January 2022

THE SECOND INTERNATIONAL CONFERENCE ON GENETIC RESOURCES AND BIOTECHNOLOGY: Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture

Committees: The Second International Conference on Genetic Resources and Biotechnology

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Preface: The Second International Conference on Genetic Resources and Biotechnology

The Second International Conference on Genetic Resources and Biotechnology, which is the continuation of the first event held in 2018, focuses on topics related to advances in biotechnology to create more opportunities for effective conservation and sustainable utilization of genetic resources for food and agriculture. This year conference's theme is Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture. The conference was organized by Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture, Indonesia, in collaboration with Indonesian Biotechnology Consortium and held on 24th-25th of May 2021virtually due to the pandemic of COVID-19.

The conference aims to share and exchange current scientific information and technological developments on biotechnology and their applications for conservation and sustainable use of genetic, to encourage and promote quality, efficiency, and modernization of management and utilization of genetic resources, and to facilitate national and international collaboration among participants. There are five scopes discussed in this conference. They are effective management of conservation and sustainable use of genetic resources for food and agriculture, application of genomics and molecular markers for genetic resource conservation and crop adaptation to climate change, application of innovative crop improvement techniques for conservation and sustainable use of plant genetic resources for food and agriculture, plant cell and tissue culture for conservation and effective utilization of genetic resources, and the use of microbial genetic resources as biological control agents of agricultural pests and diseases, and for soil bioremediation.

Five speakers from the United States of America, Japan, India and Indonesia were invited to discuss about their expertise and knowledge on relevant subjects in the plenary sessions. This conference was attended by more than 100 participants including 75 presenters and 44 listeners worldwide. They came from diverse governmental, private, or academic institutions and also scientific communities. The presented materials have undergone peer review processes and only qualified papers were selected. Furthermore, all papers were subjected to double blind peer-review and expected to meet the scientific criteria of significance and academic excellence to be published in a conference proceedings indexed in a well-known, reputable service.

We would like to express our sincere gratitude to our speakers, presenters and all participants for their contributions in this conference. We would also like to express our appreciation for the generosity of our sponsors that support this conference: PT CropLife, PT ITS Science Indonesia, PT Fajar Mas Murni and PT Prima Instrument Analitika. Lastly, special thanks to all committee members for their exceptional work and contributions in the conference and publication.

Chair of Organizing Committee

Dr. Toto Hadiarto

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Sucrose and putrescine increased callus induction in tomato anther culture

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Iswari Saraswati Dewi, Imam Nur Kholis, Bambang Sapta Purwoko, et al.

Sucrose and Putrescine Increased Callus Induction in Tomato Anther Culture

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Abstract. Androgenesis induction through anther culture technique is one of supplementary tools to accelerate selection program in classical breeding. Doubled-haploid (DH) derived-anther culture plants can be readily selected due to their high homozygosity. In this study, the effect of putrescine and various concentrations of sucrose in callus induction medium were investigated in anther culture of a tomato. Flower buds (4–6 mm) of a hybrid tomato cv. Permata containing anthers (1.5–2.5 mm) were used as anther donor material. The anthers were cultured on DBM1 callus induction medium containing sucrose at a concentration of 2.0%, 4.0%, 6.0%, and 8.0% with and without putrescine. The results showed that the onset of callus formation was affected by the concentration of sucrose, while the duration of callus formation was affected by the addition of putrescine into callus induction media. However, the number of calluses formed differed significantly with the concentration of sucrose and putrescine. The best medium to prolong anther viability and increase callus induction was DBM I + kinetin 5 mg/l + NAA 2 mg/l + 10^{-3} M putrescine + 2% sucrose.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.; 2n = 2x = 24) is an important vegetable crop only second to potato around the world, due to its unmatched culinary uses as well as its nutritive value [1, 2]. Tomato varieties consisted of inbred and hybrid varieties. However, commercially in the field and especially in the greenhouses, tomato is mainly produced via seeds of F_1 varieties possessing genes of interest derived from crossing between two homozygous parents for characteristics, such as high yield, resistance to diseases, ability to thrive and produce fruits under unfavorable conditions or in out-of-season production schemes in greenhouses [3]. In fact, the tomato is an important example of self-pollinated vegetables where heterosis is being exploited on a commercial level [4].

