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# INDUCTION OF EMBRYOGENIC CALLUS AND SOMATIC EMBRYO FROM ANTHER CULTURE IN PAPAYA (*CARICA PAPAYA* L.)

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**SUMMARY**

The androgenic callus induction and plant regeneration with the least response through anther culture are the key problems in biotechnology-based papaya (*Carica papaya* L.) breeding. Improvement of papaya response by anther culture through flower size and PGRs' combinations earnestly need investigation. The presented research aimed to determine the microspore stage and viability based on the flower size and a combination of plant growth regulators (PGRs) in MS (Murashige and Skoog) medium to induce embryogenic callus and somatic embryos in papaya. Explants used in the study were the anthers of hermaphrodite flowers. This study comprised three different experiments, i.e., microspore development and viability test, 2,4-D (2,4-Dichlorophenoxyacetic acid) and TDZ (Thidiazuron) optimization for embryogenic callus induction, and NAA (naphthalene acetic acid) and CPPU (N-[2-Chloro-4-pyridyl]-N'-phenylurea) optimization for somatic embryos' formation. Results revealed hermaphrodite flower buds were 10–25 mm in length, which can be effective for embryogenic callus induction as they contained the high percentage of uninucleate stage microspores and demonstrated high viability (>95%). The combination treatment of 2,4-D (0.1 mg $L^{-1}$) and TDZ (0.5 mg $L^{-1}$) induced the most percentage of anther forming callus (20.4%), with 58.7% of that as embryogenic callus. The combination of NAA (0.1 mg $L^{-1}$) and CPPU (0.5 mg $L^{-1}$) resulted in 70% embryogenic callus plated developed into the maximum average globular embryos (38.8).

**Keywords:** Papaya (*C. papaya* L.), callus induction, flower size, hermaphrodite flowers, microspores stage, 2,4-D, TDZ, NAA, CPPU

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**Key findings:** The induction of somatic embryos in papaya (*C. papaya* L.) anther culture can be successful through an appropriate size of hermaphrodite flowers and the use of PGRs' combination in the culture medium. This information is crucial to support papaya breeding programs to obtain double haploid lines through anther culture.

## INTRODUCTION

The papaya (*Carica papaya* L.) is an important member of the family Caricaceae and a tropical fruit grown commercially in various countries, including Indonesia. Papaya has several commercial cultivars, such as, the Caliso (Registration of Breeding Plant Cultivars No. 492/PVHP/2017). Caliso is one of the superior cultivars included in the small fruit size category from Kalimantan, Indonesia, with fruit lengths of 19.5–21.4 cm. The said cultivar has the advantage to bloom after three months of planting, harvesting made eight months after planting, and a flesh thickness (2.0–2.6 cm), with sugar content of approximately 12.0–15.2 °Brix.

Papaya propagation commercially is through seeds. However, it faces several problems, including different sex types and variations in the populations. Generally, papaya exhibits three sexual forms, i.e., male, female, and hermaphrodite, which can be noticeable after the plants bear flowers (Phuangrat *et al*., 2013). Seed-based propagation also bears some other concerns for genetic variations because of the segregation occurring during cross-pollination (Al-Shara *et al*., 2018). Efforts are ongoing to control the pollination; however, the difficulties of sexual diversity persists due to seasonal variations in papaya sex (Tamaki *et al*., 2011).

Genetic diversity of such plants can be more effective by cross-breeding with other cultivars. However, plant breeding requires homozygous plants as parental genotypes, taking more than six generations to produce through conventional breeding (Alahmad *et al*., 2018). A potential solution to overcome these problems could proceed through the implementation of haploidization technology to obtain haploid plants from diploids. The haploid plants obtained through chromosome doubling process can produce homozygous doubled haploid plants. The haploidization technology can be applicable through androgenesis (anther and microspore culture) and gynogenesis (ovule and ovary culture) (Portemer *et al*., 2015; Lentini *et al*., 2020). Androgenesis culture is better for explant availability because plants produce more anthers than ovules in the ovary.

