemented with 0.1 mM spermidine. This protocol could induce embryoids in Katumi and Bima Brebes' genotypes with the percentage of 0.89% and 0.57%. Protocol 2 showed that four genotypes produced embryo while both genotypes Bima Brebes and Katumi did not produce embryo formation. The highest percentage of embryo production by this protocol was obtained in genotype Tajuk (4.44%). In protocol 2, flower buds were subcultured to the new media after four weeks of culture in the medium one consist of BDS medium supplemented with NAA 1 mgL⁻¹ and 2iP 2 mgL⁻¹. By using protocol 2, four genotypes gave respond to embryos. By using protocols 3, flower buds were not subcultured to another media until embryo performed. Embryo-gynogenic induction media based on Protocol 3 resulted in five response genotypes in which the highest percentage of embryos production was obtained in genotypes Superphilip (6.98%) followed with Katumi (1.31%), Bima Brebes (1.13%), Trisula (0.88%) and Bauji (0.27%). Meanwhile, the genotype Tajuk did not show embryo production (0%). Protocol 3 resulted that three genotypes produced embryoids higher number than those of in Protocols 1 and 2.

Protocol 3 that employed plant growth hormone 2,4-D 2 mgL⁻¹ and BA 2 mgL⁻¹ without subculture was effectively increased the number of embryoids in shallot. Moreover protocols 3 was considered since it was more effective and simple to apply. Based on this results, the embryo-gynogenic response was influenced by their genotypes [6, 11, 14, 16] and protocols or culture media. By comparing three embryo-gynogenic induction protocols at the same genotypes, each shallot genotypes showed different responses to embryo-gynogenic in different protocols. In Protocol 1 the addition of polyamines were applied during first 15 days of culture with 2 mM putrescine, followed with 0.1 mM spermine until embryos emerged [11]. By this research, the addition of polyamine in Protocol 1 was only beneficial for few shallot genotypes (Katumi and Bima Brebes). The maximum embryo production was achieved (9.5%) in onion [11]. In contrast, the maximum embryogenic responses in the shallot were achieved...
0.89% in the genotype Katumi. However, both genotypes Katumi and Bima Brebes produced embryo-gynogenic lower number than that of conducted by Protocol 3.

According to the protocols 3 [16] reported that unpollinated flowers of three Indonesian shallot cultivars i.e Dili-white, Yogya and Dili-red resulted totally 89 embryos from 6,812 flower buds culture (1.31%). Comparing the percentage of embryos obtained in this research with previous research [16] it can be concluded that there were two genotypes produced embryos with same or higher than before [16] i.e Superphiplip (6.98%) dan Katumi (1.31%). However four genotypes produced the percentage of obtained embryos lower than previous research results[16]. Furthermore, based on their ploidy level on the research [16] using genotype of Dili-white obtained two plants were haploid indicating that embryos grow from haploid cell or ovule. In other cases, the genotype of Dili-Red obtained the highest number of directly regenerated and callus derived coming out of their somatic tissue. It was needed to distinguish the embryo coming out from ovules or their somatic tissue.

4. Conclusion
Every genotype of shallot has a different response to every three protocols. The embryos could emerge from ovaries from week 7th of cultured in all methods. The highest percentage of embryos gynogenic developed to seedlings was achieved using method 3 in Superphiplip (6.98%) followed by method 2 in Tajuk (4.44%).

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