The production of inbred parental lines is the first prerequisite and one of the most difficult steps of F_1 hybrid variety production because it takes several generations of selfing or inbreeding through conventional breeding programme. Therefore, F_1 hybrid breeding requires a long time and lots of effort, making hybrid varieties expensive. Like any other self-pollinated crops, tomato variety development may take more than 10 years after at least 5 to 7 generations of selfing. Alternatively, haploidy can be used as a strategy to significantly reduce the time in the crop breeding process [5–7]. Haploids are sporophytes carrying the gametophytic chromosome number of their parents, thus subsequently the chromosome number of such haploids can be doubled to yield 100% of homozygous inbred lines [8]. Thus, after chromosome doubling, the doubled-haploid plants can be selected and used directly as parental lines in the production of F_1 hybrids [5, 9]. Therefore, utilization of haploid in tomato breeding program can be beneficial to accelerate the obtainment of pure lines [10].

In order to obtain haploid production in various plants, embryos and plantlets of microspore origin, known as androgenesis method, are usually attained through culture of anthers or isolated microspores [9, 11, 12]. Due to its easy procedure, the application of anther culture is widely used to obtain DH plants compared to isolated microspore

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[13–16]. However, tomato has been known as recalcitrant species in *in vitro* culture [10, 17]. The results of using anther or microspore culture in tomato have remained unsatisfactory because they rarely obtained shoots either directly or indirectly through callus formation [10, 18].

Generally, androgenesis through anther culture in the tomato started with a process where cell division occurred prior to haploid calli formation from the microspores within the anther locule which later differentiate into plants [17, 19]. Among several factors affecting the success of androgenesis, initiation of androgenesis depended upon the stage development of the microspores and chemical composition of the media including the source of carbohydrate and plant growth regulator [7, 18, 20]. A carbohydrate source is the most essential in anther culture because it regulates the splitting of anther lobes to expose and release the microspores into callus induction medium. Sucrose is considered an important and the most commonly used carbohydrate source in *in vitro* culture [21–23]. Plant growth regulator such as polyamines has been used to increase callus induction and plantlet regeneration in *in vitro* culture of several crops [5, 24, 25].

While the emergence of callus marked the first phenomenon arising of most plants in anther culture before plantlet regeneration, thus in this research the effect of putrescine and various concentrations of sucrose in callus induction medium were investigated in the anther culture of a tomato.

MATERIALS AND METHODS

Materials

Plant Material

A hybrid tomato cv. Permata was used as anther donor plant because of its ability to produce the highest number of callus among several varieties in a previous experiment by Ningsih *et al*. [18]. Tomato cv. Permata was adapted to low altitude and resistance to bacterial blight and *Tomato mosaic virus* (ToMV) [26]. The collecting time and surface sterilisation of plant materials, i.e. young flower buds, were conducted according to Ningsih *et al*. [18]. Flower buds were collected during 25 days from the beginning of flowering.

Culture Media

Callus induction medium (CIM) was based on DBM1 + kinetin 5 mg/l + NAA 2 mg/l, and designated as M1 [27]. Plant growth regulator (10^{-3} M putrescine) and sucrose in several concentrations were added to the CIM. The experimental treatment consisted of 8 callus induction media: M1 + sucrose 20 g/l; M1 + sucrose 40 g/l; M1 + sucrose 60 g/l; M1 + sucrose 80 g/l; M1 + sucrose 20 g/l + 10^{-3} M putrescine; M1 + sucrose 40 g/l + 10^{-3} M putrescine; M1 + sucrose 60 g/l + 10^{-3} M putrescine; M1 + sucrose 80 g/l + 10^{-3} M putrescine. Phytagel® (3 g/l) was used as a solidifying agent in all media.

Methods

Determination of Microspore Development Stage within the Anther

Young flower buds of different lengths (2–7 mm) were collected in the morning (7–8 AM). Five buds from each length were collected and put into an ice box containing ice cubes and carried into the laboratory immediately. The anthers were measured with a caliper.

The microspore development stages within the anther was checked under the digital microscope. This was performed by removing the anthers followed by squashing one or two anthers then stained with 2.0% acetic orcein [28].