In the family Caricaceae, only a few reports exist on using *C. papaya* to produce haploids and doubled haploids. Although, they focused only on the use of anthers from male flowers as explants. Generally, male papaya is less beneficial commercially because it cannot produce fruits. In contrast, hermaphrodite papaya is more popular on the market due to its high fruit productivity with better morphology and quality (Febjislami *et al*., 2018). However, the success in anther culture has influences from several factors, such as, pretreatment (Rimberia *et al*., 2005), the genotype, physiological state of the donor plant, and medium composition (Rimberia *et al*., 2006; Gyanchand *et al*., 2015), and microspore development stage (Chong-Perez *et al*., 2018).

In papaya, the application of anther culture has specific challenges, including a low rate of androgenic callus induction and limited plant regeneration. Tsay and Sue (1985) reported a low percentage of successful embryo induction (0.7%) from anther culture on an agar medium without growth regulators. In anther culture, the induction of somatic embryos further rose to 13.8% by culturing anthers on the solid Murashige and Skoog (MS) medium with CPPU (0.01 mg $L^{-1}$) and NAA -1-Naphthalene Acetic Acid (0.1 mg $L^{-1}$) (Rimberia *et al*., 2006). Gyanchand *et al*. (2015) produced 8% embryos on MS basal medium supplemented with benzyl adenine-BA (0.1 $mgL^{-1}$), NAA (0.1 mg $L^{-1}$), sucrose (3%), and agar (0.6%). The progressive study aimed to determine the microspore stage based on

hermaphrodite flower size and a combination of growth regulators in MS medium to induce embryogenic callus and somatic embryos from anthers as explants.

## MATERIALS AND METHODS

### Experimental material and procedure

The research took place from August 2022 to December 2023 at the Tissue Culture Laboratory and Micro Techniques Laboratory, IPB University, Bogor, Indonesia. Papaya cultivar Caliso with hermaphrodite flowers were the samples used as explants, which came from the Center for Tropical Horticulture Studies (PKHT), Pasir Kuda, Bogor, Indonesia.

### Microspore development stages and viability test

The microspore development stages commenced by isolating microspores from the flower buds. The anthers' pressing used a needle to spread the microspores on the surface of the glass object, adding several drops of aceto-orcein (1%), and then, observing under a UV microscope with 40× magnification.

The iodine dye (1%) application helped microspore viability testing. The isolation of microspores from anthers ensued by pressing the anthers with a needle for the microspores to spread on the surface of a glass object. Then, adding two drops of 70% ethanol solution followed before retaining for 30 min. The removal of ethanol solution continued, followed with adding a few drops of 1% iodine solution. Observations started under a UV microscope with a 10× magnification. Viable microspores observed showed dark blue shades, while non-viable microspores had no colors.

The experiment, laid out in a completely randomized design (CRD), used a single factor of flower buds' length. The flowers used have the length of 10–15, 16–20, and 21–25 mm. The microspore stages used five replications, with each glass slide as one experimental unit, consisting of five anthers. The observations made were to count the number of microspores at the tetrad, uninucleate, and binucleate stages. The microspore viability testing used nine replications, with each slide representing one experimental unit, consisting of three fields of view. Observations continued by using the following formulas:

$$\text{Stage of microspore} = \frac{number\,of\,microspores\,ateach\,stage}{total number\,of\,microspores observed} \times 100\%$$

$$\text{Viability of microspore} = \frac{number\,of\,stained microspores}{Number\,of\,microspores observed} \times 100\%$$

### Callus induction

The anthers used came from hermaphrodite papaya flowers, of which, identifying the optimum size resulted from previous stages by having a high population of microspores at uninucleate stages. Flower buds sterilization transpired in a laminar airflow cabinet. The flower buds underwent the following steps: soaked in 70% (v/v) alcohol for 15 min, then rinsed with distilled water three times and soaked with 30% (w/v) sodium hypochlorite solution, then 10% (w/v) sodium hypochlorite solution for 10 min, and later rinsed with distilled water three times. The flowers sustained dipping in 96% alcohol before passing over a Bunsen flame several times. After sterilization, the anthers' removal from the flower buds followed, before placing them in an MS solid medium with the addition of 2,4-D (2,4-Dichlorophenoxyacetic acid) and TDZ (Thidiazuron) and exposing to a high temperature of 35 °C for seven days in an incubator. Then, moving the culture bottles for incubation in a dark culture room at 25 °C occurred.

