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In Vitro Propagation of *Anthurium adreanum* cv. Nitta through Organogenesis

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ABSTRACT

Anthurium adreanum Lindl. cv. Nitta is an ornamental plant for cut flower industries. *In vitro* propagation enables a large scale production of high quality seedlings of *A. adreanum* quickly. The aim of this experiment was to overcome some problems in the *in vitro* propagation of *A. adreanum*, such as contamination, lack of plantlets vigor and low rate of survival in the acclimatization. *In vitro* propagation of *A. adreanum* was conducted via organogenesis from leaf explant. Explant sterilization using desogerm, antioxidant, alcohol, rifampicin, and NaOCl was the best because they reduced browning and contamination until 0 %. Organogenesis of *A. adreanum* was successfully initiated using MS medium added with 2,4-D and BAP at 1 mg L^{-1}, which produced 74 shoots per explant. Shoots were best maturated in MS medium with a half strength of macro minerals added with 1 mg L^{-1} paclobutrazol. Survival rate increased by 7 and 14 days hardening treatment using double layer medium in the light culture room. Survival rate reached 89.3 % at 8 weeks after acclimatization.

Keywords: *Anthurium adreanum*; hardening; orga nogenesis; paclobutrazol; sterilization

INTRODUCTION

Anthurium adreanum Lindl. is an ornamental plant from the family of *Araceae*. This plant has been developed commercially in tropical and subtropical countries. This plant can grow on the temperature of 16 ^{o}C – 30 ^{o}C (Dufour & Guérin, 2003). Global market of *Anthurium* cultivars are the second among all tropical cut flower after orchids. Flowers of *A. adreanum* are used as cut flower because they are sufficiently thick and long lasting (Agampodi & Jayawardena, 2007). *A. adreanum* has flowers in various sizes and colors with long flower stalks. *A. adreanum* cv. Nitta is one of *A. adreanum* cultivars from Indonesia which has quite wide bright red flowers. It has a large market in Indonesia because of its bright color.

Anthurium adreanum is an evergreen plant that can be propagated by seeds. However, propagation by seeds produced a high variable offspring and very slow (Vargas, Mejías, Oropeza, & de García, 2004). Seed maturation takes about three years from the pollination. Propagation by conventional cutting also requires considerable time. Therefore, to suffice the market demand of these cut flowers, it can be done by tissue culture or called *in vitro* propagation. By *in vitro* propagation, a high quality and pathogen-free of *A. adreanum* seedling can be produced in a large scale and with the same quality efficiently.

Some *in vitro* propagation methods of *A. adreanum* had been reported successfully by using explants from leaves (Atak & Çelik, 2009; Bejoy, Sumitha, & Anish, 2008; Yu, Liu, L., Liu, J. X., & Wang, 2009), petiole (Viégas et al., 2007), shoot tips (Dhananjaya & Sulladmath, 2006), and nodes de Lima et al. (2006). *In vitro* propagation of *A. adreanum* was done via indirect organogenesis, which is initiating shoots from callus. Steps in this method are sterilization, callus induction, shoot initiation, multiplication, maturation, rooting and acclimatization.

Sterilization is the process to eliminate the contaminant from the explant. In tropical countries, contamination is a serious problem that often occurs on the *in vitro* propagation (Nurhaimi-Haris, Sumaryono, & Carron, 2009). Sterilization using a single agent mostly did not succeed. So in this research, we conducted experiment on sterilization steps using more than one sterilant agents to get the best procedure to the sterilization of *A. adreanum* leaves.

