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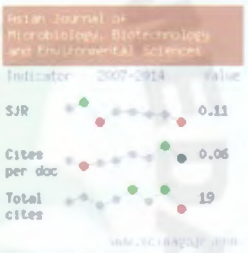

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STERILIZATION OF PINEAPPLE EXPLANT FROM SIPAHUTAR, NORTH SUMATERA, INDONESIA (*ANANAS COMOSUS* L.) AND *IN VITRO* GROWTH INDUCTION

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Key words : *Sterilization, Sipahutar pineapple, IAA, BAP*

Abstract - Sipahutar pineapple was derived from Sipahutar, North Tapanuli, North Sumatra, Indonesia, currently endangered. The aims of the research were 1) to obtain optimum sterilization techniques for pineapple explants (*Ananas comosus* L.) from Sipahutar, 2) growth induction of *in vitro* shoots with Benzyl Amino Purine (BAP) and Indole Acetate Acid (IAA) plant growth regulators (PGR). This research consists of experiment series, sterilization, growth induction, and *in vitro* propagation. Sterilization techniques used were of 9 kinds, cleaning the explants, used 5% detergent, then cleaned with water, continued using different sterilants for different durations. Between sterilan I and II, the explants were washed with sterile distilled water. Explants were cultured on MS medium+ 5 mg L^{-1} Kinetin + 0.5 mgL^{-1} IAA for induction of early growth. Growth induction continued with four concentration of Benzyl Amino Purine (BAP) (0, 2, 4, 6) mg L^{-1} and three concentration of IAA (0, 0.5, 1) mg L^{-1} were used as a treatment in Murashige and Skoog (MS) medium. The results showed: 1) Treatment by soaking the explants with a 0.008% mankozeb, 0.002% streptomycin sulphate for 1 hour followed by 1.05% NaClO, then peel the outside of explants and put on filter paper to dry, and then planted, produced high aseptic culture (55.6%) and showed the highest growth (22.2%), 2). Giving of BAP and IAA significantly affect to appearance of shoots, number of shoots, number of leaves except shoots hight. The best treatment was a combination of MS + 2 mg L^{-1} BAP + 0 mg L^{-1} IAA.

INTRODUCTION

Currently, pineapple from Indonesia a third of export fruit commodity in the world after The Philippines and Thailand. Sipahutar pineapple (Figure 1) was grown by farmers in the Sipahutar area, North Tapanuli, North Sumatra, Indonesia (Figure 2). This pineapple has the advantage that it tastes more sweet, water is low content, denser texture, yellow color and liked by the community. This fruit is one of the leading horticultural crops commodities in North Tapanuli, but production is very limited.

Farmers in Sipahutar develop these plants used crown, buds, divide old plants. The population of this pineapple was endangered. It was urgent to conduct preservation, propagation and further development. For the development of this crops, needed planlet in large quantities and uniform. One alternative to solve this problem was by tissue culture techniques. This technology has been widely used for get the uniform seedlings especially on horticulture crops (Harahap *et al.*, 2012). System to produce plantlets, through *in vitro* culture by "direct organogenesis" (Goh *et al.*, 1994). This was to prevent the occurrence of somaclonal variation, which was not desired in the propagation massal (Harahap *et al.*, 2009).

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One of the success of tissue culture was a way of sterilizing the explants before planting. Initiation of the culture that was free of contaminants is a very important step. Some chemicals can be used as a sterilizing, such as mercury chloride ($HgCl_2$), sodium hypochlorite (NaOCl) and others. These chemicals can kill external microorganisms but can not kill the internal microorganisms in plant tissues. Some laboratories use antibiotics to kill the endogenous contaminants. Propagation of *Sipahutar* pineapple with *in vitro* techniques, it need a sterile explant source. This study wanted to obtain optimum sterilization technique for pineapple explants from *Sipahutar*, growth induction and *in vitro* propagation.