The experiment arrangement in a randomized complete block design (RCBD) had two factors. The first factor was the 2,4-D different concentrations (0.1 and 0.3 mg $L^{-1}$), while the second factor was the TDZ concentrations (0.5, 1.0, and 1.5 mg $L^{-1}$).

There were six treatment combinations with 10 replications. Each experimental unit comprised one culture bottle, thus, totaling 60 experimental units. Ten anthers continued planting in each culture bottle. The variables observed were the number of anthers forming callus, callus formation time, the callus appearance (compact or friable, white, yellow or green, watery), and embryogenic callus emergence (friable, yellowish-white structure easily separated, and the callus parts with a globular structure).

**Induction of globular somatic embryo formation**

Friable callus explants (3 mm in size) bore sub-culturing in MS0 medium (MS without PGRs) for one week to eliminate the effect of the previous medium treatment. Then, planting the explants progressed in a solid MS medium supplemented with a combination of NAA (naphthalene acetic acid) and CPPU (N-[2-chloro-4-pyridyl]-N'-phenylurea) treatments. Cultures received incubation in the dark at 25 °C with 70% humidity.

For this experiment, the randomized complete block design (RCBD) used had two factors. The first factor was the NAA different concentrations (0.1, 0.5, and 1.0 mg $L^{-1}$), while the second factor was the CPPU concentrations (0.01, 0.1, and 0.5 mg $L^{-1}$). Nine treatment combinations had 10 replications to obtain the 90 experimental units. The experimental unit was a culture bottle, with three explants planted. The variables observed were the number of embryogenic callus forming somatic embryos and total number of globular embryos.

**Statistical analysis**

All the recorded data on different variables' analysis used the analysis of variance (ANOVA). The treatments with significant effects incurred further comparison with the Duncan's Multiple Range Test (DMRT) at a 5% level of probability.

**RESULTS**

**Microspore development and viability**

The microspore development stages observed were tetrad, uninucleate, and binucleate (Figure 1). The tetrad stage of microspore development, as characterized by the presence of four haploid microspore cells, had partitioned within its cell (Figure 1A). The uninucleate microspore stage showed the presence of one nucleus, with its location not in the center, but rather at the edge (Figure 1B). In the binucleate microspore stage, the microspore has two nuclei (Figure 1C). The proportion of tetrad, uninucleate, and binucleate microspores is available in Table 1. The flower bud size with 10–15 mm had the highest percentage of tetrad stage microspores (3.6%); however, it was not significantly different from the flower bud size of 16–20 mm. The flowers' bud size with 10–25 mm have the similar character of high percentage of uninucleate stage microspores (>95%). The binucleate stage of microspores was most frequent in flower buds sized 21–25 mm. However, the said stage was not significantly different from the frequency observed in flower buds sized 16–20 mm. In contrast, no microspores were visible at the binucleate stage in flower buds sized 10–15 mm.

The highest percentage of microspore viability (91.3%) was prominent in flower bud sizes of 16–20 mm, which was significantly higher than those of flower bud sizes of 10–15 mm (viability 78.8%) and 21–25 mm (viability 80.8%) (Table 1). Testing the viability of microspores with 1% iodine staining showed the viable microspores could absorb a dark blue shade. In contrast, non-viable microspores did not absorb the color; hence, they looked lighter and yellowish (Figure 2).

