

Conference Paper

In Vitro Propagation of *Lilium longiflorum* Bulbs Using NAA and BAP Plant Growth Regulator Treatment

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Abstract

Lily (*Lilium longiflorum*) is a perennial herbaceous plant with white trumpet-shaped flowers, fragrant and bulbous. *In vitro* culture through bulbs is one of way propagation of lily plants, but it requires a long time and only produces limited plants. In vitro propagation is a very promising technique for plant propagation because it can produce a lot of plant seeds in a short time. Bulbs are one of the fastest explants for growing shoots in lilies, but it is not known for certain which cuts of explants from bulb scales are best for multiplying in vitro. This study aims to determine the effect of lily bulb explants and the concentration of NAA and BAP growth regulators on the growth of lily bulb explants. The best results were obtained on the base and middle cuts explant of bulb scales compared to the tip cuts explant ones. The best results of the growing percentage, the number of shoots and the best growing time are shown in the combination treatment of growth regulator 1 mg L⁻¹ NAA and 1 mg L⁻¹ BAP. The optimum results on the number of micro bulbs were found in the treatment of growth regulators 0.5 mg L⁻¹ NAA and 1 mg L⁻¹ BAP. The best results of the average time formed micro bulb was in the treatment of 1 mg L⁻¹ NAA and BAP with middle explant cuts, and treatment concentrations of 0.5 mg L⁻¹ NAA and BAP in the base explant section. The base and middle bulb explants are able to regenerate or grow higher shoots. This is caused by the presence of endogenous natural auxin and the spread of auxin in plant parts not in the same amount. Therefore when added to the exogenous growth regulator such as auxin or cytokines to culture media will further trigger the formation of micro tubers more quickly. It can increase the concentration of endogenous growth regulators in cells, help growing process and developing tissue.

Keywords: Bulb, lily, micro bulbs, in vitro, shoots

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1. Introduction

Lily plants (*Lilium* Spp.) are plants known as cut flowers and commonly used in flower arrangements and room decorations [1]. Several types of lily such as *Lilium longiflorum*, *Lilium speciosum*, and *Lilium auratum* have long been cultivated in mountainous areas in Java as ornamental plants in the yard [2] However, the idea of lily flowers as a commercial business in Indonesia has only just begun to be developed in the last

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decade. The development is based on a very good prospect, due to the diversity of lily colors, good ability to adapt to the environment, ease of cultivation techniques, and lower production costs compared to other cut flowers, such as chrysanthemums. Along with the development of lily cut flowers, there has been an increase in demand for lily sprout [3]

Lily is a true bulb plant, in the shape of a cup surrounded by scales that functions as a food reserve containing starch, sugar and protein. Scale resembles a thin fleshy sheet and can be easily separated and grown into new shoots and plants (Crocket, 1973). Lily is commercially propagated through bulbs, while lily for bulb production is propagated through the vegetative part of the plant i.e. scales, prospective bulbs (bulblets) that grow on the rootstock of plants and by tissue culture techniques [1]. The use of tissue culture is very helpful in the commercial production process because the process of planting lily in the field can only be done in areas that support the climate during the planting process, otherwise it can prevent the infectious diseases in the propagation results.

The use of *in vitro* techniques for the development of lily cut flowers in Indonesia is greatly possible. Robb [4], and Takayama and Misawa [5] succeeded to induce bulblets in the culture of lily bulb scales. Several previous studies also reported factors influencing *in vitro* lily culture, namely the composition of growth regulators ([5];[6];[7]), explant sections ([4];[6]), and explant positions [8].

Growth induction using growth regulators is widely reported ([9];[10];[11]). It was further reported that the combination of auxin and cytokinin gave the best results in inducing micro potato bulbs of Kufru Jyoti varieties [12]

The best explants that can be used for growing shoots and micro bulbs are bulb explants. However, it is currently still uncertain which cuts of bulb scales give the best results. In addition, the effectiveness of using hormone concentration on the speed of growth of lily bulb scales is also uncertain. This study aims to determine the best potential of the bulb scales cuts and hormone concentrations, as well as the effect of temperature treatment on growing time, growing percentage, number of shoots, number of micro bulbs and the formation time of micro bulbs.

