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A REVISED METHOD FOR SUCKER STERILIZATION TO SUPPORT THE *IN VITRO* PROPAGATION OF SAGO PALM (*Metroxylon sagu* Rottb.)

Modifikasi Metode Sterilisasi Tunas Anakan Sagu (*Metroxylon sagu* rottb.) untuk Mendukung Perbanyakan Secara *In Vitro*

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ABSTRAK

Hutan sagu (Metroxylon sagu Rottb.) *dapat ditemukan dalam area yang cukup luas di wilayah Maluku dan Papua. Besarnya keanekaragaman hayati dari pohon sagu dapat dilihat di areal ini. Pohon sagu tumbuh secara alami terutama di daerah dataran atau rawa dengan sumber yang air melimpah. Tanaman sagu dapat diperbanyak dengan metode generatif melalui biji, dan vegetatif melalui tunas anakan. Dalam rangka mendukung perbanyakan pohon induk yang unggul secara* in vitro *dalam skala besar, perbaikan metode sterilisasi tunas anakan mutlak diperlukan. Tunas anakan muda (15-20 cm) yang diperoleh dari Propinsi Papua digunakan sebagai eksplan. Tujuan percobaan sterilisasi ini dilakukan untuk mendukung perbanyakan pohon sagu secara* in vitro. *Pada percobaan ini antibiotik digunakan untuk membersihkan jaringan internal eksplan dari jamur dan bakteri. Hasil percobaan ini menunjukkan bahwa campuran alkohol dan antibiotik dapat menekan pertumbuhan kontaminan.*

Kata kunci*: Antibiotik, kontaminan jamur dan bakteri, kultur* in vitro*, metode sterilisasi, sagu*

ABSTRACT

Natural sago (*Metroxylon sagu* Rottb.) forest can be found in large area in Maluku and Papua regions. There are wide genetic diversities of sago palm found in these areas. This palm grows along riverbanks and in swampy areas which are not suitable for other crops. Sago palm is propagated generatively by seed and vegetatively by suckers. With the purpose of establishing the *in vitro* culture method for a large-scale of mass clonally propagation of superior genotypes of sago palm, generating sterilized explants are very important. Young suckers (15-20 cm) obtained from areas of Papua Province were used as explants. The sterilization experiments were carrying out to support the tissue culture of sago palm. Sterilization was conducted using antibiotics in order to get rid of fungi and bacteria from inner part of explants tissues. The results showed that from all sterilization methods tested, the best result was treatment using alcohol and antibiotic as disinfectant agents.

Keywords: Antibiotics, fungi and bacteria contaminants, *in vitro* culture, sterilization method, sago palm

INTRODUCTION

Since long times ago, man uses sago palm (*Metroxylon sagu* Rottb.) trunk as a source of food starch in Southeast Asia. Sago palms grow very quickly, up to 1.5 m of vertical stem growth per year, in the fresh water swamps and lowlands in the tropics. The stems are thick and either self supporting or grow with a somewhat climbing habit, and forms suckers or tillers. The leaves are pinnate, not palmate. Formation of the inflorescence, for which the starch in the trunk is being used, begins 4 to 14 years after the start of trunk formation. They are harvested at the age of 7 to 15 years just before flowering. They only produce flower and fruit once before die (Harsanto 1986).

Sago palm has many advantages over other starch-producing crops especially for its higher yield, grows along riverbanks and in swampy areas which are not suitable for other crops. Moreover, for the benefit of plantation no regular replanted is needed. The palms are spread from Melanesia to India and from Mindanao to Java Island. Furthermore, the most wide planting areas are only found in Indonesia, Malaysia and Papua New Guinea (Johnson 1977). They normally grown from 0 to 700 m above see level, however the optimum growth is found at the height of 400 m (Haryanto and Pangloli 1999). Sago has potential and prospect for food and non-food industry, such as aceton–butanol–etanol fermentation (Gumbira *et al*. 1996), biodegredable plastic (Pramuda *et al*. 1996), sarbitol, MSG, organic acids etc. Moreover, the wood is used for pulp and paper (Muladi and Soejitno 1996).

According to Notohadipawiro dan Louhenapessy (1993), the development of sago palm can be divided into several growth stages, these are: seedling or suckers (0 – 50 cm), *sapihan* (50 – 150 cm), *tihang* (150 – 500 cm) dan tree (over 500 cm). In nurseries, the mortality rate of suckers is around 20-40%. In the dry season, higher mortality rates are common (Jong 1995). Trimming of roots to as short as 1 cm did not affect the subsequent survival of the suckers. Trimming of the rhizomes to a length close to the growing point of the sucker was deleterious. When planting of suckers was delayed, treatment with a wide-spectrum fungicide while storing the suckers in cool and moist places was shown to reduce their mortality rate. One of the vegetative propagation, which is *in vitro* culture method, is used in order to avoid above problems, as well as to obtain vigor and qualified planting materials. *In vitro* propagation of sago palm has been conducted by other laboratories (Hisajima *et al*. 1991; Tahardi *et al*. 2002).

