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Increasing Ploidy Level of Garlic (*Allium sativum* L.) "Tawangmangu Baru" *In-Vitro* Using Colchicine

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Abstract

"Tawangmangu Baru" garlic variety is known to have low productivity. The variety is still highly demanded due to its strong flavour and aroma; however, its production has not yet been able to fulfill the local needs of Central Java due to the small size and limited production area. This study aimed to determine the effect of concentration and time duration of colchicine treatment towards increasing the ploidy level of "Tawangmangu Baru" garlic variety for genetic variability. The experimental design used in this study was a complete randomized design with two factorials and 12 combinations. The first factor was concentration of colchicine, i.e. 0.00, 0.02, 0.04, 0.06, 0.08 and 0.10%, and the second factor was the immersion time, i.e. 24 and 48 hours. The result indicated that, 4.72% callus induction was obtained in BDS + 0.4 mg.L^{-1} 2,4-D + 2.0 mg.L^{-1} kinetin; and 4.0% callus proliferation were obtained in both BDS + 1.5 mg.L^{-1} 2,4-D + 1.0 mg.L^{-1} kinetin and MS +1.5 mg.L^{-1} 2,4-D and 1.0 mg.L^{-1} kinetin. The untreated plantlets showed higher mortality rate compared to the explants with 48 hours colchicine treatment. Higher number of shoots were recorded in 0.1% colchicine at 48 hours and lower shoots in 24 hours, whereas 0.1% colchicine at 24 and 48 hours showed the highest ploidy level of total nuclear DNA analyzed by flow cytometry. The genetic diversity of the "Tawangmangu Baru" garlic was successfully enhanced by colchicine and immersion treatment. Mutant lines with tetraploid and mixoploid plants were obtained. The putative lines obtained at 0.1% colchicine treatment were subcultured to produce new mutants before testing the phenotype. The application of colchicine at 24 and 48 hours treatment improved the genetic potential of "Tawangmangu Baru" garlic variety *in vitro*. The application of colchicine increased the ploidy level and an increase in ploidy is expected to make the bulb size larger. Larger tuber size will increase the tuber weight, and also the overall garlic productivity and production in the future.

Keywords: ploidy induction, 2,4-D, kinetin, colchicine, "Tawangmangu Baru"

Introduction

The production of garlic has not been able to meet the demand for food consumption in Indonesia and causing a considerable gap between consumption and domestic production (Agustina, 2019). In Indonesia, the production of garlic is limited by lack of farmers' interest on garlic farming, low prices of imported garlic, bigger clove size of imported garlic, viral diseases (Putra et al., 2015), narrow land area, high production costs, low quality of garlic seeds, high garlic consumption demand (Pharmawati and Waitiani 2013) and less competency in producing garlic with large bulb size (Nur, 2018). These constraints have led to a limited quantity of garlic in Indonesia. To fulfill the national garlic demand, the Indonesian government has been importing a large amount of garlic from China (Pharmawati and Waitiani, 2013; Nur, 2018). Garlic import in Indonesia was 94% in 2018 and 95% in 2019 due to the low price of imported garlic and reduction of importing rules of 2005 in line with the ASEAN Free Trade Area (AFTA) process (Kementerian Pertanian, 2019b; Agustina, 2019).

'"Tawangmangu Baru"' is a Javanese local garlic cultivar commonly planted in the highlands of the Tawangmangu, Central Java known by its strong flavor, low yield, good growth, and robust stems (Arisuryanti et al., 2018). The variety is grown at 1,000 meter above sea level (m.a.s.l.). It has a purplish-white clove which is suitable for cooking due to its strong aroma. The variety is widely planted in the areas of Tegal, Wonosobo, Banjarnegara, Temanggung, Magelang, Magetan, and Karanganyar (Harinta et al. 2018). The plant height reaches 60 to 80 cm and its harvesting time is in between 110 to 140 days. The bulb of the "Tawangmangu Baru" garlic variety is characterized by its oval shape, hard neck, tapered tip with a flat

base. The variety has the potential to produce about 8-12 tonnes per hectare (Samadi, 2000). The current national yield of "Tawangmangu Baru" in Indonesia is 8.7 tonnes/hectare, while the yield of garlic in China is 27 tonnes/hectare (Kementrian Pertanian, 2019a). Increasing the production of "Tawangmangu Baru" can be used to fulfill the local demand of the region and other provinces in Indonesia (Harinta et al., 2018; Kementrian Pertanian, 2019b).

Vegetative propagation of garlic offers limited chances for creating genetic variation and gives a low multiplication rate in the field. Due to the floral organ abortion and chromosomal abnormalities, pollination it is difficult to happen naturally in garlic, hence the crop is reproduced asexually, and garlic breeding mainly depends on the clonal selection and mutation breeding (Su-ping et al., 2014; Tchórzewska et al., 2017; Cheng et al., 2012; Mahajan et al., 2015).

Colchicine is an alkaloid and secondary metabolite extracted from the plant Colchicum autumnale which is the most widely used doubling agent either in garlic or other species (Alan et al., 2004; Alan et al., 2007) with an average doubling efficiency of 70% (Jakše and Bohanec, 2003). Colchicine is a chemical mutagen *senso latu*, as its main effect is on ploidy and not on genes (Spencer-Lopes et al., 2018). Colchicine affects chromosome doubling on mitosis cells by binding to the microtublin positive end leading to the disruption of the polymerization of microtublin (Roughani et al., 2017; Zhang et al., 2016; Moghbel et al., 2015). The spindle fibers consisting of microtubules used to pull the sister chromatids to opposite poles of the cell. However, without the action of spindle fibers, the mitotic process is disrupted. This disruption results in DNA replication without cell division (Roughani et al., 2017). Wei et al. (2007) reported that the increased number of chromosomes brings the change in plant morphology and functions. According to many studies, colchicine is the most effective mutagen for producing polyploid plants, but the responses to colchicine differ depending on the plant genotype (Heo et al., 2016). Colchicine is effective to induce ploidy in plant genome at a concentration of 0.001% - 1% in a period of 6–72 hours *(*Amanah et al., 2016). Ploidy induction of diploid garlic genome can be induced by treating garlic stem discs with up to 0.75% colchicine with higher duplication at 0.5% and lower duplication rate at 0.75% due to higher toxicity (Dixit and Chaudhary, 2014). Polyploidy has been induced by colchicine in several crops (Datta, 1990). Polyploid plants retain long stomata (Münzbergová, 2017), larger and thicker leaves (Jaskani et al., 2005; Moghbel et al., 2015; Roughani et al., 2017; Sajjad et al., 2013), high chloroplast number (Jaskani et al., 2005), lower number of stomata compared to control (Sajjad et al., 2013), higher secondary metabolites and increased sugar composition (Corneillie et al., 2019; Dixit and Chaudhary, 2014) used in overcoming hybridization barriers, improving stress tolerance, and restoring fertility in wide hybrids (Roughani et al., 2017). Polyploid seeds are often larger than diploid seeds because of their larger cell size (Münzbergová, 2017; Tavan et al., 2015; Moghbel et al., 2015). Moghbel et al. (2015) described polyploidy induced by colchicine *in vitro* results in an increase in DNA content and affects the epidermal surface cells. Polyploids have thicker cell size but have low cell number per leaf blade as compared to the diploids (Corneillie et al., 2019). By using this technique, through induction of ploidy to obtain polyploidy plants, it is expected that tetraploid plants which have more higher morphology and larger tubers are expected to be obtained. The main objective of this research was to improve the genetic potential of "Tawangmangu Baru" garlic variety by using colchicine treatment at different concentration and time duration of immersions *in vitro* for inducing polyploidy. The application of colchicine helps to increase the ploidy level and an increase in ploidy is expected to make the bulb size larger in "Tawangmangu Baru" garlic variety. Larger tuber size will increase the tuber weight, and also the overall garlic productivity and production.