2. Method

2.1. Time and Place of Research

The study was conducted in February 2019-July 2019 at the tissue culture laboratory at Crops Seed Center Hall, Luwus, Tabanan Regency, Bali Province.

2.2. Media Preparation

The in vitro culture media used in this experiment is Vacin and Went media supplemented with 20g.L^{-1} sucrose and 8g.L^{-1} agarose. Various combinations of NAA and BAP were used as treatments, consisted of combination of NAA and BAP growth regulators, namely a) 0.5 mg.L^{-1} NAA + 0.5 mg.L^{-1} BAP; b) 0.5 mg.L^{-1} NAA + 1 mg.L^{-1} BAP; c) 1 mg.L^{-1} NAA + 0.5 mg.L^{-1} BAP; d) 1 mg.L^{-1} NAA + 1 mg.L^{-1} BAP. The pH of the media was adjusted at 5.6-5.8 before autoclaving at 121°C and 15 psi for 15 minutes. Inoculated explants were incubated in the culture room at 25°C and cool-white fluorescent lamps with 16 h photoperiod under.

2.3. Explant Sterilization

The lily bulbs were separated by scales one by one and then washed with running water and soaked in a fungicide solution for 30 minutes. Furthermore, the explants were soaked in 20% and 10% chloric solution each for 15 minutes then rinsed with sterile aquades 3 times. Sterilized explants were planted on Vacin & Went (VW) culture media with various treatments by adding a combination of NAA and BAP growth regulators that had been sterilized with a 15 psi autoclave for 30 minutes. The culture bottles which had been planted with explants were incubated in the culture room where the temperature was maintained between $26\text{-}28^{\circ}\text{C}$ with 2000 lux irradiation for 16 bright hours and 8 hours without irradiation.

2.4. Research Design

The study used a Completely Randomized Design with Factorial pattern consisting of three factors. The first factor was explant cuts consisting of 3 parts, namely the base, middle and tip. The second factor consisted of administering a combination of NAA and BAP growth regulators, namely a) 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BAP; b) 0.5 mg L^{-1} NAA + 1 mg L^{-1} BAP; c) 1 mg L^{-1} NAA + 0.5 mg L^{-1} BAP; d) 1 mg L^{-1} NAA + 1 mg L^{-1} BAP. Therefore, there were 10 treatment combinations with 3 replications. The

observed variables were the percentage of explant growth, time of growth, number of shoots, number of micro bulbs and time of formation of micro bulbs. Visual observation to see the development of culture was carried out every day for the period of 16 weeks, while the final observation to obtain quantitative data was carried out at the end of the 16th week.

2.5. Data Analysis

The observational data obtained were analyzed to determine the effect of the treatment using a multivariate test with 2 factors at a significant level $\alpha = 5\%$. If it shows a real effect, the test is continued using Duncan test with SPSS version 17 application.

3. Results

Based on observations, micro bulbs were formed on the surface of bulb scales. In some explants, roots can grow on micro shoots and micro bulbs. At the observation of 16 weeks after culture, the micro-shoots that appeared in all treatments had an average of 2-5 leaves, while the bulblets were composed of 5-8 micro-bulb scales. The results of the study on explant cut factors are presented in Table 1 and Figure 3.

TABLE 1: Observation results are based on cuts of bulb explants.

Bulb explant cuts	Growing percentage	Growing time	Number of shoots	Number of micro bulbs	Formation time of micro bulbs
Base	100 ^b	14.30 ^a	3.07 ^{ab}	1.40 ^a	119.89 ^a
Middle	98.67 ^b	14.46 ^a	3.71 ^b	1.44 ^a	119.92 ^a
Tip	85.33 ^a	16.53 ^b	2.41 ^a	1.50 ^a	121.13 ^a

Note: The average value of the treatment followed by letters in the same column showed no significant difference based on Duncan's multiple range test at 5% level.