Some sterilization techniques of pineapple explant have been published. Sterilization of pineapple explants Simadu by isolating buds and soaking explants in alcohol, 0.2% $HgCl_2$, 15% clorox and 30% clorox, produce plantlets and a little contaminantion (Purnamaningsih *et al.*, 2009).

Other studies, pineapple crown peeled, washed in the running water, soaked in 40% alcohol for 5 minutes. Then sterilization in Laminar air flow cabinet (LAFC) by soaking explants in 10% clorox for 5 minutes, 5% clorox for 3 minutes. Pineapple crown was cut to size 1 cm, soaked in 1% clorox for 1 minutes, rinsed with sterile distilled water three times, then the explants were planted. The explants can be grown with a little contamination (Silvina *et al.*, 2007).

The type and concentration of PGR was the component that determines the success of tissue culture. At the beginning of the growth induction of pineapple from Sipahutar, the explants were cultured on MS medium + 5 mg L^{-1} Kinetin + 0.5 mg L^{-1} IAA. For further propagation, using BAP and IAA. BAP was a class of cytokines that has a role in cell division, stimulate the multiplication of shoots, and stimulate DNA synthesis process (Harahap, 2011). Auxin plays a role in the increased elongation and cell division, supporting the formation of the roots (Zulkarnain, 2009). It is expected that the development of pineapple from Sipahutar can be realized for the development of local resources.

MATERIAL AND METHODS

The study was conducted from June 2012 to December 2013 at the YAHDI Tissue Culture Laboratory and Biology Laboratory UNIMED. The materials used were pineapple crown from Sipahutar, MS medium, alcohol, sterile distilled water, detergent, $NaClO_3$, BAP and IAA. Standard tissue culture tools were used for this research.

Sterilization of explants

Explants were used for each treatment amounted to 36 explants. Explants sterilization was done by throwing the whole pineapple crown leaves, cut the bottom, washing the explants with 5% detergent and brush with a toothbrush and then washed with running water, followed soaked in different sterilizing and different times (Table 1). Between first sterilan, second and so on, the explants were washed with sterile distilled water. Tool used in sterilization procedures 1 to 8 only 1 set, especially for treatment (Yuliarti, 2009), using 3 sets of tools. Clorox was used containing 5.25% Sodium Hypocloride ($NaClO_3$).

Growth induction

After explants were sterilized with each treatments, and then planted in medium MS + 5 mg L^{-1} Kinetin + 0.5 mg L^{-1} IAA to induce early growth. The culture was maintained at 24 °C by regulating the room air conditioner. For main- taining light, flourecent light of 3000 lux tubes were used to maintain 16 hour photoperiod. When explant contamination, the percentage of aseptic cultures, number of regenerated explants, explants showed growth, the explants were necrotic, performance of explants were observed from 1 until 60 days after planting. All the results were analyzed descriptively.

Propagation of *In vitro* Shoots

Treatments applied in the experiment consisted of four Concentrations of BAP (0, 2, 4, 6) mg L^{-1} and three concentration of IAA (0, 0.5, 1) mg L^{-1}. The culture was maintained at 24 °C by regulating the room air conditioner. For maintaining light, flourecent light of 3000 lux tubes were fixed to maintain 16 hour photoperiod. This condition is same as with growth induction treatment.

Time of shoot growth, number of shoot, leaves as well as shoot height were observed weekly from 1 until 12 weeks after planting. Statistical analysis: Using Analysis of varian factorial analysis and continous with DMRT (Duncan multiple rate test) was used with significancy a 5%.