The purpose of this study was to develop the sterilization method of sago palm via *in vitro* culture derived from various types of plant for explants source (suckers) of sago palm, as initial materials for *in vitro* culture. Sterilization process is needed in plant tissue culture in order to avoid bacteria and fungi contamination during incubation.

MATERIALS AND METHODS

Plant materials

Research activity was performed in Plant Tissue Culture Laboratory, Biotech Center located in Puspiptek Serpong, Tangerang Selatan, Banten. For the purpose of *in vitro* propagation, samples of sago suckers, as long as 7-10 cm, were taken from Sentani Lake areas, Irian Jaya Province.

Sucker selection from field

Scoop and other tools were prepared for taking samples of the suckers for *in vitro* materials.

- Choose the small size sucker with basal end diameter of 4 – 5 cm
- Cut the sucker below growing point, then trim all leaves and roots, and clean up from dirt and soil
- Before taking the sucker to the laboratory, peel off the outer layer, while keeping some (2 – 3 layers) around it basal end (growing point)
- Immerse the samples into solutions, which were used as treatments for a few seconds, then leave them in room temperature until drying.
- The treatments were applied during preparation in the field, using solutions: a) aqua distillate, b) Na-hypochlorite and c) alcohol.

Sterilization treatments

The suckers were peeled off carefully in laminar airflow cabinet to obtain clean

Table 1. Using antibiotics in combination and their methods of application

Combination of antibiotics and concentration	Application
Levofloxacin 0.5% + Sanlin 0.5%	Soaked overnight
Levofloxacin 0.5% + Sanlin 1.5%	Soaked for 2 hours
Levofloxacin 0.5% + Sanlin 2.5%	Soaked for 2 hours
Levofloxacin 0.5% + Sanlin 3%	Soaked for 2 hours
Levofloxacin 0.5% + Streptomycin 0.5%	Soaked overnight
Levofloxacin 1.5% + Streptomycin 0.5%	Soaked overnight
Levofloxacin 3.5% + Streptomycin 1%	Soaked for 2 hours

explants. The explants were then surface-sterilized using bactericide and fungicide, followed by washing in running tap water for few minutes before treated with sterilization agents. The process was divided into two steps. The first step as pre-treatment and the second was used as treatment. The pre-treatments procedure was: soaking in Tween 80 for one hour, followed by washing in running water until the buble was gone. Finally washed the samples with sterilized water for three times.

To obtain the best procedure, sterilization process were used as treatments in our study. The disinfectants applied in this study were commonly used in our laboratory. The treatments employed were:

A. Soaking in alcohol 70% for 15 minutes, followed by soaking in Na-hypochlorite 2% for 2 minutes, then washed with sterilized water twice
B. Soaking in alcohol 70% for 15 minutes, followed by soaking in Povidone Iodine 0.2% for 2 minutes, then washed with sterilized water twice
C. Soaking in alcohol 70% for 15 minutes, followed by soaking in Povidone Iodine 1% for 2 minutes, then washed with sterilized water twice
D. Soaking in alcohol 70% for 15 minutes, followed by soaking in $HgCl_2$ 0,01% for 1 minutes, then washed with sterilized water twice
E. Soaking in alcohol 70% for 15 minutes, followed by immersing in antibiotic solution for 3 minutes, then washed with sterilized water twice
F. Soaking in alcohol 96% for 15 minutes, followed by immersing in antibiotic solution for 3 minutes, however without washing in sterilized water.

Subculture was carried out every month, which was transferring the explants into the new and fresh media, containing the same composition of chemical. Observations were made every week on the freshness of explants, contamination level, and the growth of culture in the incubation room.

Sterilization was also conducted using antibiotics in order to get rid of fungi and bacteria. There were two methods applied when using the antibiotics, i. e. single antibiotic and dual antibiotics used in combination. The antibiotics used were as follow: Amphicilin, Cefadroxil, Cephalexin, Ciprofloxa, Clindamycin, Doxycycline, Kanamycin, Ketoconozole, Levofloxacin, Lincomycin, Rifamycin, Sanlin, and Streptomycin. The explants were soaked in 1% antibiotic solution for 2 hours. All treatments were repeated for 20 times.

Antibiotics in combination were applied in order to remove both fungi and bacteria from inner part of explants tissues. The basal end of explant was immersed in the anti-biotics solution for 2 hours or overnight. Table 1 illustrates the use of antibiotics in combination for fungi and bacteria elimination.

RESULTS AND DISCUSSION

Sterilization treatments

After taking samples, the suckers of sago were divided into three groups. Each group was treated with aqua distillate, Na-hypochlorite or alcohol. The samples were then stored in the cartoon boxes for three days. The freshness of samples was also tested by storing them in dry and cool room for another four days. The result of treatment during sampling was exposed in Table 2. It shows that alcohol can keep the freshness of samples until three days after putting in storage.