Figure 1: Plantlets from (1) tip, (2) middle and (3) base explant cuts.



Figure 2: Micro bulbs.

Based on observations, concentrations of NAA and BAP growth regulators that the results of the growth percentage, the number of shoots and the best growing time are shown in the combination treatment of 1 mg L⁻¹ NAA and 1 mg L⁻¹ BAP. The results of the number of micro bulbs are shown in the treatment of 0.5 mg L⁻¹ NAA and 1 mg L⁻¹ BAP. The results of the average time to form micro bulbs are in the treatment of 1 mg L⁻¹ NAA and BAP with middle explant cuts and treatment concentrations of 0.5 mg L⁻¹ NAA and BAP in the base explant section are presented in Table 2 and Figure 4.

TABLE 2: Observations based on concentrations of NAA and BAP growth regulators.

Concentration of growth regulator substance (mg.L ⁻¹)	Growing percentage	Growing time	Number of shoots	Number of micro bulbs	Formation time of micro bulbs
0 NAA + 0 BAP	94 ^a	15.20 ^a	2.25 ^a	0 ^a	0 ^a
0.5 NAA + 0.5 BAP	100 ^b	14.81 ^a	3.94 ^{bc}	1.25 ^{bc}	122 ^c
0.5 NAA + 1 BAP	96.7 ^{ab}	14.71 ^a	2.46 ^{ab}	1.70 ^{ab}	128.18 ^{bc}
1 NAA + 0.5 BAP	100 ^b	14.89 ^a	3.78 ^{bc}	1.27 ^{bc}	120.57 ^{bc}
1 NAA + 1 BAP	100 ^b	14.19 ^a	4.25 ^c	1.40 ^c	128.40 ^{bc}

Note: The average value of the treatment followed by letters in the same column showed no significant difference based on Duncan's multiple range test at 5% level.

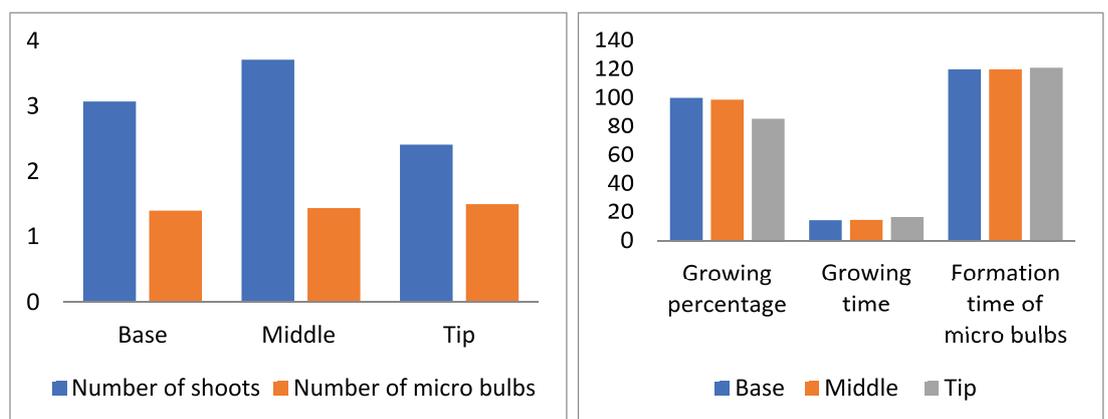


Figure 3: Graph of results based on cuts of bulb explants.