Anther Culture

The research was conducted using completely randomized factorial design with 10 replications. The treatments consisted of callus induction media with and without putrescine (0 and 10^{-3} M) and sucrose concentration (2.0%, 4.0%, 6.0%, and 8.0%). Each experimental unit was one Petri dish containing 20 anthers from 4 flower buds. Flower buds (4–6 mm) containing immature anthers (1.5–2.5 mm) were surface sterilized by washing with tap water

followed by immersion in a 20% commercial bleach for 10 minutes and rinsed three times with sterilized distilled water. Sterilized buds were placed on filter papers and let them stand until properly dried prior to culture the anther.

Tomato anther culture method followed the protocol of Ningsih *et al*. [18]. The anthers were removed aseptically by dissecting the flower buds to remove the sepals, petals, and gynoecium. While dissecting, there should be no damage to the anthers. The isolated anthers (20 anthers from 4 flower buds) were placed onto a 6.0 cm × 1.5 cm Petri dish containing 10 ml of callus induction medium and incubated in the dark at 24±2°C for 2 months. Observation was conducted on the onset of callus formation, duration of callus production, and number of callus produced. The changes in callus color were observed for 2 months after all ≤2 mm size calli were transferred to fresh medium and incubated in the light at 24±2°C under 50 μmol/m^2s light provided by a cool white fluorescent lamp for a 16/8 hours photoperiod.

Data Analysis

Statistical analysis was performed using variance analysis. Square root transformation was applied to normalize data. The differences between treatments were tested by Duncan's Multiple Range Test (DMRT) at α = 5%. Data analysis was carried out using the Statistical Tool for Agricultural Research (STAR) version 2.0.1.

RESULTS AND DISCUSSIONS

Microspore Development Stage within the Anther

The developmental stage of microspore or pollen grain is crucial in affecting the success of anther culture [5]. Therefore, the relative frequency of each of the stages of microspore development within the anther should be determined since microspores in the anthers are at various stages of development. The observation on the tomato cv. Permata showed that the flower buds length of 2.0 mm to 7.0 mm, containing anthers of approximate length of 0.5 mm to 3.2 mm, covered the different stages of microsporogenesis between meiosis and mid-stage of unicellular microspore (Fig. 1). The optimal microspore stage for callus induction through anther culture in tomato was varied depending on the anther-donor genotypes. Many researchers working in anther culture have stated that the meiocyte could be used as a starting point where normal anther development can be interfered with in order to induce callogenesis, for example was prophase I [29], tetrad phase [30], metaphase I to telophase II [31], and telophase II to unicellular phase [10].

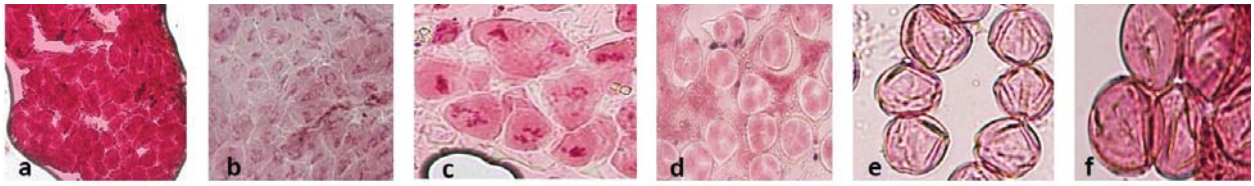

FIGURE 1. Microspores development in anther of tomato cv. Permata flower buds. (a–c) Meiosis stage at flower buds of 2.0 mm, 3.0 mm, and 4.0 mm, respectively. (d) Tetrad phase at flower buds of 5.0 mm. (e, f) Unicellular young microspore at flower buds of 6.0 mm and 7.0 mm, respectively.

Adhikari and Kang [32] found that there was correlation between flower buds size and anther length parameters. However, the strongest correlation occurred between anther length and microspore development indicating that this parameter was more suitable to predict the microspore developmental stage than the floral bud size parameter. Therefore, in this research 4.0 mm to 6.0 mm flower buds containing anthers with the length at 1.5–2.5 mm were chosen to be used as anther donor for anther culture of tomato cv. Permata because they represented the optimal microspores stage for anther culture in several other crops [10, 33].