Callus of *A. adreanum* can be induced by the addition of auxin on the medium, or combination of auxin and cytokinin. Medium for callus induction of *A. adreanum* from leaf explant was already well established based on many reports. Bejoy, Sumitha, & Anish (2008) succeeded in inducing

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callus by 53 % from leaf explants using MS medium supplemented with BAP (6-benzylaminopurine) at 1 mg L^{-1} and 2,4-D (2,4-dichlorophenoxy acetic acid) at 0.5 mg L^{-1}. Zhao, Guo, Wang, & Wen (2004) succeeded in inducing callus from leaf explants by using MS medium supplemented with BAP at 1 mg L^{-1} and 2,4-D at 0.1 mg L^{-1}. Atak & Çelik (2009) also succeeded in inducing callus by 81.25 % from leaf explants of "Arizona" and "Sumi" cultivars by using BAP at 1 mg L^{-1} and 2,4-D at 0.6 mg L^{-1} on the MS basal medium with a half strength of macro minerals.

Initiation and multiplication of *A. adreanum* shoots can be done by using cytokinin or its combination with auxin. Medium for shoot initiation of *A. adreanum* was also already well established based on many researches. It used hormones such as BAP (Vargas, Mejías, Oropeza, & de García, 2004), Thidiazuron (Yu, Liu, L., Liu, J. X., & Wang, 2009), and Kinetin (Dhananjaya & Sulladmath, 2006). The multiplication rate of *A. adreanum* shoots was very high. About 33-44 shoots could be produced per explant (Atak & Çelik, 2009; Vargas, Mejías, Oropeza, & de García, 2004).

The problem is that because of the high multiplication, the shoot vigor was poor. Vigorous plantlets are needed in the acclimatization in order to survive. Plantlet vigor can be maintained by using minerals and hormones in the right concentration (Hardarani, Purwito, & Sukma, 2012), modifying culture system, or using retardants such as paclobutrazol or ancimidol (Sumaryono & Sinta, 2011). There were also some reports regarding the effect of carbon and light intensity on the growth of *A. adreanum* plantlets (Gu et al., 2012; Stancato & Tucci, 2010). However, the information regarding the role of mineral concentration in culture medium to the plant growth is still limited. A plant needs enough minerals to grow well. Macro minerals are needed in high concentration, while micro minerals are needed in low concentration but must exists. Plants with minerals deficiency will show many symptoms, such as lack of growth, chlorosis, and susceptible to disease. However, over concentration of minerals can be toxic to the plant growth (Hodges & Constable, 2010). In this experiment, to increase the vigor of *A. adreanum* plantlets we used paclobutrazol added in the MS basal media with various concentrations of macro minerals and studied its effect on the vigor of *A. adreanum* plantlets.

Beside maintained plantlets vigor, acclimatization is also the important and critical step on the propagation of *A. adreanum* by tissue culture. Acclimatization is a step when plantlets are transferred from *in vitro* culture in the laboratory to *ex vitro* environment in the greenhouse. *In vitro* environment is very humid. In the acclimatization, plantlets must adapt and survive in the *ex vitro* environment with a low humidity. Hardening treatment is needed for plantlets before acclimatization to increase the survival rate. Hardening can be done by planting plantlets on the gas-permeable vessels (da Silva, Nagae, & Tanaka, 2005) or incubate the culture in the greenhouse (Cardoso, Rossi, Rosalem, & da Silva, 2013) in order the plantlets to adapt with the environment in a lower humidity. In this experiment, we studied the effect of hardening treatment in laboratory and in the greenhouse and the optimum periods to increase survival rate of *A. adreanum* plantlets.

MATERIALS AND METHODS

Preparation and Sterilization

Experiment was conducted in Laboratory of Cell Culture and Plant Micropropagation, Indonesian Research Institute for Biotechnology and Bioindustry. Newly opened leaves with light green color were used as explants. Mother plants were planted in the greenhouse to reduce contamination before using as explant source. Explants were cut and sterilized with three methods, (1) rinsed with desogerm, soaked in antioxidant solution for 2 hours, soaked in 50 % Benomil solution for 15 minutes, and soaked in 20 % of NaOCl solution for 15 minutes; (2) rinsed with desogerm, soaked in antioxidant solution for 2 hours, alcohol for 3 seconds, rifampicin solution for 30 minutes, and then 20 % of NaOCl solution for 15 minutes; (3) rinsed with desogerm, soaked in alcohol for 3 seconds, rifampicin solution for 30 minutes, and then 20 % of NaOCl solution for 15 minutes.