**Callus induction**

The callus succeeded induction in all the 2,4-D and TDZ treatments, with the anthers previously incubated at a high temperature of

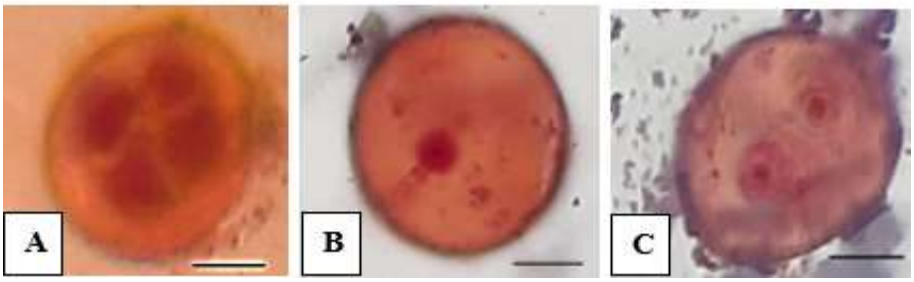


**Figure 1.** Determination of microspore developmental stages. Aceto-orcein stain showing A) tetrad, B) Uninucleated, and C) Binucleated microspores (Bar = 10 µm).

**Table 1.** Microspore developmental stage distribution and viability test in anthers with different flower size in papaya cv. Caliso.

| Flower Size (mm) | Tetrad (%) | Uninucleate (%) | Binucleate (%) | Viable Microspore (%) |
|---|---|---|---|---|
| 10 – 15 | 3.6 ± 2.5 b | 96.4 ± 2.2 a | 0.0 ± 0.0 a | 78.8 ± 4.3 a |
| 16 – 20 | 1.9 ± 2.9 ab | 97.3 ± 2.9 a | 0.9 ± 1.2 ab | 91.3 ± 2.3 b |
| 21 – 25 | 0.0 ± 0.0 a | 96.7 ± 3.2 a | 3.2 ± 3.1 b | 80.8 ± 4.6 a |

Values followed by the same letter in a column showed no significant difference at a 0.05 level with the Duncan's Multiple Range Test.

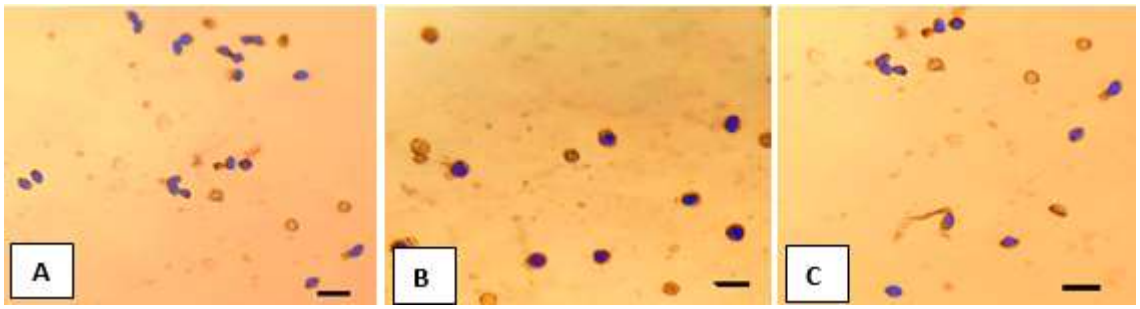


**Figure 2.** Iodine viability test from isolated microspore of hermaphrodite papaya flower, with flower size: A) 10-15 mm, B) 16-20 mm, and C) 21-25 mm (Bar = 100 µm).

35 °C for seven days (Table 2). The explants responsive to 2,4-D and TDZ treatments formed callus at 15–38 days after planting. The lowest percentage of anther forming callus (9.3%) appeared with the treatment of 2,4-D (0.1 mg $L^{-1}$) and TDZ (1.5 mg $L^{-1}$). However, the highest percentage of anther developing callus (20.4%) emerged with the treatment of 2,4-D (0.1 mg $L^{-1}$) and TDZ (0.5 mg $L^{-1}$) at 14 weeks of culture initiation. The interaction of the 2,4-D and the TDZ presented significant differences in the percentage of anthers' formed callus.