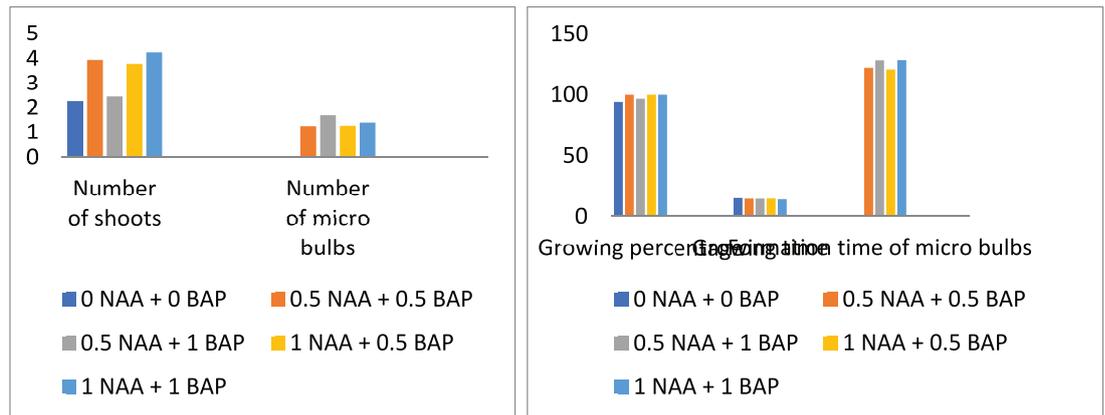


Figure 4: Graph of results based on concentrations of NAA and BAP growth regulators.

Interactions between explant cuts and growth regulator substance are presented in Table 3. In the test results, the relationship between explant cuts and concentration of growth regulators used the best percentage of growth in the treatment of middle bulb explant cuts with all concentrations of growth regulators. The interactions between the explant parts and the concentration of growth regulators in the variable of growing time show significantly different results from the average number of shoots at the 1 mg treatment. The interaction between bulb explant cuts and the concentrations of growth regulators showed significantly different results with the average number of shoots at the highest concentration of 1 mg. The interaction bulb explant cuts with concentrations of growth regulators, the average number of micro bulbs was found in the treatment of 0.5 mg L⁻¹ NAA and 1 mg L⁻¹ BAP with middle explant cuts. Based on the interactions between bulb explant cuts and concentrations of growth regulators, the average result of micro bulbs was formed in the treatment of 1 mg L⁻¹ NAA and BAP in middle bulb explant cuts, and in the treatment concentrations of 0.5 mg L⁻¹ NAA and BAP in the base explant cuts.

4. Discussion

4.1. Growing percentage

The best explants that can be used for growing shoots and micro bulbs are bulb explants, but which cuts of bulb scales give the best results and the use of hormone concentration on the speed of growth of lily bulb scales it is not known for certain. This statement confirmation with the earlier study from Kanchanapoom et al. [13] that bulb scales of lily have a high regeneration potential hence they are most commonly used as explants for vegetative propagation and an efficient protocol for shoot regeneration

TABLE 3: Interactions between explant cuts and growth regulator substance.

Explant cuts	Concentration of growth regulators	Growing percentage	Growing time	Number of shoots	Number of micro bulbs	Formation time of micro bulbs
Base	0 NAA+0 BAP	96.67 ^a	14.50 ^b	2.50 ^a	0 ^a	0 ^a
	0.5NAA+0.5 BAP	100.00 ^b	14.50 ^b	3.00 ^{bc}	1.00 ^b	121.67 ^b
	0.5 NAA + 1 BAP	96.67 ^a	14.50 ^b	3.00 ^{bc}	1.80 ^c	128.00 ^{bc}
	1 NAA + 0.5 BAP	100.00 ^b	14.17 ^b	4.17 ^c	1.25 ^b	129.60 ^c
	1 NAA + 1 BAP	100.00 ^b	13.53 ^a	4.67 ^c	1.50 ^{bc}	129.33 ^c
Middle	0 NAA+0 BAP	100.00 ^b	14.75 ^b	2.38 ^a	0 ^a	0 ^a
	0.5NAA+0.5 BAP	100.00 ^b	14.67 ^b	2.50 ^{ab}	1.00 ^b	123.00 ^b
	0.5 NAA + 1 BAP	100.00 ^b	14.50 ^b	3.00 ^{bc}	2.00 ^c	128.00 ^c
	1 NAA + 0.5 BAP	100.00 ^b	14.33 ^b	2.83 ^{ab}	1.50 ^{ab}	124.00 ^b
	1 NAA + 1 BAP	100.00 ^b	13.75 ^a	3.25 ^c	1.67 ^{bc}	121.67 ^b
Tip	0 NAA+0 BAP	83.33 ^a	16.50 ^d	1.83 ^a	0 ^a	0 ^a
	0.5NAA+0.5 BAP	93.33 ^{ab}	15.50 ^c	2.17 ^b	1.00 ^b	0 ^a
	0.5 NAA + 1 BAP	100.00 ^b	15.33 ^c	2.83 ^c	1.40 ^{bc}	124.40 ^b
	1 NAA + 0.5 BAP	100.00 ^b	15.50 ^c	2.33 ^c	1.00 ^b	128.50 ^c
	1 NAA + 1 BAP	100.00 ^b	14.83 ^b	2.83 ^c	1.75 ^c	129.33 ^c