Table 1. sterilization procedure with sterilant name and duration of soaking

Procedures	Treatment	Sterilizing Name and Treatment	Duration (minutes)
1	Outside LAFC	30% Alcohol	5
		0.26% NaClO	3
		0.21% NaClO	3
		0.16% NaCLO	2
	In LAFC	0.05% NaClO (explants cut, 1 cm x 1 cm size)	1
		Sterile distilled water (explants were washed 3 times and planted)	10
2	Outside LAFC	0.04% Mancozeb as Fungicide, 0.01% Streptomycin sulphate as Bactericidal	120
	In LAFC	0.53% NaClO	10
		Sterile distilled water (explants were washed 3 times)	10
		Explants were cut, the size of 1x1 cm, soaked 0.5% amoxilin antibiotics	120
3	Outside LAFC	1.05% NaClO	7 menit
	In LAFC	0.02% HgCl	3 menit
		Sterile distilled water (explants were washed 3 times)	10 menit
		Explants were cut with a size of 1x1 cm, planted	
4	Outside LAFC	1.05% Clorox	7 menit
	In LAFC	0.02% HgCl	10 detik
		Sterile distilled water (explants were washed 3 times)	10 menit
		Explants were cut, the size of 1x1 cm, planted	
5	Outside LAFC	30% Alcohol	5 menit
	In LAFC	0.26% NaClO	3 menit
		0.21% NaClO	3 menit
		0.16% NaClO	2 menit
		10% Alcohol (Explants were cut, the size of 1x1 cm)	1 menit
		Sterile distilled water (explants were washed 3 times) and planted	
6	Outside LAFC	0.04% Mancozeb as fungicide	60 menit
		0.01% Streptomycin sulphate as bactericidal	
		5% Detergen 3 times	15 menit
	In LAFC	1.6% NaClO	10 menit
		Sterile distilled water (explants were washed 5 times)	25 menit
		Whole explant peeled, dried on filter paper and then planted	
7	Outside LAFC	5% Detergen	20 menit
		0.0056% Mancozeb as fungicide	
		0.002% Streptomysin sulphate as bactericidal	
	In LAFC	0.53% NaClO	3 menit
		0.26% NaClO	5 menit
		Sterile distilled water (explants were washed 3 times), explant cut into four and planted	
8	Outside LAFC	0.008% Mancozeb as fungicide	60 menit
		0.002% Streptomysin sulphate as bactericidal	
		5% Detergen, explants were washed 3 times	15 menit
	In LAFC	1.58% NaClO	30 menit
		Sterile distilled water	20 menit
		Sterile distilled water (explants were washed 5 times), peeled surface of explant and planted	
9	Outside LAFC	0.008% Mancozeb as fungicide	60 menit
		0.002% Streptomysin sulphate as bactericidal	
	In LAFC	1.05% NaClO	10 menit
		Sterile distilled water (explants were washed 5 times), peeled surface of explant, dried on filter paper followed planted	

RESULTS AND DISCUSSION

Sterilization of explants and growth induction: Nine sterilization procedure was performed on *Sipahutar* pineapple crown. Various chemical compounds, antibiotics showed an influence on the time of contaminated, the percentage of aseptic culture, explants were necrotic, performance of the explants and growth of explant (Table 2). The type and concentration of antibiotics to reduce contamination affected the development of *Heliconia psittacorum* explants (Cantika, 2006).

The larger explants, reducing damage of tissue, due to the sterilizing treatment. Explants that small size greater damage during handling and culture (Figure 3), because more sensitive to failure during early phase of planted (Harahap *et al.*, 2012). In contrast, larger explant size can reduce the wound explants. The larger explants have supplies of food and growth regulator that helps to initiate growth (Zulkarnain, 2009).

The highest percentage of aseptic cultures (86.1%) was obtained from 1.05% $NaClO_3$ treatment for 7 minutes, followed by 0.02% $HgCl_2$ for 3 minutes and rinsed sterile distilled water 3 times (Procedure 3), but the whole explant was necrosis and no growth.