In papaya cv. Caliso anther culture, three types of calli developed. The type 1 was a friable callus, yellowish and slightly watery. Type 2 was a white-compact callus like cotton. Type 3 was a brownish yellow-compact callus. A callus with a friable, yellowish-white structure can easily separate, and the callus parts with a globular structure were most probably embryogenic callus (Figure 3A). Meanwhile, a non-embryogenic callus with a compact structure was brownish and wet (Figures 3B, 3C). Therefore, type 1 callus was the option chosen as the material for

**Table 2.** Effects of 2,4-D and TDZ on callus induction and embryogenic callus formation on papaya cv. Caliso anthers at 14 weeks of culture initiation.

| 2,4-D (mg $L^{-1}$) | TDZ (mg $L^{-1}$) | Anthers forming callus (%) | Callus formation time (days) | Embryogenic callus (%) |
|---|---|---|---|---|
| 0.1 | 0.5 | 20.4 ± 1.7 f | 15.9 ± 1.3 a | 58.7 ± 16.9 b |
| 0.1 | 1.0 | 15.7 ± 1.4 d | 17.5 ± 1.6 a | 30.1 ± 5.7 a |
| 0.1 | 1.5 | 9.3 ± 0.8 a | 37.9± 2.4 b | 33.6 ± 11.8 a |
| 0.3 | 0.5 | 18.4 ± 0.8 e | 14.6 ± 1.1 a | 33.1 ± 8.3 a |
| 0.3 | 1.0 | 12.3 ± 0.8 b | 15.5 ± 2.0 a | 34.4 ± 11.2 a |
| 0.3 | 1.5 | 14.3 ± 0.8 c | 16.5 ± 1.2 a | 28.4 ± 4.3 a |

Values followed by the same letter in a column show no significant difference at a 0.05 level with the Duncan's Multiple Range Test. Value ± SE. A total of 100 anthers were in each treatment.

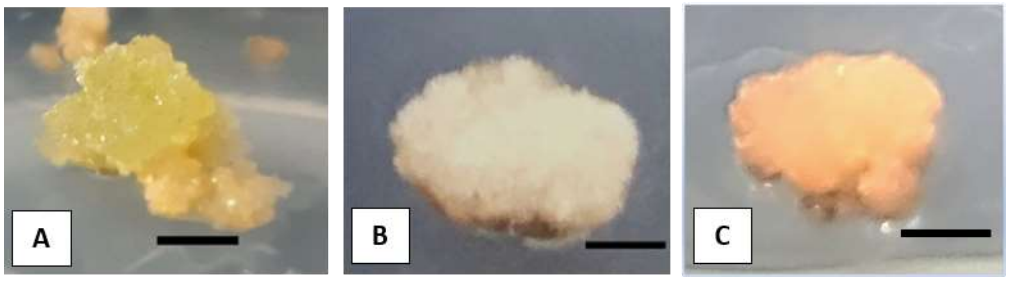


**Figure 3.** Types of callus on papaya cv. Caliso from anther culture. A) friable, brownish white, and slightly watery, B) compact and white like cotton, and C) compact, brownish yellow, and slightly watery (Bar = 3 mm).

experiment of somatic embryo induction. In this research, the concentration of 2,4-D and TDZ significantly affected the formation of callus type 1 (embryogenic callus). The highest proportion of embryogenic callus (58.7%) resulted in the treatment combination with 2,4-D (0.1 mg $L^{-1}$) and TDZ (0.5 mg $L^{-1}$) (Table 2).

**Induction of globular somatic embryo**

The friable, and yellowish-white calli, obtain ed from the previous experiment (Figure 3A), reached culturing in a treatment medium for somatic embryo induction. The used MS medium had supplements with a combination of NAA (0.1, 0.5, and 1.0 mg $L^{-1}$) and CPPU (0.01, 0.1, and 0.5 mg $L^{-1}$). The interaction of NAA and CPPU significantly affected the percentage of embryogenic callus-forming globular phase somatic embryos. The treatments NAA (0.1 mg $L^{-1}$) and CPPU (0.5 mg $L^{-1}$) were the best plant growth regulators (PGRs) combination for developing globular phase somatic embryos, with an average friable calli (70%), forming globular embryos at 20 weeks of culture initiation (Figure 4).