Note: The average value of the treatment followed by letters in the same column showed no significant difference based on Duncan's multiple range test at 5% level.

from bulb scales in *L. longiflorum* has been developed. The shoots occurred directly via organogenesis and without forming callus on MS medium containing BA and NAA.

Duncan's test shows significantly different results in the treatment of the explant cuts. The average percentage of the best growth from the treatment of explant cuts that is at the base and middle bulb explants compared to the tip explant cuts. It is suspected that endogenous auxin and cytokinin content in lily scales differ between tissue parts, so the percentage of regeneration produced in this study also varies depending on the direction of the explant cuts. This assumption is supported by the opinion of Mantell et al.[14] explaining that cell differentiation is influenced by the relative balance of auxins and cytokines both in culture media and which are naturally contained in plant tissue. This is in confirmation with the earlier study by Reshi et al. [15] observing the *in vitro* Micropropagation of *Anisochilus carnosus* (L) wall and revealed that more effect can be gained through combinations of BAP and auxin on multiple shoot induction, compared to BAP or auxins when used separately.

In the test result, the relationship between explant cuts and concentration of growth regulators used, the best percentage of growth is seen for the treatment of middle bulb explant cuts with all concentrations of growth regulators (Table 3).

4.2. Growing time

Further test shows significantly different results with the fastest growing time on the base and middle explant cuts compared to the tip cuts. Growing time on bulb explants is related to the presence of natural food reserve substances and natural endogenous growth regulators found in the bulbs. The success of a plant in growth is influenced by genetic and environmental factors. Genetic factors are related to the inheritance of plant traits derived from the parent plant, while environmental factors are related to the environmental conditions in which the plants grow [16]

In the treatment of growth regulators, the treatments for growing time variables showed that there was no significant difference in growing time based on differences in concentration.

The linkage testing of the explant parts and the concentration of growth regulators in the variable of growing time showed significantly different results from the average number of shoots at the 1 mg treatment.

4.3. Number of shoots

The average results of number of shoots in the treatment of bulb explant cuts show that the most shoots can be found in the middle and base explant cuts compared to the tip ones (Table 1). Mantell et al. [14] reported that many micro lily shoots were formed at the base of the bulb scales. Furthermore, Liu and Burger [6] reported differences in the response of the formation of micro shoots in explants of lily stalks in the pedicle and receptacle parts. Micro lily shoots are more common in the receptacle than in the pedicle.

The formation process of shoots begins with a rounded swelling on the bulb scale cuts due to internal changes and cell division in parenchymatic sub-epidermal tissue [4]. Leaf tips continue to elongate to form leaf blade (lamina) and leaf midrib, followed by the formation of new leaf strands. Furthermore, in a short time (about 2 weeks) after the appearance of the first leaf blade, roots emerge at the base of the micro shoots. Morphologically, the composition and shape of leaf strands in micro shoots resulting from in vitro culture are similar to shoots produced from lily bulbs in the field. However,

there are fundamental differences in the pattern of its formation. Lily shoots in the field are produced from bulbs which are preceded by the formation of bulb scales, but micro shoots are produced in vitro without preceding the formation of bulbs or micro bulbs.