Antioxidant solution was made from ascorbic acid (1 mg L^{-1}) and citric acid (1 mg L^{-1}). Each sterilization step from one sterilant to other sterilant was followed by rinsing with sterile aquadest. Afterwards, the sterile explants were cut (0.5 cm x 0.5 cm), then inoculated at medium for callus induction on the petridish. Medium were sterilized after pH adjustment on autoclave 1 kg cm^{-3} for 20 minutes. Each petridish contain 5 pieces of explants. Each treatment has 10 replications.

Callus Initiation and Shoot Regeneration

In this experiment, callus initiation and shoot regeneration were only used single composition of media based on the established method reported. A callus initiation followed the method by Atak & Çelik (2009). Sterile explants were inoculated on the MS basal medium with a half strength of macro minerals enriched with 2,4-D (0.6 mg L^{-1}); BAP (1 mg L^{-1}); thiamine-HCl (0.5 mg L^{-1}); folic acid (0.5 mg L^{-1}); biotin (0.05 mg L^{-1}); and sucrose (20 mg L^{-1}). The culture was incubated in the dark room until callus was formed. The time of callus formation and color of callus were observed.

Formed callus was subcultured to the medium with the same composition as previous, but with the less concentration of 2,4-D. Culture was incubated on the light room with 36watt fluorescent tube on the temperature of 25 ± 1 ^{o}C, and 12 hours photoperiod until shoot was formed on the callus propagule. Number of shoots per propagule (2 x 1 cm) was calculated using handy counter with a destructive observation. It used 20 propagules as a replication.

For shoot multiplication, propagules were cut in size of 0.5 x 0.5 cm and subculture to medium with the same compositions as those for shoot initiation on the bottle with 30 ml volume of medium. Culture bottle was incubated in the light room and continuously sub-cultured every 6 weeks.

Maintaining Plantlets Vigor

Plantlets with a minimum height of 2 cm were separated from the propagule and sub-cultured to maturation medium. Five plantlets were sub-cultured to MS basal medium with 0.5; 1.0; 2.0; 4.0 time(s) concentration of macro minerals, enriched with sucrose (30 g L^{-1}), and paclobutrazol (0; 1 mg L^{-1}). Each treatment has five of replications. Culture was incubated in the light room for 4 weeks. Parameters of plantlet vigor such as height, number of node, stem diameter, number, color, and size of leaves were calculated from mature plantlets after 4 weeks of incubation on the maturation medium. Plantlets height was measured from the basal stem to the shoot tip. Stem diameter was measured about 0.5 cm from the basal stem using calipers. Size of leaf was the length multiplied with the width.

Hardening

Mature plantlets were hardened on the MS medium with sucrose (30 g L^{-1}), IBA (indole-3- butyric acid) (1 mg L^{-1}), and active charcoal (4 g L^{-1}). It used double layer medium (10 ml of solid medium on the bottom, and 2.5 ml of liquid medium on the top) on the test tube, covered with two layers of plastic wrap. Culture was incubated in the light culture room and in the green house for 0; 4; 7; 14 days. Each treatment has 28 of replications.

Acclimatization

Plantlets from hardening medium were removed from the test tube and were cleared with tap water. Plantlets, which have been cleaned, were then soaked in fungicide solution for 5 minutes. Afterwards, plantlets were planted on *multitray* with a hole size of 3 cm x 3 cm containing soil, dung manure, cocopeat (1:1:1). The seedlings were then placed in the greenhouse and covered with UV plastic lid for 4 weeks. After 4 weeks, the lid was opened gradually and seedlings were then grown in the nursery. Survival rate was observed by the number of plantlets survived for each treatment.