In vitro Response of Cultured Anthers

After a few weeks of culture, white and friable callus masses could be observed to emerge from the lobes of swollen anthers (Fig. 2). Callus formation appeared in all media. At the beginning, callus was white in color and friable (Fig. 2a) and then turned slightly yellow in color and more compact in appearance (Fig. 2b and 2c). All calli turned to brown when reached more than 2 mm size in the callus induction media (Fig. 2d). This suggested that the calli approximately 2 mm in size should be immediately transferred to regeneration medium. According to Dewi *et al*. [34] in rice anther culture, the small calli as small as 2 mm in diameter should be immediately transferred to increase plant regeneration.

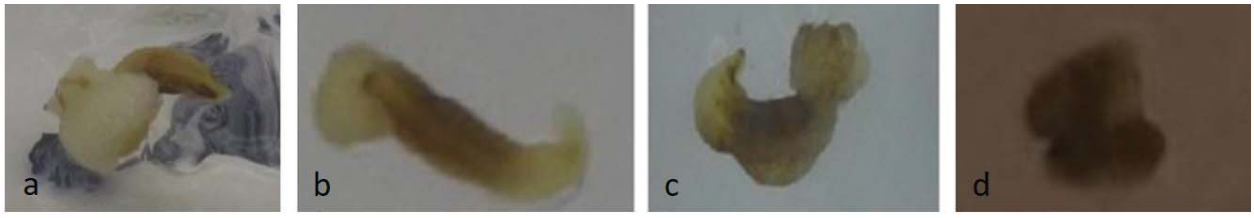

FIGURE 2. Callus appearance in callus induction medium. (a) White in color, friable. (b, c) Yellowish in color, compact. (d) Brownish in color, compact.

The analysis of variance revealed significance of mean squares due to sucrose treatments on the onset of callus induction, putrescine treatment on the duration of callus formation, and their interaction on the number of callus formation (Table 1). The results indicated that the onset of callus formation was affected by concentration of sucrose, while the duration of callus formation was affected by the addition of putrescine into callus induction media. However, the number of calli formed was differed significantly with both concentration of sucrose and putrescine.

TABLE 1. Analysis of variance for the onset of callus formation, duration of callus production, and number of callus in tomato cv. Permata anther culture.

Source of variance	Mean square values		
	Onset of callus formation	Duration of callus production[a]	Number of callus[b]
Sucrose	6.0**	1.1	0.3
Putrescine	2.7	16.2*	0.3
Sucrose × putrescine	0.5	4.8	1.0*

[a]The data were transformed by using √x+1. [b]The data were transformed by using √x+2. *Significant at $P<0.05$. **Significant at $P\leq0.01$.

The fastest callus formation occurred in callus induction media with 6% sucrose at 20.6 days after anther inoculation (DAI). However, this is not significantly different with 2% sucrose treatment which reached 21.4 DAI (Table 2). This result is better than that of Ningsih *et al*. [18] when the onset of callus formation from anther culture of the same cultivar of tomato took longer, i.e. 27.7 DAI. Previously, other research, using tomato cv. Roma [35], cv. Maya, cv. Rio Grande, and T-55 [36], observed that the onset of callus formation ranged from 30–50 DAI. The mechanism that regulates the splitting of anther lobes heavily depends on the osmotic pressure [24]. In rice anther culture a high level of carbohydrate source in the callus induction media (6.0–6.5%) is required [23]. However, in this research, even a low level of sucrose (2.0–4.0%) in callus induction medium can cause the osmotic pressure needed for splitting the anther lobes of tomato cv. Permata.

TABLE 2. Effect of different sucrose concentration on the onset of callus formation in callus induction media of tomato anther culture.

Sucrose (%)	Onset of callus formation (DAI)
2	21.4 b
4	30.6 a
6	20.6 b
8	29.4 a

DAI = days after inoculation of the anther. Numbers in the same column followed by the same letter are not significantly different by DMRT at $P \leq 0.05$.

The addition of 10^{-3} M putrescine greatly prolongs the duration of callus formation in callus induction media compared to those without putrescine (Table 3). Similar finding from Dewi and Purwoko [37] showed the addition of putrescine in the anther culture of *indica* rice did not only delay senescence process of the anther so that callus production may prolong, but also improved green plant regeneration. Putrescine as one of the main polyamines has potent biological activity in inhibition of ethylene, a growth hormone that causes senescence [25, 38].

TABLE 3. Effect of the addition of putrescine on the duration of callus production in callus induction media of tomato anther culture.