Materials and Methods

Planting Material

The research increasing ploidy level of "Tawang mangu Baru" garlic variety by colchicine *in vitro* was conducted in the Micro Technic Laboratory, Department of Agronomy and Horticulture from May 2019 to March 2020. The incubation culture room was arranged with temperature 22-24 ^{o}c with a 24 hours photoperiod per day from fluorescent lamps with 1000 lux light intensity. Bulbs of six-months-old "Tawangmangu Baru" garlic variety were purchased from Maju Karanganyar, Solo Farmers' Group. The explant preparation and sterilization process for planting was conducted through the procedures as described in Sinha et al. (2016), Khan et al. (2017) and Salam et al., (2018). Clumps of garlic bulbs were separated into individual clove during the start of the tissue culture process. The outermost layer of the bulbs was carefully peeled, washed with detergent three times followed by rinsing in 2.0 mg.L^{-1} of Dithane (a.i. Mancozeb) and Agrimycin (a.i. Oxytetracyline) for 20 hours. After the treatment by fungicide and bactericide, the surface of the cloves were washed with sterile water three times followed by sterilization with 50% of Clorox for the whole bulb, 30% for the half-disc, and 20% of Clorox for the explant (0.5 cm) at 30 minutes, 30

minutes and 20 minutes respectively. The bulbs were taken out and chopped using a sterile surgical blade after treated by 50% and 30% Clorox. Immediately after Clorox treatment, five explants per bottle were then cultured in prepared MS0. The size of the explants was measured about 0.5 cm length with intact meristem. These segments were used as explants for increasing the induction of polyploids. Explants were placed with the abaxial side on a 200 ml bottle composed of MS macro- and micronutrients, 30 $g.L^{-1}$ sucrose, 7 $mg.L^{-1}$ agar with pH 5.8. Five-hundred $mg.L^{-1}$ cefotaxime was used in the MS to control the fungal contamination of the media. After MS culture, the explants were induced at 0, 0.02, 0.04, 0.06, 0.08 and 0.10% concentrations treated at 24 and 48 hours for all treatments. After 24 and 48 hours of colchicine treatment, the MV0 garlic explants were cultured in complete BDS media and 0.4 $mg.L^{-1}$ 2,4-D for two months to produce callus. Due to zero formation of callus after MV0, the MV0 explants again were sub-cultured after two months interval for callus formation and regeneration in fresh BDS media supplemented with 2,4-D (0.4 $mg.L^{-1}$) and kinetin (2.0 $mg.L^{-1}$) and stayed for two months. The third sub-culture were performed for all explants in another fresh BDS media supplemented with 2,4-D (0.4 $mg.L^{-1}$), kinetin (2.0 mg. L^{-1}), and asparagine (25 $mg.L^{-1}$) to ensure the somatic embryogenesis for a month. After the 3rd sub-culture, all the explants were transferred into another fresh BDS and MS media with increased 2,4-D (1.5 $mg.L^{-1}$) and kinetin (1.0 $mg.L^{-1}$) levels to ensure the proliferation and regeneration of callus for polyploid analysis. For this research, two trials were performed to get a large number of calluses after the "Tawangmangu Baru" garlic variety failed to form callus in BDS + 0.4 $mg.L^{-1}$ 2,4-D in the first trial. The second trial also performed on BDS + 0.4 $mg.L^{-1}$ 2,4-D and 0.5 $mg.L^{-1}$ kinetin to get enough callus for shoot proliferation and regeneration for ploidy analysis. The cytological analysis was conducted at the Micro-technic Laboratory, Department of Agronomy and Horticulture, and flow cytometry analysis at LIPI Research Center.

The materials used in this research include Basal Dunstan Short (BDS) media, Murashige and Skoog 1962 (MS) media, sterile water, aqua desitillata sterile (distilled water with more quality), Dithane, Agrimycin, cefotaxime, rubber, micropipette with different sizes, measuring glass, 70 and 96% alcohol, volumetric flash, 15-mm Petri dishes, rectangular buckets, plastics wrap, shelves, growth chamber or a windowless room with environmental control. The tools used in tissue culture include; digital pH meter, micro-organism killing autoclave, pressurized autoclave, laminar airflow cabinet, sensitive balance, dissecting instruments (scalpels with removable blades, and forceps). Photos were conducted using flow cytometry (complete set of BD Accuri C6 Plus, Partec, Germany), bi-nuclear microscope, and digital camera to display the status of the cells and structures of callus.

Experimental Design and Analysis

The experimental design used in this study was a factorial completely randomized design with two factors and 12 treatments. The first factor was concentration of colchicine in percentage, i.e. 0.00, 0.02, 0.04, 0.06, 0.08 and 0.10%. The second factor was the time duration (immersion time) at 24 and 48 hours. In this research, there were four replications with 180 explants in each replication. ANOVA for each experiment and means were compared using the Fisher test ($p< 0.05$) through Minitab 2018 software. The LC 20%, LC 30%, and LC 50% of the dead plants were calculated by probit analysis in Minitab software. Callus induction and proliferation efficiency were performed through direct observation; while the nuclear DNA status in each putative line was analyzed through flow cytometry analysis.

Callus Induction and Proliferation

After the basal disc meristem grown in MS0 (MS basal medium without growth hormone) for a week, followed by colchicine treatment at 24 and 48 hours, the explants were cultured in BDS media supplemented with 0.4 $mg.L^{-1}$ 2,4-D for two months. After two months, all the explants were transferred into BDS media augmented with 0.4 $mg.L^{-1}$ 2,4-D and 2.0 $mg.L^{-1}$ kinetin to induce callus and organogenesis in two months. After four months from colchicine treatment, all the explants were sub-cultured into BDS media supplemented with 0.4 $mg.L^{-1}$ 2,4-D, 2.0 $mg.L^{-1}$ kinetin, and 25 m. L^{-1} asparagine for a month. After five months of *in vitro* culture, all the explants were transferred to another fresh MS and BDS mediums supplemented with 1.5 $mg.L^{-1}$ 2,4-D and 1.0 $mg.L^{-1}$ kinetin. The percentage of callus induction, shoot proliferation, regeneration, and the number of shoots was calculated based on the following formula:

$$\text{Callus induction (\%)} = \frac{\text{No. of plants produced callus per treatment}}{\text{Total No. of planted explants}}$$

$$\text{Callus proliferation (\%)} = \frac{\text{No. of callus proliferated per treatment}}{\text{Total no. of callus/treatment}}$$

$$\text{Shoot regeneration from callus (\%)} = \frac{\text{No. of explants produced shoot/treatment}}{\text{Total no. of proliferated callus/treatment}}$$

The number of surviving and dying plants in each treatment were counted every week after colchicine treatment. Data collection was taken every week to know the number of dying and surviving explants at different colchicine concentrations and time intervals.