Explants were treated with $HgCl_2$ necrotic even with low concentrations and short time, all explants were necrotic, brown and undeveloped. Browning was generally a sign of physiological deterioration, this condition ends with the death of explant (Yuliarti, 2010). Treatment by soaking the explants with 0.008% mancozeb as fungicide, 0.002% streptomisin sulphate as bactericide for 1 hour followed by 1.05% $NaClO_3$, then peel the outside explants and put the explants on filter paper to dry, then planted, produced a high number of aseptic culture (55.6%) and showed the highest growth of 22.2% (procedure 9th). High aseptic culture were also obtained from treatment 5th by soaking the explants with 30% alcohol for 5 minutes, 0.26% $NaClO_3$ for 3 minutes, then lowered to 0.21% for 3 minutes, then lowered again to 0.16% for 2 minutes followed by 10% alcohol for 1 minute. Aseptic culture that produced by 52.7% and the explants showed growth of 22.2% (Table 2).

This research showed that the pineapple from *Sipahutar* could not stand with the $HgCl_2$ sterilizing even very low dose (0.02%) for 10 seconds. This research showed that $HgCl^3$ could be damage explants and cause maturating tissue (Figure 4). In contrast to studies of culture propagation through meristem culture, treatment of 0.1% mercuric chloride for 4 minutes most effectively sterilized the surface of the explants, explants produce maximum of live explants and minimum of damaged explants (Rattanpal *et al.*, 2011). Strawberry cultivation using shoot tips and nodal segments, surface sterilization with 0.1% mercuric chloride for 4 minutes was the optimum time to produce a high percentage of explants (Jan *et al.*, 2013).

Treatment peel the outer explants after sterilization (procedure 8th), resulted an increase the number of sterile explants (47.2%), reducing necrosis and explants showed growth of 19.4% (Table 2). If the treatment followed by drying explants on filter paper before being planted, sterile explant increased to 52.7% and that showed growth to 22.2% (procedure 9th, Table 2).

This study showed that contamination of pineapple explants from indigenous Sipahutar was very high. Contaminants indegenous too high. Explants were initially sterile, after 40 days of planting, still experiencing contamination, the mucus comes out from the inside of the explants. Mucus was a highly concentrated of complex phenolic compounds, which causes the explant

Table 2. Results of sterilization with the percentage of aseptic cultures, explants that growth and necrosis explant

Prosedures	Contamination (Day)	Aseptic culture (%)	Explant Growth (%)	Explant necrosis (%)	Perfomace of exsplants
1	14	38.89	5.56	11.1	Brown explants
2	10	-	-	-	The whole explant moldy
3	35	86.1	-	86.1	Brown explants
4	42	41.67	-	41.67	Most explants brown wrinkled
5	40	52.7	22.2	0	Swelling and greenish
6	34	44.4	-	66.7	At the day 5th brown explants
7	30	38.9	5.6	55.6	day 8th brown explants
8	32	47.2	19.4	0	Swelling and greenish
9	40	55.6	22.2	0	Swelling and greenish

Fig. 1 Map of Sipahutar District of North Tapanuli, North Sumatra, Indonesia

Fig. 2 Pineapple (*Ananas comosus* L.) plant from *Sipahutar*

was difficult to be cultured. So that explant will be browning. Browning was an adaptive change due to the influence of the physical and biochemical effects (Zuraida *et al.*, 2011). $NaClO_3$ 5%, 3% and 1% was used to keep the surface of the explants, so it did not show browning (Zulkarnain, 2009).

Sterilization treatment causes the explants to turn brown and show no growth (procedures 1, 2, 3, 4, 6, 7) unless the sterilization procedure 5, 8 and 9 (Figure 4). Browning was generally a sign of physiological deterioration of explants, this condition will be ends with explant death (Yuliarti, 2010).

Shoots appeared from all types of explants treated with BAP faster (2-3 weeks after planting) than that of the untreated explant (3-6 week after

Fig. 3 Pineapple explant from *Sipahutar* after sterilization.
a. Explants measuring 1cm, **b.** Explants were quartered, **c.** The whole explant