In the treatment of growth regulator substance concentration on the variable of the number of shoots, the highest number of shoots was found in 1 mg L⁻¹ NAA and 1 mg L⁻¹ BAP followed by 0.5 mg L⁻¹ NAA and BAP and treatment of 0.5 mg L⁻¹ NAA and 1 mg L⁻¹ BAP (Table 2 and Fig. 1). Sun et al. [17] also showed that in the culture of micro bulb explants in *Lilium lancifolium*, successful growth of shoots were observed in a combination of MS media treatment with the addition of growth regulators of 1 mg L⁻¹ NAA and 0.1 mg L⁻¹ BA (auxin and cytokinin). Application of 6-BA-benzylaminopurine (6-BA) as a cytokinin application can increase meristem formation and shoot proliferation in many species. Likewise, Yun et al. [18] studied that administration of 6-BA, α-naphthaleneacetic acid (NAA) and sucrose in the development of *L. lancifolium* was very effective in producing regeneration and induction of shoot propagation.

Auxin growth regulator substances such as NAA functions in increasing osmotic pressure, cell permeability, protein synthesis, plasticity and developing cell walls and reducing pressure on cell walls. In addition, Auxin plays a role in stimulating cell lengthening and enlargement, while cytokinin such as BAP functions in cell division. In conjunction with cell permeability, auxin increases the diffusion of water into the cell. The combination of auxin and cytokinin will stimulate cell division and affect the path of differentiation [19]. Fønnesbech [20] states that auxin does not function if it does not interact with other hormones. Meanwhile, Morel [21] states that the best concentrations used are 1 ppm cytokinin and 1 ppm NAA.

The concentration of growth regulators with the lowest average number of shoots was in the treatment of 0 NAA and BAP and 0.5 mg L⁻¹ NAA and 1 mg L⁻¹ BAP. This shows that BAP accumulation in mini bulb scales is very high so that it inhibits the formation of micro shoots. Winarsih et al. [7] reported that high BAP concentrations inhibited the formation of micro shoots but encouraged bulb formation in the culture of lily bulb scales. Joseph et al., [25], showed that the study a procedure for in vitro production of bulbs of *Cyrtanthus mackenii* was developed from triscales has been reported that auxin is an effective for shoot induction and in combination with a cytokinin is essential for shoot induction.

Duncan's test results show that interaction between bulb explant cuts and the concentrations of growth regulators showed significantly different results with the average number of shoots at the highest concentration of 1 mg. Because of the natural content

of growth regulators in the bulbs, especially most collected in the middle and base cuts which are close to the meristematic part of the tissue can trigger better growth. In addition, adding appropriate synthetic growth regulators will encourage the growth of more shoots. Winarsih et al. [7] state that the content and relative balance of endogenous growth regulators (auxin and cytokinin) on lily bulb scales have high potential to regenerate to form shoots and bulblets, so that adding appropriate growth regulators in the culture medium, the potential can be expressed.

4.4. Number of micro bulbs

Duncan's test results on the number of micro bulbs in the explant cuts show no significant difference. Formation of micro bulbs is characterized by the formation of small yellowish white spots. The spots are enlarged into small brownish spheres. Furthermore, small bulb scales begin to appear when the small dots have a diameter of 2-3 mm. The outer bulb scales continue to enlarge and are followed by the formation of new bulb scales from the point of growth. Along with the long culture, the micro bulbs turned green. Thus, the resulting bulb is composed of several micro bulb scales. The shape and arrangement of the mini bulb scales on the bulblets is the same as the shape and arrangement of the bulb scales on the lily bulbs that are produced naturally in eight ponds [3].

In the treatment of growth regulators concentration on the variable number of micro bulbs, Duncan test results show significantly different results, with the best result was found on the concentration of growth regulators 0.5 mg L^{-1} NAA and 1 mg L^{-1} BAP (fig. 2). Growth regulators of low concentration BAP was combined with NAA for the formation of micro shoots, while high concentrations of BAP both with and without the addition of NAA was used for micro bulb formation [7]. Plant tissue respond differently to auxin and cytokinin, this situation can be used in the regeneration process. Decruse et al. state that the combination of a comparative treatment between auxin and cytokinin is very influential in determining the type of morphogenesis.