The number of dead and survived explants as a result of colchicine treatment and immersion time were calculated through the method used by (Sajjad et al., 2013). The brown and non-growing explants were considered as dead explants, whereas, the live explants were considered as survived plants. To evaluate the number of dying and surviving plants at different time intervals, the data analysis and comparison for dying and surviving plants as a result of colchicine treatment were taken at 2, 4, 5, and 7 months.

Flow Cytometry Analysis

After the colchicine treated plants had grown for 60 days on BDS medium and were induced by colchicine at 24 and 48 hours in vitro, putative polyploids of plants were investigated by flow cytometry. Putative polyploid shoots were selected based on morphology, as they appeared to be thicker, darker and showed slow growth. Flow cytometric analysis was carried out using a BD Accuri C6 Plus (Partec, Germany). The different flow cytometer parameters were adjusted with untreated control to secure well defined and reproducible readings. Twenty-four shoots were selected from 12 treatments (0, 0.02, 0.04, 0.06, 0.08, and 0.10%) induced at 24 and 48 hours. From each treatment, 2 plants were detected by flow cytometry to know the total nuclear DNA status of the induced plants. The middle dark green part of the leaf of the plants was taken for sample preparation. The plant part taken for sample preparation was chopped manually through a razor blade approximately for 2 minutes to release the intact nuclei of the plant tissue. 0.5 cm^2 of leaf was taken from the middle part of the leaf to extract the nuclei of the plant tissue. The leaf sample was poured by 250 µl CyStain PI Absolute P (nuclei extraction buffer) to extract the intact nuclei representative of the cells within the plant tissues. After the tissues ground well, the solution was transferred to the puppet filtered by 30 µm filter size and added 300 CyStain PI Absolute P (staining buffer) for colouring. The staining buffer was prepared from 60 µl propidium iodide (a red fluorescent stain that preferentially intercalates the double-stranded DNA with 540 nm wavelength excitation and 615 nm emission), 20 µl CyStain PI Absolute P, and 60 µl Ribonuclease (RNase) to degrade the RNA molecule from the sample tissue as the target to study in the plant tissue was the nuclear DNA status of plant tissue. From the solution that contains propidium, staining buffer, and RNase, 300 µl solution was added to the extracted sample tissue for colouring of the plant tissues. Immediately after adding the staining buffer, the homogenized sample tissue was detected by BD Accuri C6 Plus flow cytometry (Partec, Germany) to determine the amount of nuclear DNA.

Result and Discussion

Callus Induction and Shoot Proliferation with 2,4-D and Kinetin Treatment

After the basal disc meristem grown in MS0 for a week followed by colchicine treatment at 24 and 48 hours, the explants were cultured in BDS media supplemented with 0.4 $mg.L^{-1}$ 2,4-D alone for two months (1st *in vitro* culture). After two months all the explants were transferred to BDS media augmented with 0.4 $mg.L^{-1}$ 2,4-D and 2.0 $mg.L^{-1}$ kinetin supposed to produce callus and organogenesis in two months. After four months from colchicine treatment, all the explants were transferred into BDS media supplemented with 0.4 $mg.L^{-1}$ 2,4-D, 2.0 $mg.L^{-1}$ kinetin, and 25 $mg.L^{-1}$ Asparagine for a month to ensure somatic embryogenesis. All the explants were unable to produce callus at 0.4 $mg.L^{-1}$ 2,4-D alone. There was no response of 2,4-D alone to callus production and organogenesis. 4.72% explants were produced callus in 0.4 $mg.L^{-1}$ 2,4-D and 2.0 $mg.L^{-1}$ kinetin combination within two weeks as shown in Figure 1. The produced calluses were embryogenic callus with white, friable, and transparent characteristics. However, the produced calluses were failed to proliferate into shoot and regenerate under these concentrations of growth hormones.

The meristematic basal discs of “Tawangmangu Baru” garlic variety induced callus in 0.4 $mg.L^{-1}$ (2,4-D) and 2.0 $mg.L^{-1}$ kinetin combination after the explants failed to induce callus in 2,4-D alone. Auxins and cytokinins in combination are effective in producing callus instead of auxins alone in “Tawangmangu Baru” garlic. However, at low concentrations of auxins (0.4 $mg.L^{-1}$) and kinetin (2.0 $mg.L^{-1}$), the induced callus were unable to proliferate and regenerate. Some calluses were produced somatic embryo; however, the produced somatic embryos were died due to the low concentration of 2,4-D after four months. After five months of *in vitro* culture, all the explants were transferred to another fresh complete MS and BDS mediums supplemented with 1.5 $mg.L^{-1}$ 2,4-D and 1.0 $mg.L^{-1}$ kinetin. After transferring the explants into these growth hormones, 4.0% of the failed callus to proliferate in 0.4 $mg.L^{-1}$ (2,4-D) and 2.0 $mg.L^{-1}$ kinetin, started to proliferate rapidly in BDS+1.5 $mg.L^{-1}$ 2,4-D + 1.0 $mg.L^{-1}$ kinetin and MS+1.5 $mg.L^{-1}$ 2,4-D + 1.0 $mg.L^{-1}$ kinetin after two weeks. The callus proliferation occurred very fast in BDS medium supplemented with 1.5 $mg.L^{-1}$ 2,4-D + 1.0 $mg.L^{-1}$ kinetin compared to MS medium supplemented with 1.5 $mg.L^{-1}$ 2,4-D + 1.0 $mg.L^{-1}$ kinetin. The development of a somatic embryo into a whole plant is likely affected by the non-optimal growth hormone or media composition during the callus growth. Somatic embryogenesis

can be affected by ethylene concentration, where low concentrations can stimulate, while high concentrations inhibit (Trigiano and Gray, 2004).

The Effect of Colchicine Treatment on Mortality and Survivability of Garlic "Tawangmangu Baru"

Based on the total number of plantlets, the colchicine treated plantlets at 24 and 48 hours have no significant difference in mortality and survivability compared to the untreated plantlets as indicated in Tables 2 and 3. The untreated plantlets showed a higher mortality rate compared to the treated explants at 48 hours. The explants induced at colchicine 0.08% for 24 hours also resulted in high mortality compared to the other treated explants. The high mortality rate in control plantlets and 0.08% at 24 hours was may be due to the genetic and growth medium condition

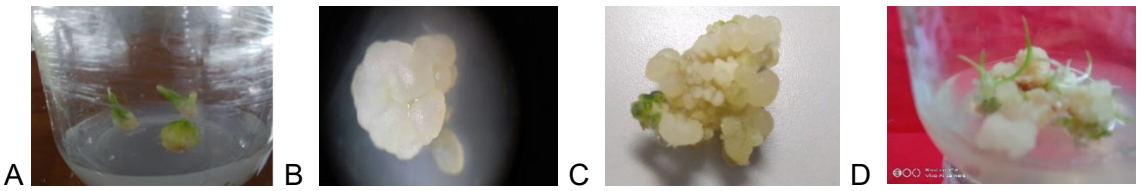

Figure 1. The effect of 2,4-D and kinetin on "Tawangmangu Baru" somatic embryogenesis; A) BDS + 0.4 mg. L^{-1} 2,4-D+ 0.06% colchicine after 3 weeks; B) BDS + 0.4 mg.L^{-1} 2,4-D + 2.0 mg.L^{-1} kinetin + 0.02% colchicine after two weeks; (C) BDS + 1.5 mg.L^{-1} 2,4-D + 1.0 mg.L^{-1} kinetin + +0.02% colchicine; (D) MS + 1.5 mg.L^{-1} 2,4-D+1.0 mg.L^{-1} kinetin after two weeks