Statistical Analysis

Completely randomized design was used for this experiment. Data were then subjected to analysis of variance (ANOVA), followed by Duncan Multiple Range Test (DMRT), which differences at $p \leq 0.05$ considered to be significant. Analysis was conducted using SPSS Statistics software package.

RESULTS AND DISCUSSION

Sterilization

Sterilization process on the *in vitro* culture of *Anthurium adreanum* cv. Nitta was determined by sterilant agent, steps of sterilization, and the explants. It used newly opened young leaves instead of leaf buds to ensure that all sterilant could reach the entire surface of explants. Young leaves are susceptible to browning, so antioxidant solution was used on the sterilization to prevent browning. However, in this experiment browning did not occur whether antioxidant was used or not (Table 1).

Based on Table 1, the first sterilization method using desogerm, antioxidant, benlate fungicide and NaOCl was not effective as 100 % contamination occurred after 4 weeks. However, the second method was the most effective for sterilization of *A. adreanum* leaf explants as it reduced contamination until 0 % after 2 weeks and 4 weeks. The second method used desogerm, antioxidant, Rifampicin and 20 % NaOCl. The third method, although used Rifampicin, it could not reduce contamination. The use of antioxidant with Rifampicin was effective for sterilization of *A. adreanum* leaf explants.

Table 1. Effect of sterilant agent on the sterilization of Anthurium adreanum cv. Nitta leaf explants

Sterilant agent	Browning (%)	Contamination on 2nd week (%)	Contamination on 4th week (%)
Desogerm, antioxidant, Benomil, NaOCl	0	60	100
Desogerm, antioxidant, alcohol, Rifampicin, NaOCl	0	0	0
Desogerm, alcohol, Rifampicin, NaOCl	0	20	90

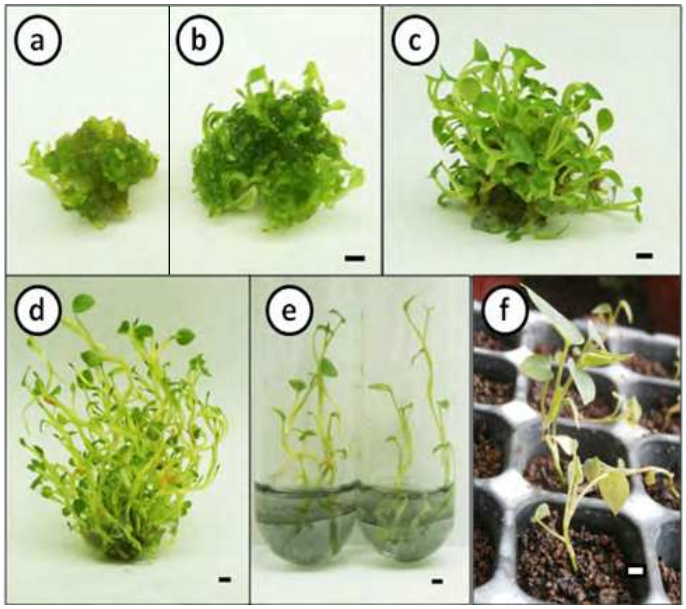


Fig. 1. Organogenesis of *A.adreanum* cv. Nitta : Green callus (a), early shoot (b), shoot multiplication (c), shoot ready for maturation (d), hardening on the double layer medium (e), seedling after 8 weeks of acclimatization (f). Bar = 0.5 cm

Rifampicin is an antibiotic that is often used for sterilization on the in vitro culture (Eziashi et al., 2014). Rifampicin was used for sterilization of *A. adreanum* leaf explants to prevent contamination from endogenous bacteria. Low concentration of antibiotics could prevent contamination without causing damage to explant cells. However, if it was used in high concentration more than 1 0.5 mg L^{-1} and long duration exposure, antibiotics could inhibit the growth of explant cells. Therefore, in this experiment, explants were exposed on the rifampicin solution (0.5 mg L^{-1}) only for 30 minutes. Also, the use of antibiotics was better than a very harmful sterilant agent such as $HgCl_2$. da Silva, Winarto, Dobránszki, & Zeng (2015) reported that the sterilization of *A. adreanum* explants using 0.01 and 0.05 % $HgCl_2$ could minimize contamination until less than 10 %. However, because of its toxicity, it needed to be extra careful using this sterilant agent. Therefore, the use of antibiotics instead of $HgCl_2$ was safer in the explants sterilization.