Based on the results of the interaction test of 2 factors, bulb explant cuts with concentrations of growth regulators, the average number of micro bulbs was found in the treatment of 0.5 mg L^{-1} NAA and 1 mg L^{-1} BAP with middle explant cuts. With the presence of endogenous growth regulators in bulbs and the addition of exogenous growth regulators, it is able to trigger the growth of micro bulbs. Exogenous growth regulator concentrations that support the growth of micro bulbs are cytokinin. Phytohormone works synergistically with other growth hormones in stimulating growth.

Natural cytokinin produced in plant tissues work synergistically with NAA and which are synthetic auxins and BAP which are synthetic cytokinin given in this experiment are important in regulating cell division and play an important role in the formation of direct somatic embryogenesis [22]

4.5. Formation time of micro bulbs

Duncan test results at the time the micro bulbs were formed in the explant cuts show that there was no significant difference. Lily tuber or bulbs scale organs already have natural endogenous growth regulators to trigger growth, but in pieces the bulbs parts there was no significant difference on the time of the formation of the tubers. Therefore when added to the exogenous growth regulator will further trigger the formation of micro tubers more quickly. Addition of auxin or cytokines to culture media can increase the concentration of endogenous growth regulators in cells, so that it becomes a trigger factor growth in the process of growing and developing tissue [23].

In the treatment of growth regulators concentration for the variable of formation time of micro bulbs, the Duncan test shows significantly different results with the best results at the concentration of growth regulators 1 mg L^{-1} NAA + 0.5 mg L^{-1} BAP. Based on the results of the interaction test of 2 factors for bulb explant cuts with concentrations of growth regulators, the average result of micro bulbs was formed, namely in the treatment of 1 mg L^{-1} NAA and BAP with middle bulb explants cuts and treatment concentrations of 0.5 mg L^{-1} NAA and BAP in the base explant cuts.

In all geophytes like tulip, lily, hyacinths and iris, the formation of storage organs or growth *in vitro* is a slow and has problematic process. Although micro propagation protocols have been developed, most are not quite workable in commercial laboratories. Therefore, more research is needed to study and improve *in vitro* storage organ formation [24]. This study shows an excellent regeneration capacity for *L. longiflorum* success with *in vitro* formation shows that the micro bulb culture is an effective and useful technique for propagation of this species. Mass production through tissue culture can also be a desirable alternative for multiplication of seedlings in a short amount of time. The conventional method through seed multiplication requires a longer time, has a low germination rate and a slow growth process.

5. Conclusions

Based on the research results of *in vitro* propagation of *Lilium longiflorum* bulbs using various treatments of administering growth regulators and bulb explant cuts, it can be concluded that the explants with the best growth response are base and middle bulb explant cuts compared to the tip cuts. The results of the growth percentage, the number of shoots and the best growing time are shown in the combination treatment of 1 mg L⁻¹ NAA and 1 mg L⁻¹ BAP. The results of the number of micro bulbs are shown in the treatment of 0.5 mg L⁻¹ NAA and 1 mg L⁻¹ BAP. The results of the average time to form micro bulbs are in the treatment of 1 mg L⁻¹ NAA and BAP with middle explant cuts and treatment concentrations of 0.5 mg L⁻¹ NAA and BAP in the base explant section. Based on the results of this study, the method of culture propagation of base or middle bulb explant cuts combined with the administration of growth regulator substance can be used as a comparison method for the propagation of lily or other plant species. Therefore when added to the exogenous growth regulator such as auxin or cytokines to culture media will further trigger the formation of micro tubers more quickly. It can increase the concentration of endogenous growth regulators in cells, help growing process and developing tissue. The micro bulb culture is an effective and useful technique propagation for *L. longiflorum*. Mass production through tissue culture can also be a desirable alternative for multiplication of seedlings in a short amount of time.

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