Table 1. Callus induction on BDS medium supplemented with 2,4-D and kinetin after colchicine treatment of "Tawangmangu Baru" explant *in vitro* culture

Growth medium	Colchicine (%)	Callus induction (%)	Callus proliferation (%)	Shoot regeneration from callus (%)	No. of shoots (%)(direct without callus	No. of roots (%)
BDS + 0.4 mg.L^{-1} 2,4-D	0	0	0	0	100	0
	0.02	0	0	0	100	0
	0.04	0	0	0	100	0
	0.06	0	0	0	100	0
	0.08	0	0	0	100	0
	0.10	0	0	0	100	0
BDS+0.4 mg.L^{-1} 2,4-D + 2.0 mg.L^{-1} kinetin	0.00	0.80	0	0	100	0
	0.02	1.56	0	0	0	0
	0.04	1.56	0	0	0	0
	0.06	0.50	0	0	0	0
	0.08	0	0	0	100	0
	0.10	0.30	0	0	0	0
BDS+1.5 mg.L^{-1} 2,4-D + 1.0 mg.L^{-1} kinetin	0.00	0.50	0	0	100	0
	0.02	1.0	100	0	0	0
	0.04	1.0	100	0	0	0
	0.06	0.30	100	0	0	0
	0.08	0	0	0	100	0
	0.10	0.30	100	0	0	0
MS+1.5 mg.L^{-1} 2,4-D + 1.0 mg.L^{-1} kinetin	0.00	0.30	0	0	100	0
	0.02	0.50	100	0	0	0
	0.04	0.50	100	0	0	0
	0.06	0.30	100	0	0	0
	0.08	0	0	0	100	0
	0.10	0	0	0	0	0
Total (%)		4.72	4.00	0	95.30	0

Note: *The total percentage of induced callus, callus proliferation, shoot regeneration from callus, number of shoots, and number of roots that are produced in different growth mediums and growth hormones.

(stress), light, temperature, and other cellular and physiological phenotypic traits of the explants. The quick growth of untreated plants at the beginning of *in vitro* culture may lead to higher death as compared to the treated plants.

Based on the total number of survivability of the mutagenic plantlets, the colchicine treated plants survived well compared to the untreated plants. The mean of the colchicine treated plantlets was higher in all treatments except the control and the plantlets induced at 0.08% for 24 hours (Table 3). This showed that there was lower survivability in control diploid plants and slightly at higher colchicine treatment.

The Lethal Concentration (LC)

The LC 20%, LC 30%, and LC 50% were calculated at 2, 4, 5, and 7 months from colchicine treatment because the number of dying and surviving explants were observed after two months from colchicine treatment. The colchicine concentration needed for LC 20%, LC 30%, and LC 50% for the experiment at two and four months were 0.03%, 0.08%, and 0.16% respectively. There was no difference at two and four months for the lethal concentration. Whereas, at 7 months, the LC 20%, LC 30%, and LC 50% were 0.01%, 0.08%, and 0.2% respectively. There was a high mortality of plants at the early stages and lower mortality after four months at LC 20% due to the stability of the plantlets. However, there was high mortality of plants as the time increases from 2 months to 7 months *in vitro* culture at 0.1%.

In vitro plants had decreasing survival as the concentration of colchicine and *in vitro* culture increases with time. Colchicine treated explants can survive even at higher colchicine concentrations, but there is also a higher mortality of explants *in vitro* culture. This is also supported by Dixit and Chaudhary (2014), Amanah et al. (2016) that colchicine is effective in inducing diploid plants at a concentration of 0.001 - 1% in a period of 6–72 hours depending on the response of each plant species.

The untreated plants had a greater number of shoots compared to the treated plants at 24 hours (Table 5). However, a lower number of shoots were observed in untreated plants in 48 hours. The higher colchicine concentration resulted in a lower number of shoots

Table 2. Mortality rate of garlic “Tawangmangu Baru” explants after colchicine treatment

Months	Colchicine	Immersion time (hours)	
	(%)	24	48
2	0	1.25 ± 0.96a	3.00 ± 4.76a
	0.02	0.25 ± 0.50a	0.5 ± 0.58a
	0.04	0 ± 0a	1.00 ± 1.41a
	0.06	0.25 ± 0.50a	0.25 ± 0.50a
	0.08	2.50 ± 4.36a	0 ± 0a
	0.10	0.50 ± 0.58a	0 ± 0a
4	0	1.25 ± 0.96a	3 ± 4.76a
	0.02	0.25 ± 0.50a	0.5 ± 0.58a
	0.04	0 ± 0a	1.00 ± 1.41a
	0.06	0.25 ± 0.50a	0.25 ± 0.50a
	0.08	2.50 ± 4.36a	0 ± 0a
	0.10	0.50 ± 0.58a	0 ± 0a
5	0	1.75 ± 1.71a	3.25 ± 4.57a
	0.02	0.50 ± 0.58a	1.00 ± 0.82a
	0.04	0 ± 0a	1.25 ± 1.50a
	0.06	0.50 ± 1.00a	0.25 ± 0.50a
	0.08	2.75 ± 4.27a	0.50 ± 1.00a
	0.10	0.50 ± 0.58a	0.50 ± 1.00a
7	0	2.00 ± 1.41a	3.25 ± 4.57a
	0.02	0.50 ± 0.58a	1.00 ± 0.82a
	0.04	0.25 ± 0.50a	1.25 ± 1.50a
	0.06	0.50 ± 1.00a	0.25 ± 0.50a
	0.08	3.00 ± 4.24a	0.75 ± 0.96a
	0.10	0.75 ± 0.50a	0.75 ± 1.50a

Note: *Values followed by the same letters on the same column showed no significant difference based on the Fisher test at α=5%. Values are means ± SD.

Table 3. Survivability rate among the "Tawangmangu Baru" explants after colchicine treatment

Months	Colchicine	Immersion time (hours)	
	(%)	24	48
		Survivability rate	
2	0	13.75 ± 0.50a	12.0 ± 0.58a
	0.02	14.75 ± 0a	14.50 ± 1.41a
	0.04	15.00 ± 0.50a	14.00 ± 0.50a
	0.06	14.75 ± 4.36a	14.75 ± 0a
	0.08	12.50 ± 0.58a	15.00 ± 0a
	0.10	14.50 ± 4.76a	15.00 ± 0a
4	0	13.75 ± 0.5a	12.00 ± 0.58a
	0.02	14.75 ± 0a	14.50 ± 1.41a
	0.04	15.99 ± 0.50a	14.00 ± 0.50a
	0.06	14.75 ± 4.36a	14.75 ± 0a
	0.08	12.50 ± 0.58a	15.00 ± 0a
	0.10	14.50 ± 4.76a	15.00 ± 0a
5	0	13.25 ± 0.58a	12.00 ± 0.82a
	0.02	14.50 ± 0a	14.00 ± 1.5a
	0.04	15.00 ± 1.00a	13.75 ± 0.96a
	0.06	14.50 ± 4.27a	14.25 ± 1.00a
	0.08	12.25 ± 0.58a	14.50 ± 1.00a
	0.10	14.50 ± 4.76a	14.50 ± 0a
7	0	13.00 ± 0.58a	11.75 ± 0.82a
	0.02	14.50 ± 0.50a	14.00 ± 1.50a
	0.04	14.75 ± 1.00a	13.75 ± 0.96a
	0.06	14.50 ± 4.24a	14.25 ± 0.96a
	0.08	12.00 ± 0.50a	14.25 ± 1.50a
	0.10	14.25 ± 4.57a	14.25 ± 0a

Note: *Values followed by the same letters on the same column showed no significant difference based on the Fisher test at α=5%. Values are means ± SD

Table 4. The lethal concentration* of colchicine treatment at different time intervals after colchicine treatment *in vitro*

Lethal concentration (%)	Time after *in vitro* culture (months)			
	2	4	5	7
20	0.03	0.03	0.01	0.01
30	0.08	0.08	0.06	0.08
50	0.16	0.16	0.15	0.20

Note: *The percentage of lethal concentrations were calculated by Probit analysis in the Minitab software.

due to the higher toxicity of colchicine at 24 hours. High colchicine concentration leads to higher plant death as compared to the untreated plants. The colchicine treated explants showed greater and thicker shoot size after 5 weeks. There was no root production in all the colchicine treated and untreated explants.