The globular somatic embryos further developed onto green structure. The treatments NAA (0.1 mg $L^{-1}$) and CPPU (0.5 mg $L^{-1}$) were the best medium for developing globular embryos, with an average of 38.8 at 20 weeks of culture initiation (Figure 5). The embryogenic structure changes color from brownish/yellow to brownish white, then becomes greenish. The embryogenic callus, which forms somatic globular embryos, displayed the formation of round nodules. Visual observations showed the production of globular phase of somatic embryos. In the presented experiment, the globular embryo did not clearly develop into mature somatic embryos through the heart, torpedo, and cotyledon stages of development.

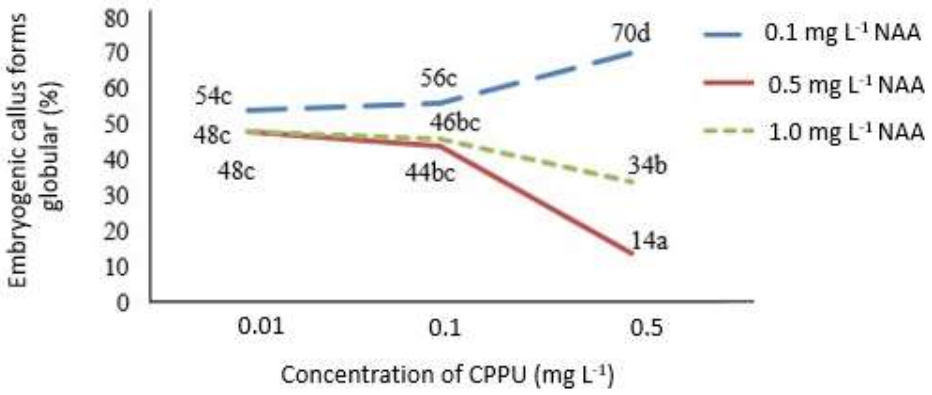


**Figure 4.** Percentage of embryogenic callus that formed globular phase somatic embryos at 20 weeks of culture initiation.

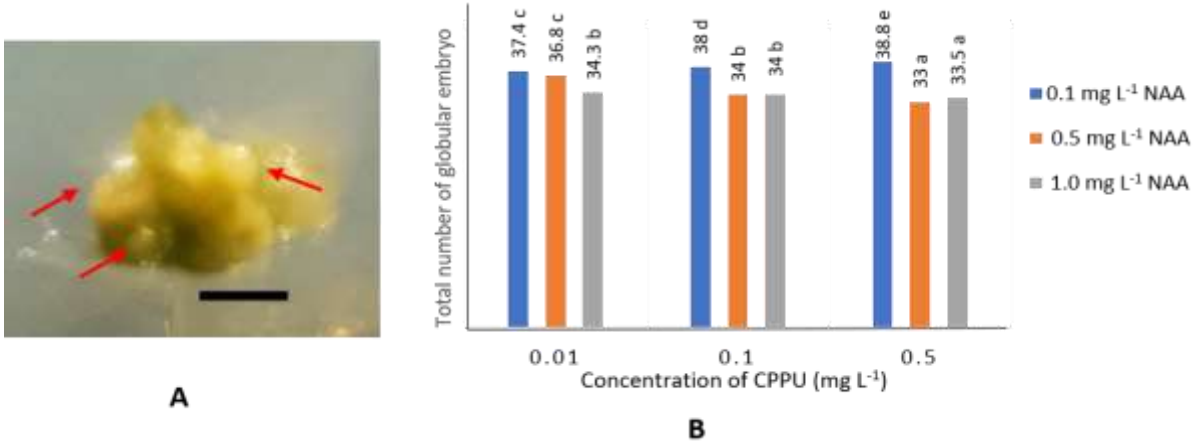


**Figure 5.** A) Globular embryos resulting from anther culture on somatic embryo induction medium (Red arrows: globular embryo; Bar = 5 mm) and B) Total number of globular phase somatic embryos at 20 weeks of culture initiation.