Callus and Shoot Formation

Callus were formed after 8 weeks of incubation in the dark room. Callus were formed on the edge of explants. Callus then transferred to the light culture room soon proliferate into greenish propagule (Fig. 1a). Afterwards, shoots were formed from the propagule after 4 weeks (Fig. 1b). One hundred percent of propagule formed shoots. Shoot formation was induced by a combination of auxin and cytokinin. Experiment by Vargas, Mejías, Oropeza, & de García (2004) used combination of NAA and BA could produce on average 44 shoots per explant of *A. adreanum* cv. Rubrun. While in this experiment, the combination of 2,4-D and BAP at 1 mg L^{-1} effectively stimulated shoot formation up to on average 75 shoots per explant (2 x 1 cm) of *A. adreanum* cv. Nitta (Fig. 1c). Experiment conducted by Atak & Çelik (2009) reported that with the same composition of medium for shoot formation, produced on average 34 shoots per explant of *A. adreanum* cv. Arizona and 26 shoots per explant of *A. adreanum* cv. Sumi. Thus, *A. adreanum* cv. Nitta produced twice more shoots on the same medium composition.

The shoots have poor vigor because of their high rate of multiplication (Fig. 1d). The shoots have an average height of 1.69 cm, 0.07 cm of stem diameter, and 3 leaves with a size of 0.3 x 0.2 cm (0.06 cm^2). That poor vigor affected survival rate in the acclimatization. Plantlet vigor related to how well the plantlets grow in the culture medium and environment. Plantlets which were able to grow optimally in the in vitro culture, their organs also grew well such as having a larger stem diameter, more nodes and relatively larger and greener leaves. Good vigor was needed not only for acclimatization purpose but also to keep in vitro culture stock remain sustainable. So, plantlet vigor of *A. adreanum* cv. Nitta was maintained in this experiment by using combination of macro minerals concentrations of the MS basal medium and paclobutrazol to increase plantlet vigor of *A. adreanum* cv. Nitta.

Plantlet Vigor

In this experiment, plantlet vigor was maintained in the maturation medium using different concentrations of MS macro minerals and paclobutrazol. MS basal medium with half (1/2x) concentration of macro minerals was used as control because it had been used for callus initiation until shoot formation. Concentration of macro minerals and paclobutrazol affected vigor of *A.adreanum* plantlets. Plantlets which had a good vigor were mainly indicated from the big green leaves and wide stem. Paclobutrazol were used to inhibit stem elongation to make plantlets more vigor. The use of paclobutrazol on the maturation medium only reduced plantlets height on the 1/2x concentration of MS macro minerals (Table 2).

The highest average number of nodes were grown on the full (1x) concentration of MS macro minerals with paclobutrazol. The highest average number of leaves were grown on the 1x concentration of MS macro minerals with or without paclobutrazol. The highest average size of leave was grown on the 1/2x concentration of MS macro minerals with paclobutrazol, and 2x concentration of MS macro minerals without paclobutrazol. However, on the double (2x) concentration of MS macro minerals without paclobutrazol, the leaves color was green to yellow, while the 1/2x concentration of MS macro minerals with paclobutrazol the leaves color was fresh green (Fig. 2). The yellow color of leaf could be the indication of leaf senescence, mineral deficiency, or toxicity due to excess mineral on the plant.