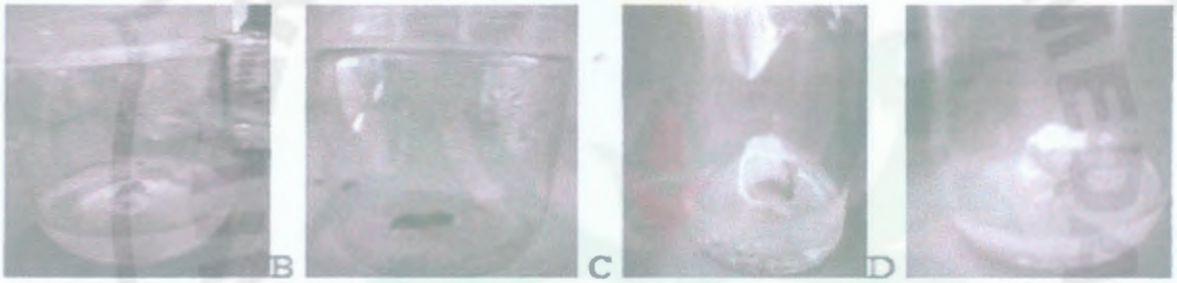

Fig. 4 a) Explant contamination, b). Explants survived until 7 week after planting, browning and no grow, c). Explants began to swell, from 9th procedures, d) explants began to swell, from 5th procedures.

planting). The smaller the explant the longer time for shoot appearance.

Time of shoot induction - Giving BAP accelerate the appeared of shoots. Shoots appeared 2 weeks after planting, resulting from the treatment of 2 mg L^{-1} BAP, faster than the other treatments, started at 3 to 7 weeks after planting (Figure 5). Growth of shoot occurs as a response to plant growth regulator substances that contained in explants and in the media.

Number of shoot: was significanly affected by interaction between BAP and IAA concentrations at 12 weeks after planting. The highest number of shoots produced from the treatment of MS medium + 2 mg L^{-1} BAP + 0 mg L^{-1} IAA which were 4.5 shoots (Figure 6). This research showed that BAP is a plant growth regulator for shoots induction. Cytokinin can stimulate an increase of the shoot number (Harahap *et al.*, 2012). This observation showed that, high concentration (6 mg L^{-1}) BAP, generally caused swelling explants before growth and produced many initial bud, but shoots appeared stunted (Figure 7). There was an optimum concentration of BAP for induction of pineapple shoot from *Sipahutar*. For mangosteen, 2 mg L^{-1} BAP Stimulates the formation of shoots with 2 cm in size explant (Harahap *et al.*, 2012). Other research reported that, the high BAP concentration induced more shoot pineapple than the lower concentration (Zuraida *et al.*, 2011). MS medium supplemented with 5 µM BA gave the highest number of pineapple shoots (Usman *et al.*, 2013). High frequency plant regeneration of pineapple (*Ananas-comosus* (L.) Merr, was affected by cytokinine (Akbar *et al.*, 2003), as well as micro-propagation of pineapple hybryd pexsc-52 and cultivar smooth cayenne were affected by cytokinine (Barboza *et al.*, 2004). MS medium with 0.5 mgL^{-1} BAP gave highest shoot proliferation of *Pelargonium radula* from nodal segments (Zuraida *et al.*, 2012). WPM medium with 0.5 mg L^{-1} BAP concentration gave the highest percentage mangosteen shoot from nodular callus (Qosim, 2007). BAP medium with 1 mg L^{-1} or 2 mg L^{-1} combined with *Ruscus* 0.2 mg L^{-1} produced the highest shoot of *Ruscus*. Increasing concentration of BAP more than 4 mg L^{-1} decreased number and size of adventitious shoots (Agus *et al.*, 2005). IAA and BAP affected development of RAJA NANGKA banana nodul (Musa AAB) by *in vitro* (Rainiyati and Kristiana, 2009).