Shoot and Root Growth Responses to Growth Hormones and Colchicine Treatments

There was no root production in all the colchicine treated and untreated explants. After Colchicine treatment at 24 and 48 hours, the treated and untreated explants were planted in BDS media supplemented with 0.4 $mg.L^{-1}$ 2,4-D and 2.0 $mg.L^{-1}$ of cytokinin; BDS + 0.4 $mg.L^{-1}$ 2,4-D and 0.5 $mg.L^{-1}$ of cytokinin *in vitro* supposed to produce callus, shoot

Table 5. The average number of shoots per treatment after seven months from colchicine treatment *in vitro*

Concentration (%)	Immersion time (hours)	
	24	48
	No. of st	
0.00	7.25 ± 0.975a	4.75 ± 1.893ab
0.02	6.75 ± 1.500ab	3.25 ± 3.770b
0.04	6.00 ± 1.826ab	4.00 ± 3.650ab
0.06	5.75 ± 2.870ab	6.75 ± 0.957ab
0.08	3.00 ± 3.830b	6.75 ± 1.500ab
0.10	4.00 ± 3.270ab	7.00 ± 0.816a

Note: Values followed by the same letters on the same column showed no significant difference based on the Fisher test at α=5%. Values are means ± SD

proliferation, and regeneration at different trials. For this research, two trials were performed to get a large number of calluses in different growth hormones concentrations. However, there was no formation of callus at BDS media supplemented with 0.4 mg.L^{-1} 2,4-D alone (trial 1) and BDS + 0.4 mg.L^{-1} 2,4-D and 0.5 mg.L^{-1} of cytokinin (trial 2) as indicated in Figure 2. This indicated that “Tawangmangu Baru” garlic cannot form a callus at a low concentration of 2,4-D alone and in combination with a low concentration of kinetin. The genotype should be tested in other auxins and cytokinin other than 2,4-D and kinetin. The explants did not produce callus and root within the seven months *in vitro* culture due to the inappropriate 2,4-D and kinetin combinations and concentrations in the BDS medium. The results from the *in vitro* culture showed that the interaction between the concentration and immersion time of colchicine did not have a significant effect on the root production of the "Tawangmangu" baru garlic variety. The failure of root production in this genotype may result from the concentration and combination of the growth hormones, genotype, and other *in vitro* culture conditions or through endogenous ethylene production. The colchicine treated explants showed greater and thicker shoot size after 5 weeks as shown in Figure 2. The control diploid plants showed the fastest growth over the colchicine treated explants. While the induced plants showed higher leaf and shoot size with slow growth over the control of diploid plants after treated by colchicine in vitro. There was also a clear difference in terms of shoot size within the colchicine treated plants as shown in Figure 2. Colchicine causes an increase in ethylene content. Ethylene is a hormone that stimulates cell elongation and cell division through the action of gibberellin. Ethylene itself may inhibit the auxin and cytokinin action so that the plantlets did not demonstrate fast growth and development (Ayu et al., 2019).

The explants treated at 0.06% colchicine showed larger and thicker leaf and shoot size over the other treatments. The explants treated at 0.06% produced a larger shoot size level over the other treatments. A higher concentration of colchicine leads to more negative effect on the treated plants due to the disruption of the genetic content of the plants and cellular inhibition to certain physiological processes in plants (Ayu et al., 2019).

Ploidy Verification through Flow Cytometry

Flow cytometry measures DNA quantity in nuclei from fresh leaves. Ploidy level analysis is laborious and time-consuming. Although it is a quick and hardly destructive technique, it measures nuclei in phases of high and low ploidy levels (Foshi et al., 2013; Yenchon, 2014). After the colchicine treated plants had grown for 60 days on BDS medium and were induced by colchicine at 24 and 48 hours in vitro, putative polyploid plants were investigated by flow cytometry. After two months, the induced plants were selected based on their larger shoot size to know the ploidy level of the different treatments. 24 plants were selected from 12 treatments (0, 0.02, 0.0, 0.06, 0.08, and 0.10%) induced at 24 and 48 hours. From each treatment, 2 plants were detected by flow cytometry to know the total nuclear DNA status of the induced plants. The nuclear DNA contents of leaves derived from the cultures were measured with flow cytometry. Nuclear suspensions of leaf cells were obtained from 0.5 cm^2 pieces of fresh leaves cut with a razor blade in a buffer solution using the CyStain UV Precise P kit following the manufacturer’s protocol. Leaves of “Tawangmangu Baru” (2n= 2x = 16) were used as an internal standard. Each sample was first analyzed alone to identify its fluorescence intensity profile and then analyzed with an internal standard to measure their ratio of mean cell fluorescence intensities. The dark green part of the leaf of the plants was taken for sample preparation. The plant part taken for sample