The highest average diameters of stems were grown on the 4x concentration of MS macro minerals with paclobutrazol. It has the highest average height of plantlets and stem diameters, but in the Fig. 2, showed that the leaves were mostly small and browning. Double concentration of MS macro minerals without paclobutrazol also increased stem diameters, number and size of leaves, but some leaves were yellow. Based on all parameters measured, plantlets of *A. adreanum* cv. Nitta had the best vigor when grown on the 1/2x concentration of MS macro minerals with 1 mg L^{-1} paclobutrazol. Plantlets grown in this medium composition had the lowest plantlets height, but with relatively more nodes, large stem diameters, highest number and size of leaves and greener.

Table 2. Effect of paclobutrazol and macromineral concentration on the *Anthurium adreanum* cv. Nitta plantlets vigor after four weeks of culture

Concentration of paclobutrazol (mg L-1)	Concentration of macro mineral	Height (cm)	Number of nodes	Number of leaves	Size of leaves $(cm)^2$	Colour of leaves	Stem diameter (mm)
0	1/2x (control)	3.2 ab	5 b	6 abc	0.4 abc	Green-yellow	0.09 ab
	1x	3.3 ab	5 ab	6 bc	0.4 abc	Green	0.08 b
	2x	3.1 ab	5 ab	7 a	0.5 ab	Green-yellow	0.10 ab
	4x	3.4 ab	4 b	5 c	0.3 c	Yellow-brown	0.07 b
1	1/2x	3.0 b	5 ab	7 ab	0.5 a	Green	0.09 ab
	1x	3.5 ab	6 a	7 ab	0.4 abc	Green-yellow	0.08 b
	2x	3.4 ab	5 ab	7 a	0.3 bc	Green-yellow	0.09 ab
	4x	3.6 a	5 ab	6 abc	0.4 abc	Green-brown	0.12 a

Remarks: Same letters in the same column indicates no significant means differences according to Duncan's multiple range test at P = 0.05

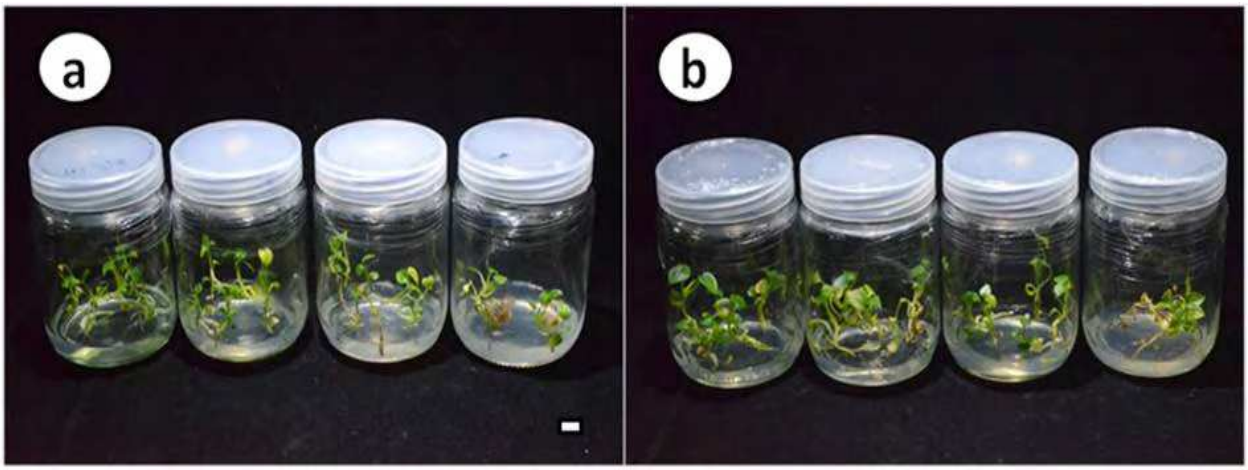


Fig. 2. Effect of paclobutrazol and macro mineral concentration on the *A. adreanum* plantlets vigor after 4 weeks of culture: without paclobutrazol (a) and with paclobutrazol (1 mg L-1) (b). Each picture, from left to right: 1/2x macro, 1x macro, 2x macro, and 4x macro. Bar = 1 cm.