## DISCUSSION

Successful in vitro androgenesis depends upon various factors, such as, genotype and physiological state of the donor plant, stage of microspore development, culture medium, and the pre-treatment. The uninucleate and binucleate stages were the microspore stages found most responsive. These stages induced the microspores to convert from gametophyte development to sporophyte. This conversion results in the formation of embryos and calluses. Induction of embryogenic callus requires cellular dedifferentiation and repression of gametophyte developmental pathways. The description of microspore transcriptome was more similar to that of a cell suspension than a mature microspore. The uninucleate stage has a transcriptional status that was still proliferative and not fully differentiated (Honys and Twell, 2004). At the uninucleate stage, the microspores occurred more receptive to transitioning into the sporophyte development pathway than the more mature gametophyte tissue (Bhowmik *et al.*, 2011). In the late binucleate stage until maturity, microspores begin to accumulate storage reserves, causing them to lose their embryogenic capacity and follow the gametophyte development pathway (Ibrahim

*et al*., 2014). The responsiveness and conversion process can also gain influences from several treatments (Shumilina *et al*., 2020).

Microspore development showed positive correlation with flower size. The larger the flower size, the more mature the microspore development stage (Chong-Perez *et al*., 2018). The study results established most microspores were in the uninucleate stage in flower buds, ranging from 10 to 25 mm in length. However, the late uninucleate microspore stage had a larger vacuole size, thus, pushing the nucleus to the peripheral side (Purnamaningsih *et al*., 2024). Reports of these uninucleate microspores have stated as those in the most responsive state to induce the production of embryogenic or organogenic calli in several plant species, such as, *C. papaya* L. (Chong-Perez *et al*., 2018), *c*itrus (Cimo *et al*., 2016), and *O. sativa* L. (Mayakaduwa and Silva, 2021).

For estimating the viability of microspores, the staining methods are a widely used technique. The typically used dyes include acetocarmine, carmine, aniline blue, and IKI (iodine). One of the dyes, namely, iodine, served to detect the presence of sugar/starch content. Viable microspores were characteristics of the ability of the microspores to form the pollen grains, ably absorbing the dyes. Pollen grains function as the carriers of male gametes to the embryo sac, and the viable microspores showed the good cells' activity. Non-viable microspores also resulted from the microspores unable to create pollen grains, and the length of the pollen grains was less than the diameter of the microspore, thus, unable to absorb the dye properly (Sari *et al*., 2010). Microspore viability testing aims to determine the percentage of microspores with good metabolic activity (Indrianto *et al*., 2014). The size and viability of microspores play an important role in ensuring the microspores can take up nutrients from the culture medium and then proliferate (Mulyana *et al*., 2023).

In the presented study, the pretreatment of anthers with high temperature (35 °C) for seven days induced the embryogenic callus. A high temperature (35 °C) was more effective in inducing embryogenic callus than the lower temperature (25 °C) in papaya (Gyanchand *et al*., 2015). Callus induction also incurred influences from the growth regulator substances auxin (2,4-D) and cytokinin (TDZ), which play a vital role in cell division and elongation, and their activity causes callus formation. Providing auxin 2,4-D in the medium is crucial in inducing genes to respond to stress conditions, which activates signal transduction; hence, cells can express the genes needed to induce callus (Feher, 2015). The 2,4-D was a widely used PGR to induce the embryogenic callus, which can develop into somatic embryos (Cipriano *et al*., 2018; Juzon *et al*., 2022). The determination of callus initiation speed resulted from the initial response of the explant to the provided growth regulators. The almost equal levels of exogenous TDZ and 2,4-D seemed to be the trigger of earlier explant dedifferentiation, and the callus formed earlier. The higher the concentration of TDZ combined with 2,4-D would slow down the callus formation (Lizawati *et al*., 2023).