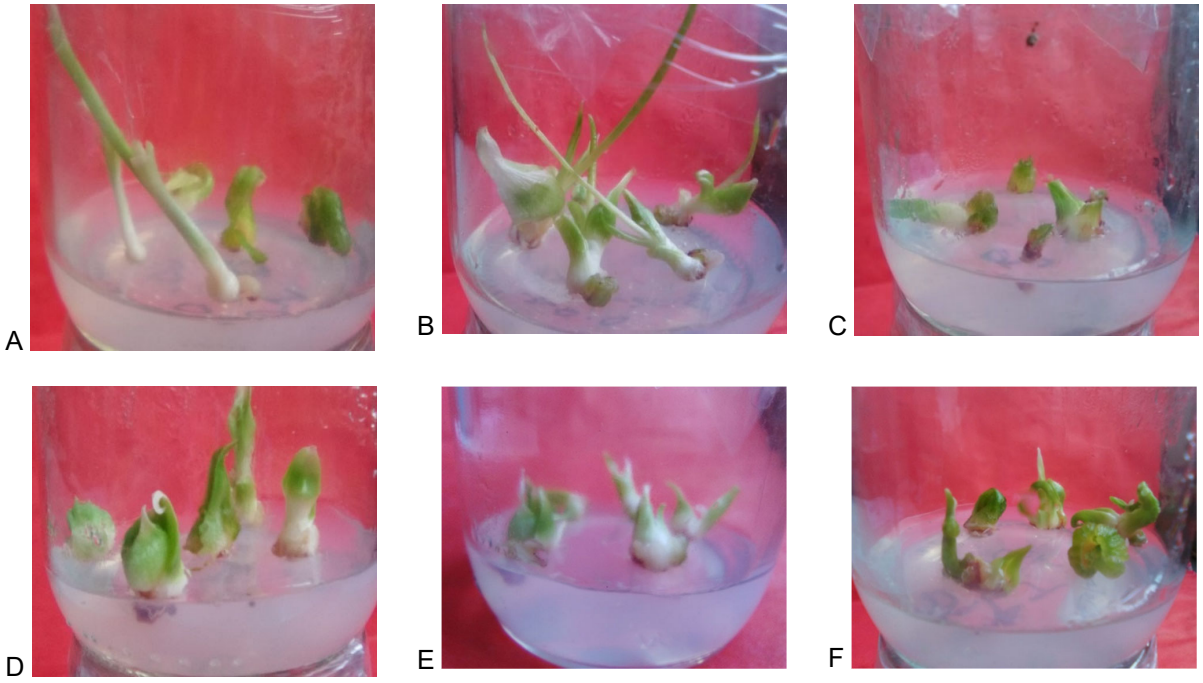

Figure 2. The shoot and root growth and development of garlic explants treated at 24 and 48 hours *in vitro*; A. Control (BDS + 0.4 mg.L^{-1} 2,4-D + 0.5 mg.L^{-1} kinetin; B: BDS + 0.4 mg.L^{-1} 2,4-D + 0.5 mg.L^{-1} kinetin + 0.02% colchicine; C: BDS + 0.4 mg.L^{-1} 2,4-D + 0.5 mg.L^{-1} kinetin+ 0.04% colchicine; D: BDS + 0.4 mg.L^{-1} 2,4-D+ 0.5 mg.L^{-1} kinetin +0.06 % colchicine; E: BDS + 0.4 mg.L^{-1} 2,4-D + 0.5 mg.L^{-1} kinetin + 0.08% colchicine; F: BDS + 0.4 mg.L^{-1} 2,4-D + 0.5 mg.L^{-1} kinetin + 0.1% colchicine

Table 6. The ploidy level of "Tawangmangu Baru" putative lines after flow cytometry analysis

Colchicine (%)	Immersion time (hrs)	Chromosomal counting	Ploidy level
0.00	24	16	diploid
0.02	24	mixoploid	mixoploid
0.04	24	16	diploid
0.06	24	16	diploid
0.08	24	mixoploid	mixoploid
0.10	24	32	tetraploid
0.00	48	16	diploid
0.02	48	mixoploid	mixoploid
0.04	48	16	diploid
0.06	48	16	diploid
0.08	48	mixoploid	mixoploid
0.10	48	32	tetraploid

preparation was chopped manually through a razor blade approximately for 2 minutes to release the intact nuclei of the plant tissue. 0.5 cm^2 of the leaf was taken from the middle part of the leaf to extract the nuclei of the plant tissue. The leaf sample was poured by 250 µl CysStain PI Absolute P (nuclei extraction buffer) to extract the intact nuclei representative of the cells within the plant tissues. The extraction was made manually by grinding the plant tissues through a laser blade in the Petri dish. After the tissues ground well, the solution was transferred to the puppet filtered by 30 µm filter size and added 300 CysStain PI Absolute P for colouring. The staining buffer was prepared from 60 µl propidium iodide, 20 µl CysStain PI Absolute P, and 60 µl Ribonuclease (RNase) to degrade the RNA molecule from the sample tissue as the target to study in the plant tissue was the nuclear DNA status of plant tissue. From the solution that contains propidium, staining buffer, and RNase, 300 µl solution was added to the extracted sample

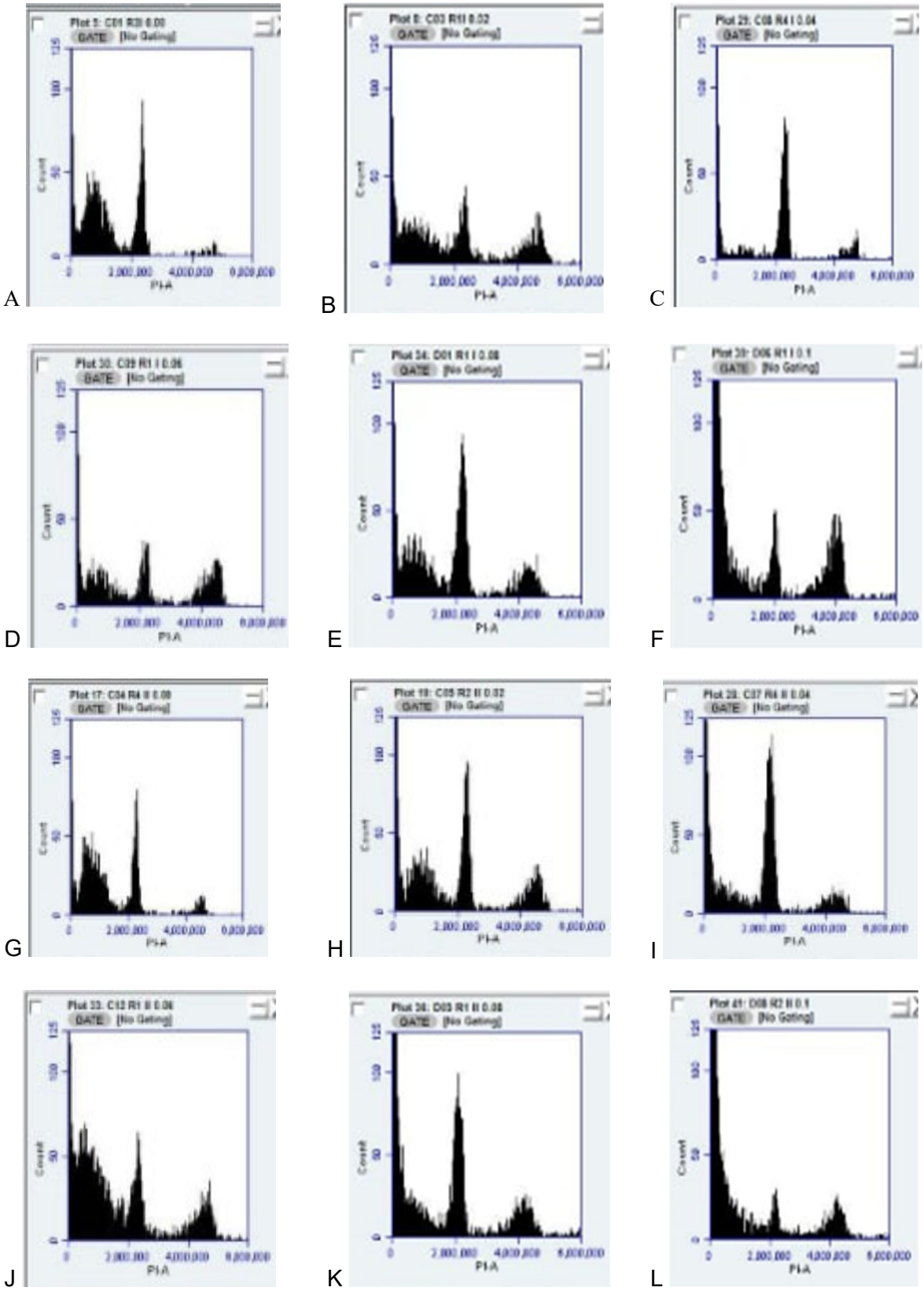

Figure 3. Flow cytometric analysis of nuclei of "Tawangmangu Baru" garlic induced by colchicine; A, G: control; B, H: 0.02% colchicine; C, I: 0.04% colchicine; D, J: 0.06% colchicine; E, K: 0.08% colchicine; F, L : 0.1% colchicine at 24 hours (A to F) and 48 hours (G-L).

tissue for colouring of the plant tissues. Immediately after adding the staining buffer, the homogenized sample tissue was detected by BD Accuri C6 Plus flow cytometry (Partec, Germany) to determine the amount of nuclear DNA in each treated line rapidly and efficiently.