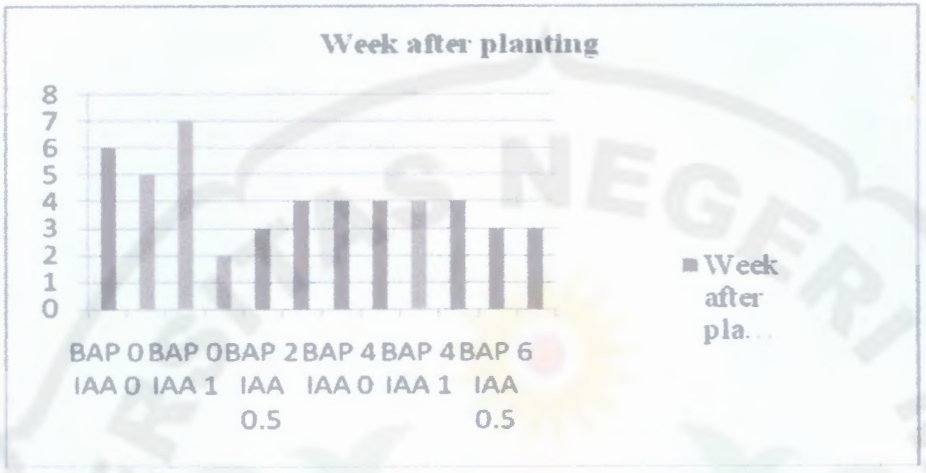

Fig. 5 Time of bud appearance with BAP and IAA treatment on the pineapple explant from *Sipahutar* (*Ananas comosus* L.)

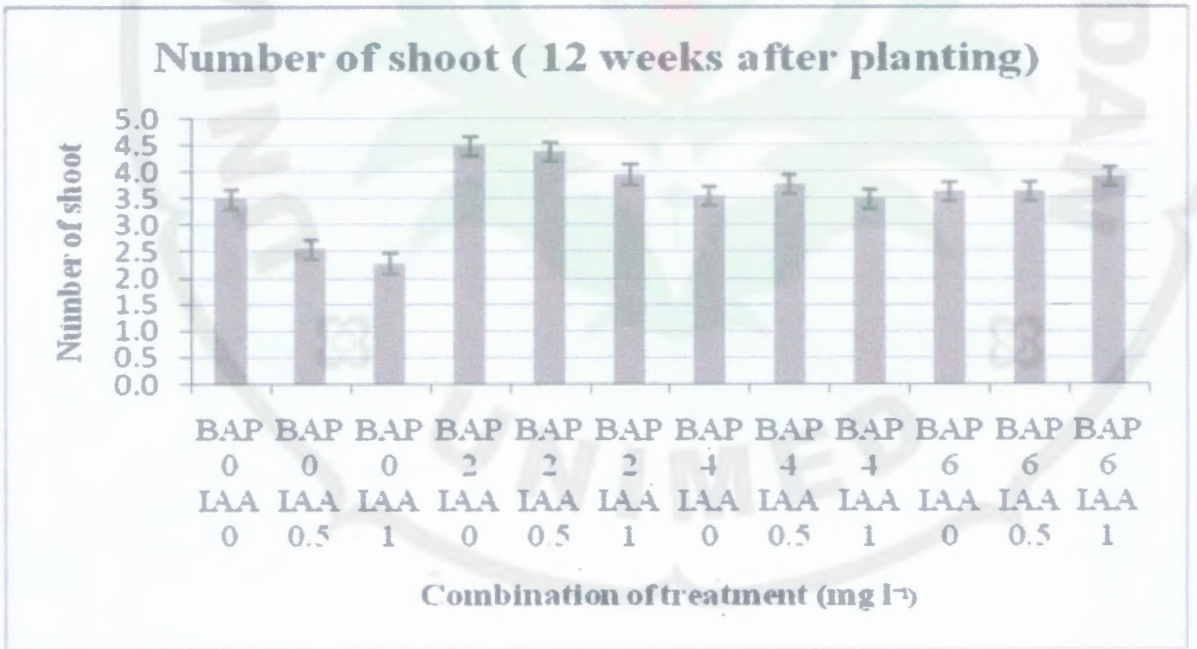

Fig. 6 The number of shoots results BAP and IAA treatment on the pineapple explant from *Sipahutar* (*Ananas comosus* L.)