Treatment	Duration of callus production (DAI)
Without putrescine	33.1 b
With 10^{-3} M putrescine	43.7 a

DAI = days after inoculation of the anther. Numbers in the same column followed by the same letter are not significantly different by DMRT at $P \leq 0.05$.

Observation on callus formation showed that sucrose and putrescine interaction significantly affected the number of callus (Table 4). The media without putrescine gave an average number of callus in the range of 1.9 to 3.5, while in media with putrescine the average number of callus ranged from 3.1 to 4.8. The best callus induction medium to increase the number of callus was DM1 + kinetin 5 mg/l + NAA 2 mg/l + 10^{-3} M putrescine + 2% sucrose, although statistically not a difference from those with 6% sucrose. Sucrose was added to regulate the osmotic pressure in callus induction media, stimulate cell dedifferentiation during callus induction and prevent callus formation from somatic anther tissue with respect to the number of embryo-like structure, while polyamines, such as putrescine, spermidine and spermine, have been applied in anther culture of food and horticulture crops in order to prevent anther senescence, increase the number of callus formation and microspore-derived embryo [24, 25, 39].

TABLE 4. Sucrose and putrescine interaction on the number of callus in callus induction media of tomato anther culture.

Sucrose (%)	Putrescine (M)	
	0	10^{-3}
2	1.9 b	4.8 a
4	3.5 a	3.1 bc
6	3.1 ab	3.6 ab
8	3.1 ab	1.9 c

Numbers in the same column followed by the same letter are not significantly different by DMRT at $P \leq 0.05$.

The changes in callus color, recorded at 4 weeks to 8 weeks, are presented in Fig. 3. The callus began to proliferate and become more friable and granulate in appearance after being transferred to fresh media. New callus budded from the old one and turned to green color after 4 weeks. However, the callus began to turn brown at 6 weeks. The last observation at 8 weeks showed that callus with black color were abundant. This observation indicated that after 4 weeks in the same media, the callus should be transferred to regeneration media to induce shoot formation. The delay in transferring the callus will affect plant regeneration and also hamper the normal development of the regenerated plantlet as has been stated previously by Ningsih *et al*. [18].

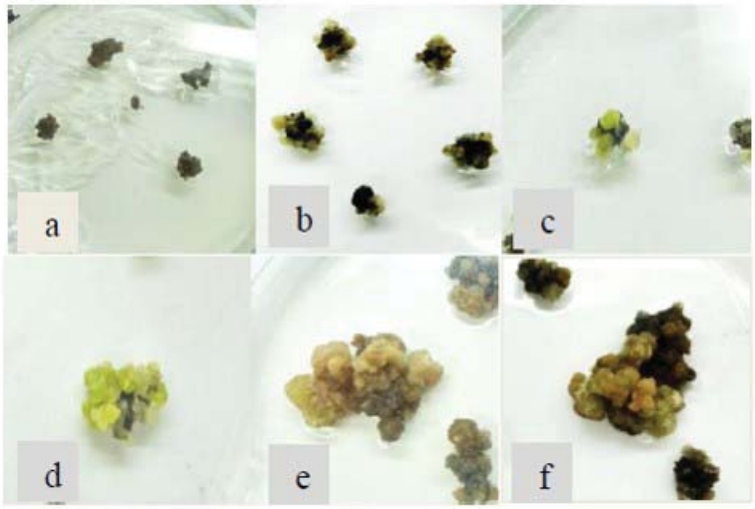

FIGURE 3. The appearance of calli after being transferred to fresh media and incubated under the light. (a) The calli began to proliferate after 2–3 weeks. (b, c) New callus emerged and some turned to green color at 4 weeks. (d) More friable callus with green color appeared after 4 weeks. (e) Calli at 6 weeks, some green color could be spotted, but more dark color were also spotted. (f) At 8 weeks, callus with dark color was abundant.

CONCLUSION

The onset of callus formation was affected by 2% and 6% sucrose concentration, but the duration of callus production was significantly longer with the addition of 10^{-3} M putrescine than without the addition of putrescine in the induction medium. The highest number of callus formation occurred in callus induction medium of DM1 + kinetin 5 mg/l + NAA 2 mg/l + 10^{-3} M putrescine + 2% sucrose, although statistically not a difference from those with 6% sucrose.

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