Discussion

After colchicine treatment, all the explants were unable to produce callus in BDS basal medium with 0.4 $mg.L^{-1}$ 2,4-D alone. After failing the explants to produce callus in BDS medium and 0.4 $mg.L^{-1}$ 2,4-D, the explants were sub-cultured into BDS medium supplemented with 0.4 $mg.L^{-1}$ 2,4-D and 2.0 $mg.L^{-1}$ kinetin and the explants were produced an embryogenic callus with very white, friable, and globular structure. However, the produced calluses were failed to proliferate and regenerate under these growth hormones. At BDS medium with 2,4 D (0.4 $mg.L^{-1}$) and kinetin (2.0 $mg.L^{-1}$), the callus was unable to achieve the somatic embryogenesis and organogenesis. However, the calluses were proliferated in 1.5 $mg.L^{-1}$ 2,4-D and 1.0 $mg.L^{-1}$ kinetin rapidly after two weeks. The response for callus proliferation was higher in the BDS medium supplemented with 1.5 $mg.L^{-1}$ 2,4-D + 1.0 $mg.L^{-1}$ kinetin compared to MS medium supplemented with 1.5 $mg.L^{-1}$ 2,4-D + 1.0 $mg.L^{-1}$ kinetin. The developmental processes, such as callusing and proliferation are controlled by plant growth regulators (PGRs). PGR concentration is vital for an optimum response as too low concentration may not trigger the callus induction and shoot proliferation from callus. The combination of Kinetin and 2,4-D causes slightly better responses in callus induction than the use of 2,4-D alone in the “Tawangmangu Baru” garlic. The initiation of embryogenic cells requires an appropriate medium that contains specific plant growth regulators.

The meristematic basal discs of “Tawangmangu Baru” garlic induced callus in the first sub-culturing in 0.4 $mg.L^{-1}$ (2,4-D) and 2.0 $mg.L^{-1}$ kinetin combination after the explants failed to induce callus in 2,4-D alone. Auxins and cytokinins in combination are effective in producing callus instead of auxins alone in “Tawangmangu Baru” garlic. However, at low concentrations of auxins (0.4 $mg.L^{-1}$) and kinetin (2.0 $mg.L^{-1}$), the induced callus unable to proliferate and regenerate. After five months of *in vitro* culture, all the explants were transferred to another fresh MS and BDS mediums supplemented with 1.5 $mg.L^{-1}$ 2,4-D and 1.0 $mg.L^{-1}$ kinetin. After transferring the explants into these growth hormones, the failed callus to proliferate in 0.4 $mg.L^{-1}$ and 2.0 $mg.L^{-1}$ kinetin started to proliferate rapidly after five weeks. The response for callus proliferation was seen very fast in BDS medium supplemented with 1.5 $mg.L^{-1}$ 2,4-D + 1.0 $mg.L^{-1}$ kinetin compared to MS medium supplemented with 1.5 $mg.L^{-1}$ 2,4-D + 1.0 $mg.L^{-1}$ kinetin.

Mortality is a very important indicator when evaluating the efficiency of the induction of polyploidy plants after soaking bulblets in colchicine. Explants can show stress symptoms due to colchicine concentration but the symptoms are variable based on the colchicine treatment. Based on the total number of plantlets, the most suitable treatment to induce polyploidy was at 0.06% colchicine treatment for 24 and 48 hours because it did not cause the death of the plants compared to the other treatments. As described in Table 3, the colchicine treated plantlets survived well in all treatments except the control, and the plantlets induced at 0.08% for 24 hours. Untreated plantlets showed a higher mortality rate compared to the treated plantlets at both 24 and 48 hours. Moreover, the explants induced at colchicine 0.08% for 24 hours resulted in high mortality as indicated in table 2. The high mortality rate in control plantlets and 0.08% at 24 hours was perhaps due to the genetic and physiological state of the explants. Heo et al. (2016) obtained a 50% survival rate at 0.05% concentration of colchicine immersed at 9 hours and he reported that low survival rate and decreased root formation at higher colchicine concentration and longer immersion time were perceived. The meristematic basal disc of tawangmnagu baru garlic explants could survive even at higher colchicine concentration and immersion time if the other growth medium and hormonal compositions were contented. Amanah et al. (2016) reported colchicine as an effective mutagen to induce plants at a concentration of 0.001% - 1% in a period of 6–72 hours, depending on the response of each plant species.

LC50 (lethal concentration 50 %) is used to determine the optimum concentration to be used in the mutagenesis experiment; it causes 50 % lethality in the plant organism used for irradiation in defined time duration. LC50 varies with the plant species, the type and status of the material, and the stage where the lethality is measured (Bahadur et al., 2015). According to Amanah et al. (2016) colchicine is effective to induce plants at a concentration of 0.001% 0.001 to 1% in a period of 6–72 hours depending on the response of each plant species. Dixit and Chaudhary (2014) observed the maximum toxicity of garlic at 0.75% (w/v) colchicine and a maximum number of putative tetraploids at 0.5% (w/v) colchicine in 36 hours treatment. Dixit and Chaudhary (2014) reported all concentrations of colchicine proved lethal to garlic meristematic basal disc of explants when applied for 48 h or 72 hrs after one-week post-treatment incubation.

Polyploid plants possess large and thicker leaves with a low number of stomata and high chloroplast numbers compared to the diploid plants (Ayu et al., 2019; Münzbergová, 2017; Sajjad et al., 2013; Roughani et al., 2017; Jaskani et al. 2005). Tuwo and Indrianto (2016) stated that plantlets at a lower colchicine level grew faster than plantlets which have higher colchicine levels cultured at the same time. According to Widoretno (2016) research study, colchicine has also a significant effect on patchouli growth and shoot regeneration cultured on MS medium. As shown in Figure 2, the colchicine treated explants possess larger and thicker leaves with curled shoots compared to the control diploid plants. Dixit and Chaudhary (2014) suggested polyploid plants possess thick green leaves, wider, and greener leaves with larger stomata compared to the control diploids. According to many researcher's investigation, colchicine is an effective mutagen for producing polyploid plants, but they showed that responses to colchicine differ depending on the plant genotype (Heo et al., 2016). The degree of genetic variability obtained with *in vitro* induction depends on the genotype, type of explant, growth hormone, and the culture conditions used (Luciani et al., 2006; Mubarrat et al., 2018; Hassan et al., 2014).