Number of leaves was significanly affected by interaction between BAP and IAA concentrations at 12 weeks after planting. The highest number of leaves produced from the treatment of BAP 2 mg L^{-1} + IAA 0 mg L^{-1} with 7.79 number of leaves (Figure 8). Addition of BAP 3.54 and 2.98 ppm without IAA to *in vitro* MS medium in the first experiment, significantly increased number of leaves (52.53) at 5 weeks after planting (Nirwan and Aziz, 2010). WPM Medium with 2.0 mg L^{-1} BAP induced shoot and leaf better than the control (Lizawati *et al.*, 2009). Plant growth regulator could improved of *Catharanthus roseus* micropropagation (Rupesh *et al.*, 2013).

Shoots height- BAP affected to shoots height, IAA had no effect on shoot height (12 weeks after planting). The highest shoot resulting from the treatment of 4 mg L^{-1} BAP + 0 mg L^{-1} IAA with 1.7 cm shoot height (Figure 9).

Fig. 7 Performance of *in vitro* pineapple shoots from *Sipahutar*, **a).** 0 mg L^{-1} BAP, 0 mg L^{-1} IAA, **b).** 2 mg L^{-1}BAP, 0 mg L^{-1} IAA, **c).** 6 mg L^{-1} BAP, 1 mg L^{-1} IAA, the shoots appeared stunted (12 weeks after planting)

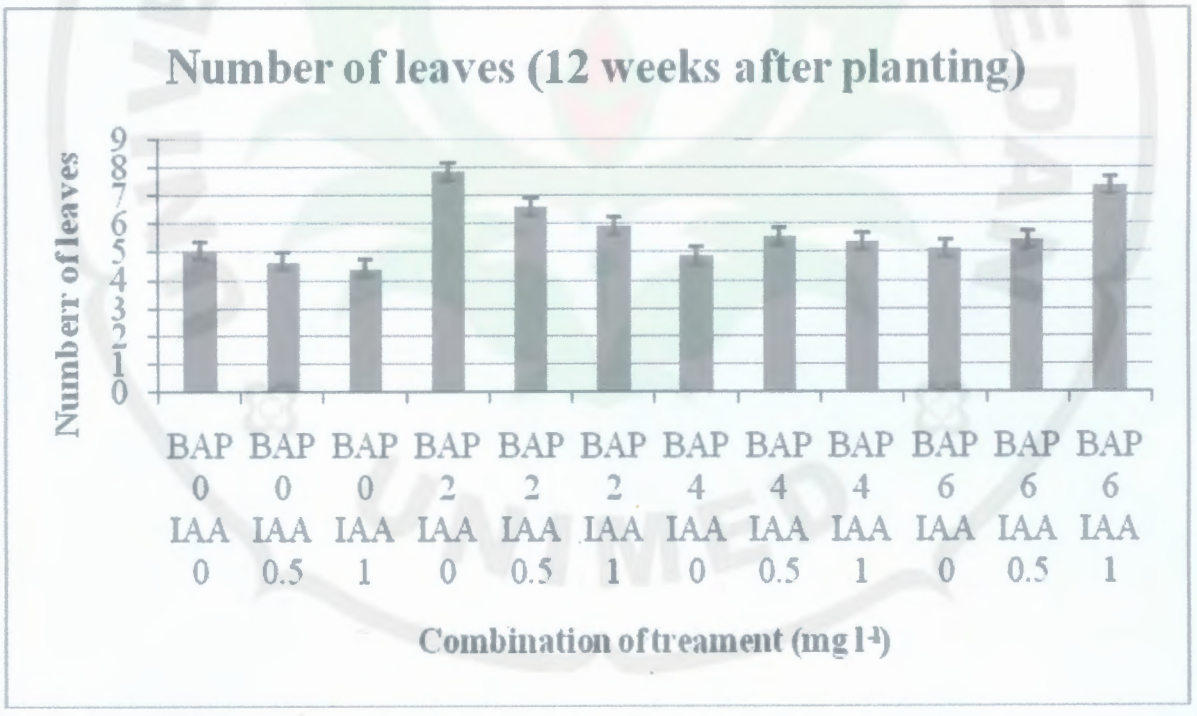

Fig. 8 The number of leaves results BAP and IAA treatment on the pineapple explant from *Sipahutar* (*Ananas comosus* L.)