Half concentration of MS macro minerals was an optimal concentration to maintain plantlet vigor of A. adreanum cv. Nitta compared to other concentrations. The result was similar to an experiment by Hardarani, Purwito, & Sukma (2012) that plantlets of Hydrolea spinosa L. planted on the MS medium with 1/2 concentration of macro minerals have the highest biomass. When paclobutrazol was supplemented to MS medium with 1/2 concentration of macro minerals, it produced the best plantlet vigor of A. adreanum.

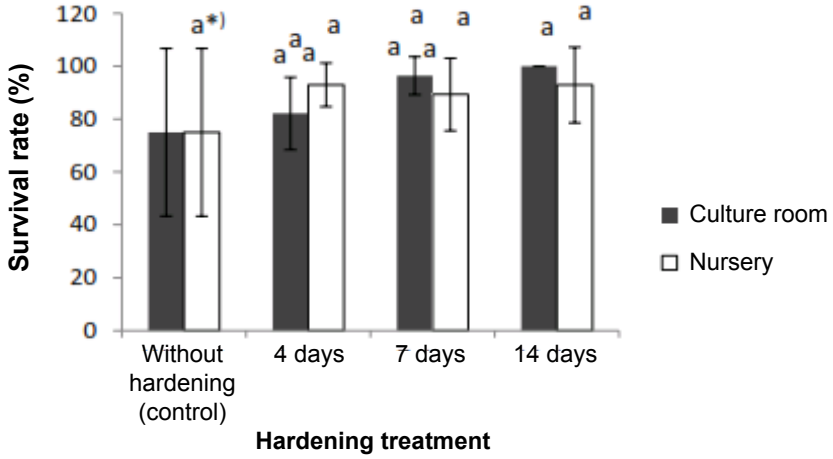
Paclobutrazol is a plant growth retardant which can cause dwarf in plants. Dwarf plants usually have a better vigor than normal plants. Paclobutrazol inhibited biosynthesis of gibberellic acid (GA) that regulated stem elongation on plants (Hedden & Graebe, 1985). The stem cannot elongate by the inhibition of GA in its cells but it could be more vigor. Paclobutrazol has some positive effects on plants. Nouriyani, Majidi, Seyyednejad, Siadat, & Naderi (2012) reported that application of paclobutrazol on the wheat increased stem diameter, produced more green leaves by increasing the chlorophyll content. Hendrati (2008) also reported that in Eucalyptus, application of paclobbutrazol increased biomass and induced reproductive phase. The use of paclobutrazol on the in vitro culture to maintain plantlet vigor can increase survival rate on the acclimatization and keep sustainability of in vitro culture stock.

Survival Rate

Hardening on the double layer medium did not have a significant effect on the survival rate of *A. adreanum* cv. Nitta plantlets after four weeks of acclimatization in the nursery (Fig. 3). Survival rate was about 75-100 %. After that, the survive seedlings from nursery were transferred to the greenhouse. On the 8 weeks after acclimatization, survival rate decreased for all treatments (Fig. 4). The lowest survival rate was from 4 days hardening in nursery (32.2 %), in the culture room (42.9 %), and 7 days hardening in nursery (46.5 %), while without hardening survival rate was 53.6 %. The highest survival rate was from 7 days hardening in the culture room (89.3 %) and 14 days hardening in the culture room (82.1 %). Overall, hardening of *A. adreanum* cv. Nitta plantlets in the culture room produced higher survival rate than in the nursery.