The root meristem helps to have continuous growth as a result of cellular extension in the root system (Ayu et al., 2019). Limiting the growth and development of roots may affect the plant's ability to absorb nutrients from the growth media leading to a hindered growth due to cell division failure. Cytokinins inhibit rooting and affect treatment efficiency in the growth medium. Many economically important plant species cannot induce root easily. The reluctance to root could be due to a high level of cytokinin persisting in stem tissues from previous treatments (Trigiano and Gray, 2004). However, rooting can be induced by the transfer of the shoots once or twice to medium without PGRs to allow cytokinin levels to drop. The degree of genetic variability obtained from *in vitro* culture extremely depends on the genotype, type of explant, growth hormone (particular chemical structure and composition), and the culture conditions used (Luciani et al., 2006; Mubarrat et al., 2018; Hassan et al., 2014). The results from the *in vitro* culture showed that the interaction between the concentration and immersion time of colchicine did not have a significant effect on the root production of the "Tawangmangu Baru" garlic. The failure of root production in this genotype may result from the concentration and combination of the growth hormones, genotype, and other *in vitro* culture conditions. For this research, two trials were performed to get a large number of calluses, shoot, and root induction. One trial was on BDS with 0.4 $mg.L^{-1}$ 2,4-D alone (1st sub-culture) and the second trial was on BDS with 0.4 $mg.L^{-1}$ 2,4-D and 0.5 $mg.L^{-1}$ kinetin for two months (1st subculture). In the first trial, after two months all the explants were transferred into BDS media augmented with 0.4 $mg.L^{-1}$ 2,4-D and 2.0 $mg.L^{-1}$ kinetin (2nd subculture) supposed to produce callus and organogenesis in two months. In the second sub-culture, 4.72% explants were produced callus within two weeks after 1st sub-culture. After four months, all the explants were transferred into BDS media supplemented with 0.4 $mg.L^{-1}$ 2,4-D, 2.0 $mg.L^{-1}$ kinetin, and 25 $mg.L^{-1}$ asparagine for a month to ensure somatic embryogenesis. Still, all the explants were unable to produce callus, shoot, and root at 0.4 $mg.L^{-1}$ 2,4-D alone and in combination with kinetin. There was no response of 2,4-D alone to callus production and organogenesis. The second trial was sub-cultured again in the same hormonal combination and concentration to ensure somatic embryogenesis, however, there was no somatic embryogenesis at all. Occasionally mixtures of auxins usually at lower concentrations than those used singly can promote root initiation whereas single auxins have no activity (Trigiano and Gray, 2004). Plant species or genotype has an adverse effect in shoot and root growth and differentiation, and adventitious root induction in plant tissue culture. In some genotypes, endogenous ethylene causes leaf crinkling, folding, restriction of root growth, and reduces tendril growth after a month in plant tissue culture (Trigiano and Gray, 2004).

The flow cytometry analysis showed a diploid level of chromosome in all treatments except the plants induced in 24 hours with 0.1% colchicine concentration as shown in Figure 3. The cytological analysis with flow cytometry revealed that tetraploid "Tawangmangu Baru" garlic variety (2n=4x=32) stained with Propidium Iodide showed higher chromosome number compared to the control (2n=2x=16). The tetraploid "Tawangmangu Baru" garlic plants were characterized with thick dark-green leaves and showed slow growth compared to the other treatments. The counted nuclear DNA events with a rate of 50 nuclei per second resulted in a short peak in all the polyploidy plants. The shortest peak in the DNA histogram may represent the total nuclei in G_0 and G_1 phases. Mechanical chopping does not produce significant DNA hydrolysis or nuclear degradation. The colchicine treated "Tawangmangu Baru" garlic showed in growth reduction and most of the clones were diploids. Dixit and Chaudhary (2014) reported that the regenerated garlic plantlets showing reduced growth were all haploids, while those with frizzed leaves were all polyploids. The growth abnormalities as a result of colchicine treatment indicate the success of polyploid induction (Dixit and Chaudhary, 2014). In treatment 0.02% at 24 hrs, 0.08% at 24 and 48 hrs resulted in mixoploid plants

that produced due to the incidence of chimera. Some cells may not be affected by colchicine treatment and remain as diploid (2n). Only partial induction of chromosome cells remain doubled by colchicine treatment (Amanah et al., 2016).

Colchicine causes microtubule depolymerization and inhibits cell division by blocking the microtubule development (Zhang et al., 2016; Roughani et al., 2017; Moghbel et al., 2015; Amanah et al., 2016). Colchicine inhibits the addition of tubulin subunits which merge at the end of the microtubule polymers (Amanah et al., 2016). Microtubule depolymerization causes the disturbance of the spindle thread formation. The disturbance of spindle fibers results in the separation of chromosomes but the cells fail to produce new cells and cause the doubling of chromosomes (Wiendra et al., 2011). Results in garlic are promising and research on in vitro mutation breeding technology has been established (Broertjes, 2012); however, all cells exposed to mutagen do not incur mutations (Amanah et al., 2016). But, those that do incur mutations will give rise to cells reveals the mutation (Bahadur et al., 2015; Amanah et al., 2016). The research showed that 50% of the putative plants were diploid and 33.3% of the plants were mixoploid in both 24 and 48 hours, and that 16.6% of the putative "Tawangmangu Baru" plants duplicated their genome at 0.1% colchicine in both 24 and 48 hours. This ploidy value indicates that polyploid garlic candidates have been successfully induced using the meristematic basal discs as an explant. The application of colchicine increased the ploidy level, and an increase in ploidy is expected to make the bulb size larger in the "Tawangmangu Baru" garlic variety. Larger tuber size will increase the tuber weight and overall garlic productivity and production.

Conclusion

The meristematic basal disc of "Tawangmangu Baru" garlic variety can be propagated by *in vitro* tissue culture. However, the degree of genetic variability obtained from *in vitro* culture depends on the plant genotype, type of explant, growth hormone, and the culture conditions. The genotype failed to form callus on BDS medium supplemented with 0.4 $mg.L^{-1}$ 2,4-D alone and BDS with 0.4 $mg.L^{-1}$ 2,4-D and 0.5 $mg.L^{-1}$ kinetin, but it formed a transparent, friable, and white callus within two weeks after culturing on 0.4 $mg.L^{-1}$ 2,4-D and 2.0 $mg.L^{-1}$ kinetin on BDS medium. Cell and shoot proliferation were better on BDS + 2,4-D (1.5 $mg.L^{-1}$) and 1.0 $mg.L^{-1}$ kinetin. The LC 20%, LC 30%, and LC 50% for the concentration of colchicine in "Tawangmangu Baru" garlic at two months were 0.03%, 0.08%, and 0.16% respectively. There was no difference at two and four months for the lethal concentration, whereas, the LC 20%, 30%, and 50% at 7 months was 0.01%, 0.08%, and 0.2% respectively. There was a high mortality of plants at the early stages and lower mortality after four months at LC 20% due to the stability of the plantlets. The "Tawangmangu Baru" basal disc explants treated by colchicine showed a higher survivability rate in the range of 0.00% - 0.1% with immersion time of 24 and 48 hours. The genetic diversity of the "Tawangmangu Baru" garlic was successfully enhanced by colchicine concentration and immersion treatment. The flow cytometry analysis indicated that 50% of the putative plants were diploid and 33 % of the plants were mixoploid in both 24 and 48 hours. Seventeen percent of the putative "Tawangmangu Baru" duplicated their genome at 0.1% colchicine treatment for 24 or 48 hours, indicating that polyploid garlic have been successfully induced using the meristematic basal discs as an explant. The tetraploids possessed thicker and darker green leaves with slow growth compared to the untreated control plants. The application of colchicine treatment at 24 or 48 hours improved the genetic potential of "Tawangmangu Baru" garlic variety *in vitro*.

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