Cytokinin can increase cell division, but for the elongation of cells required addition auxin, auxin must be present. In this research, IAA had no effect on shoot height. The possibility needed combination of a certain concentration between BAP and IAA to induce and increase research shoot height of pineapple from *Sipahutar*. Proliferation and growth of pineapple (*Ananas comosus* L. Merr cv smooth cayenne was affected by BAP (Hamad and Taha, 2008). Another research reported, NAA and BAP affected to growth of Garlic meristem tissue on B5 mediun (Karjadi and Buchori, 2007). Several on banana (*Musa paradisiaca* L.) have been done treatment with NAA and Kinetin and showed that NAA and kinetin affected length of banana (Nisa and Rodinah, 2005).

CONCLUSION

Various sterilization treatments yielded aseptic cultures. Treatment by soaking the explants with a 0.008% mankozeb, 0.002% streptomycin sulphate for 1 hour followed by 1.05% $NaClO_3$, followed peel the outside of explants and put on filter paper to

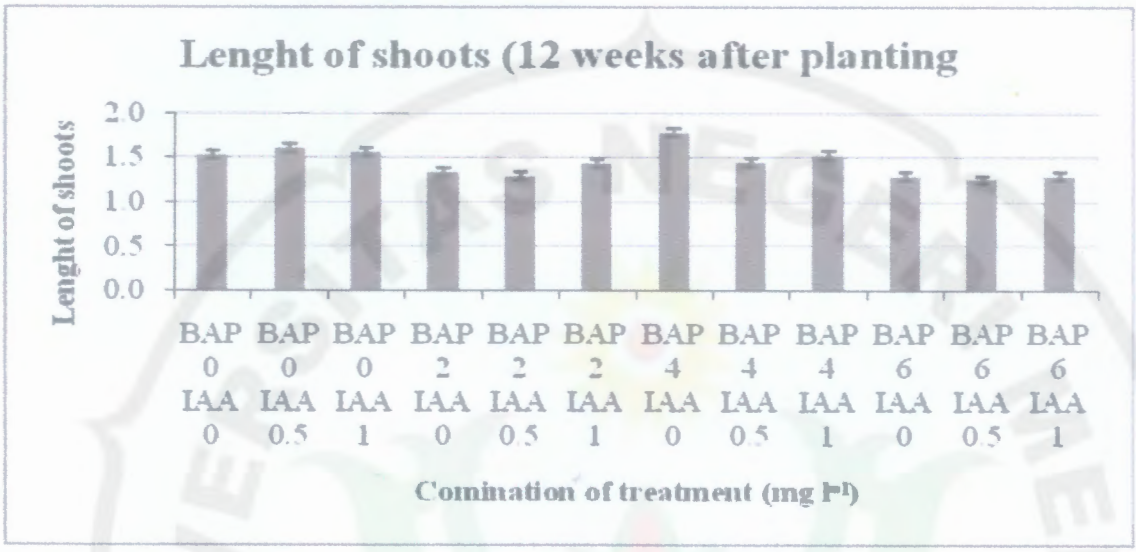

Fig. 9 Average shoot height results BAP and IAA treatment on the pineapple explant from *Sipahutar* (*Ananas comosus* L.)

dry and then planted, produced high aseptic culture and highest growth.

Pineapple explants from *Sipahutar* treated with $HgCl_2$ even with low concentrations and short time, all explants were necrotic, brown and undeve- loped.

Giving of BAP and IAA Significantly affected the appearance of shoots, number of shoots, number of leaves except shoots high. The best treatment was a combination of MS + 2 mg L^{-1} BAP+0 mg L^{-1} IAA.

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