In papaya, the embryogenic callus, as characterized by a crumbly callus structure with yellow color, had a considerable lower growth rate than the non-embryogenic callus (Heringer *et al*., 2013). In contrast, non-embryogenic callus showing a milky white to brownish yellow color, wet and soft, had a compact callus structure, making it difficult to distinguish (Alcantara *et al*., 2014). Generally, the embryogenic callus morphology included an obvious white to yellowish color, which showed a shiny and friable structure. Past findings also disclosed somatic embryos development correlated with embryogenic callus having friable structures and comprising dark-colored cells (Rivai *et al*., 2014).

The previous research also showed there were four types of callus in papaya cv. Caliso culture using zygotic embryo explants. These comprised type 1 (compact and brownish yellow callus), type 2 (compact and white callus like cotton), type 3 (friable, yellowish white and slightly watery), and type 4 (friable, brownish yellow and slightly watery) (Lolliani *et al*., 2021). Variations in the calluses structure induced from explants were notable.

However, this study only observed three types of calli, i.e., 1, 2, and 4 from the anther culture. The differences in the explant used might have caused the differences in the type of callus formed. Callus induction commenced by swelling on the anthers' surface. Following from this, the anther wall showed amorphous structure, and finally, the callus mass covered the whole anthers. The growing callus develops from within the anther after the anther wall swelled and ruptured. This indicates the callus originated from the microspores within the anther (Zulkarnain, 2014; Sudirga *et al*., 2022).

The embryogenic callus forming somatic embryos was in the globular phase, which was the initial stage of somatic embryo development. Globular phase somatic embryos can develop to shape the mature somatic embryos through the heart, torpedo, and cotyledon phases. The PGR treatments used for embryogenesis of anther culture may interact with several other factors, such as, plant genotype, tissue type and development, culture environmental conditions, concentration, and types of endogenous plant hormones (Zur *et al*., 2015). Auxin treatment alone or in combination with cytokinin can also be effective for induction and initiation of somatic embryogenesis (Tanida and Shiota, 2019). The use of cytokinin as PGRs causes reduced embryogenic callus formation (Saenz *et al*., 2010). However, the use of combination of high concentrations of cytokinin with low auxin can encourage the induction of somatic embryos (Gyanchand *et al*., 2015). The cytokinin CPPU proved successful in inducing embryogenesis (Zhang *et al*., 2005; Aalifar *et al.,* 2019; Heedchim *et al*., 2020).

Previous research enunciated the best PGRs for somatic embryo induction was the combination of NAA (0.1 mg $L^{-1}$) and CPPU (0.1 mg $L^{-1}$) (Rimberia *et al*., 2006), with the present result also authenticating the best PGRs combination was NAA (0.1 mg $L^{-1}$) and CPPU (0.5 mg $L^{-1}$). The difference in optimum CPPU concentration for somatic embryo induction could be because of the differences in the genotypes and the flower types. With the NAA and CPPU treatment, the development of embryogenic callus showed the presence of green spots on the embryonic callus. CPPU, substituted phenylurea compounds, was considerably more effective in promoting in vitro morphogenesis than several other cytokinins in plants (Huang *et al*., 2015). CPPU remarkably promoting nodular induction was evident on the surface of the embryogenic callus and differentiation from the callus; but, the combination of CPPU and NAA showed negligible effect on morphogenesis (Sudha *et al*., 2015; Xu *et al*., 2022).

## CONCLUSIONS

In papaya cv. Caliso, the anthers of hermaphrodite flower buds (10–25 mm) can be applicable for embryogenic callus induction because these buds contained a high percentage of uninucleate stage of microspores (>95%), while the flower buds measuring 16–20 mm have the highest percentage of viable microspore (91.3%). In papaya cv. Caliso, for embryogenic callus induction, the best treatment combination of PGRs was 2,4-D (0.1 mg $L^{-1}$) and TDZ (0.5 mg $L^{-1}$) in MS medium, resulting in 58.7% embryonic callus. The friable callus (70.0%) emerged from the combination of NAA (0.1 mg $L^{-1}$) and CPPU (0.5 mg $L^{-1}$) treatment and produced the most globular embryos, with an average of 38.8.

## ACKNOWLEDGMENTS

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