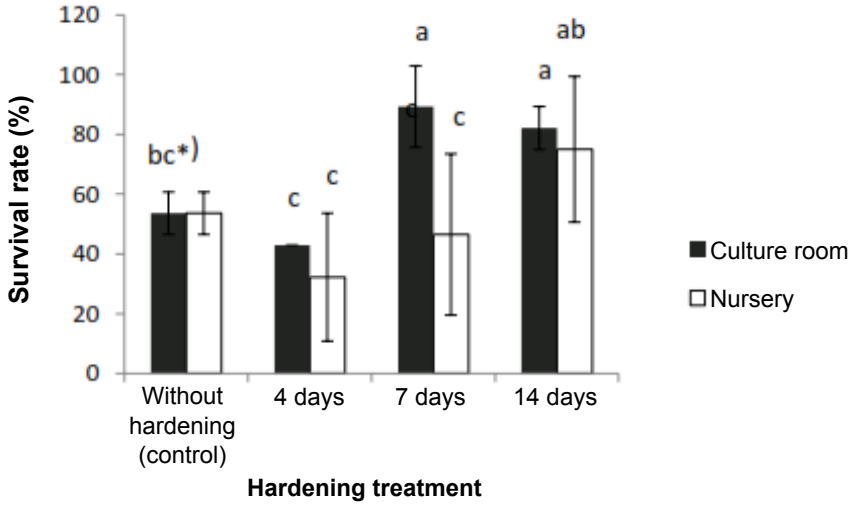
Hardening is a treatment to conditioning *in vitro* planlets before acclimatization. In the *in vitro* condition, sugar as an energy source for the plant metabolism is already available in the culture medium. So that *in vitro* plants do not need to make sugar from CO_2 and water to produce energy. Moreover, the environment of *in vitro* culture has its temperature optimized for plant growth. The microclimate on the vessels is very humid, different from natural condition where the humidity is very fluctuating. In the *in vitro culture*, plants do metabolism as heterotrophic organism. But,

when *in vitro* plants or plantlets are acclimatizated to the *ex vitro* environment, they should start doing autotrophic metabolism and adapt to the environment with high temperature and low humidity that causes high evaporation. This different condition can make plantlets stressed and fail to survive on the nursery. Therefore, hardening can help plantlets to begin the adaptation to the *ex vitro* environment. In this experiment, plastic wrap was used as culture closures. Based on the experiment by Sinta, Riyadi, & Sumaryono (2011), the use of plastic wrap as culture closures provided the best aeration in the *in vitro* culture of oil palm. It made gas exchange from the inside to the outside of culture vessels to be more intense, so that the moisture could be reduced. However, to compensate with the high evaporation, double layer medium was used for hardening (Fig. 1e) so that plantlets were not completely dried. Seven days of hardening in the culture room was the best treatment to increase survival rate of *A. adreanum* cv. Nitta plantlets. A longer time of hardening decreased survival rate due to higher evaporation rate. Plantlets that were not completely adapted, experienced a water loss, some dropped their leaves, which caused a low survival rate on the acclimatization.



Remarks: *) Same letters indicates no significant differences according to Duncan's multiple range test (P = 0.05)

Fig. 3. Survival rate of *A.adreanum* plantlets after four weeks of acclimatization



Remarks: *) Same letters indicates no significant differences according to Duncan's multiple range test (P = 0.05)

Fig. 4. Survival rate of *A.adreanum* plantlets after eight weeks of acclimatization

CONCLUSION

From the experiment it can be concluded that organogenesis of *A. adreanum* cv. Nitta could be induced from leaf explant in the MS medium added with 2,4-D and BAP (1 mg L^{-1}); contamination of explants could be reduced to 0 % with gradual sterilization using desogerm, antioxidant, Benomil and NaOCl; MS medium with half concentration of macro mineral added with 1 mg L^{-1} paclobutrazol produced the most vigorous *A. adreanum* plantlets; and survival rate of *A. adreanum* plantlets could be increased by 7 and 14 days hardening treatment in the culture room.

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