Table 2. Condition of sago sucker in boxes 3 days after treatments during sampling in the field

Parameter	Treatments During Sampling		
	Aqua Distillate	Hypochlorite	Alcohol
Suckers condition	Not fresh	Not fresh	Fresh
Suckers color	Pale brown	Browning	Green
Leaves/shoot of suckers	Rotten	Rotten	Fresh
Basal end (Growing point) of suckers	Not fresh	Not fresh	Fresh
Percentage of sterilized suckers	80 %	62 %	90 %

Table 3. Condition of sago suckers in boxes 4 days after treatments

Parameter	Treatments		
	Aqua Distillate	Hypochlorite	Alcohol
Suckers condition	Not fresh	Not fresh	Fresh
Suckers color	Pale brown	Brown	Green
Leaves/shoot of suckers	Rotten	Rotten	Rotten
Basal end (Growing point) of suckers	Browning	Browning	Pale brown
Percentage of sterile suckers	30 %	40 %	80 %

The percentage of sterilized sucker in the incubation room was also the highest after treated with alcohol. The result of storing samples in dry and cool room was exhibited in Table 3. Treatment the sago suckers with alcohol showed the best method to obtain the fresh materials. The suckers still fresh four days later and the sterilized culture obtained also high. Long period of storage is important to our study, since the samples were taken in large amount and from long distance places. Only few samples can be processed every day in the laboratory, while the freshness of samples is the first priority in plant tissue culture propagation.

All fresh samples were then sterilized with similar method, and the result was shown in Table 4. Alcohol treatment produces the highest sterilized explants in the culture room. The other two treatments resulted in non fresh state, browning and rotten explants started from the bottom, while the number of sterilized cultures obtained was very low.

Table 4. Percentage of sterilized sago suckers in the incubation room 1 week after culture

Treatment	Sterilized Suckers
Water	60 %
Na-hypochlorite	50 %
Alcohol	70 %

Various sterilization procedures showed the results one week after incubation (Table 5). From all methods tested, the best result was obtained from the treatment F (70.38%), followed by treatment E (58.78%). Both methods used alcohol and antibiotic as disinfectant agents. Since most of the suckers explants were obtained from the underground, where the bacteria and fungi are easily found, the disinfectant used should be systemic types, instead of surface-sterilized types. Na-hypochlorite, Povidone Iodine and $HgCl_2$ are surface-sterilized type disinfectants, while antibiotics attack bacteria and fungi systemically.

The percentage of contamination level was increased during rainy season. The rainy season resulted in increasing the humidity at the sampling area. Such

Table 5. The results of several sterilization treatments on the growth of sago explants *in vitro*

Treatment Code	Sterilized Suckers
A	0 %
B	4.50 %
C	20.96 %
D	10.00 %
E	58.78 %
F	70.38 %

Note: Treatment code is referred to sterilization treatments on Materials and Methods

condition affected the cleanness of sampling materials. The sampling materials were wet and easily contaminated by fungi and bacteria. The best sampling materials, with dry and good condition, were normally obtained during March to September. During this time, the percentage of contamination was very low.

During rainy season, various kinds of antibiotics were used to eliminate the contaminant, fungi and bacteria, systemically. The explants were soaked into antibiotics solution for 2 hours. The culture was observed after 10 days incubated in culture room. The result is revealed in Table 6. It shows that Levofloxacin gave the best result by producing high percentage of survival explants. It was followed by Rifamycin and Amphicilin, with percentage of survival around 50%. While the others resulted in low percentage of survival explants.

Table 6. Effect of antibiotics to reduce contamination on Sago explants after 10 days incubation, with initial number of 20 explants

Kinds of Antibiotics	Contaminated Explants	Sterilized Explants	Condition of Sterilized Explants		% Survival Explants
			Fresh	Dried	
Amphicilin 1%	10	10	9	1	45
Cefadroxil 1%	18	2	2	0	10
Cephalexin 1%	13	3	3	0	15
Ciprofloxa 1%	1	19	3	16	15
Clindamycin 1%	18	2	1	1	5
Doxycycline 1%	0	20	0	20	0
Kanamycin 1%	1	19	0	19	0
Ketoconozole 1%	20	0	0	0	0
Levofloxacin 1%	0	20	17	3	85
Lincomycin 1%	20	0	0	0	0
Rifamycin 1%	0	20	10	10	50
Sanlin 1%	0	20	0	20	0
Streptomycin 1%	8	12	0	12	0

Table 7. The percentage of survival explants after treated with mixture of antibiotics

Combination of Antibiotics	Methods	Initial No. of Explants	Sterile Explants	Condition of Sterile Explants		% Survival Explants
				Fresh	dried	
Levofloxacin 0.5% + Sanlin 0.5%	Soaked overnight	273	137	107	30	39
Levofloxacin 0.5% + Sanlin 1.5%	Soaked for 2 hrs	230	143	100	43	43
Levofloxacin 0.5% + Sanlin 2.5%	Soaked for 2 hrs	166	72	60	12	36
Levofloxacin 0.5% + Sanlin 3%	Soaked for 2 hrs	196	172	80	92	41
Levofloxacin 0.5% + Streptomycin 0.5%	Soaked overnight	250	80	45	35	18
Levofloxacin 1.5% + Streptomycin 0.5%	Soaked overnight	255	220	100	120	39
Levofloxacin 3.5% + Streptomycin 1%	Soaked for 2 hrs	285	270	250	20	88

The percentage of survival was increased when antibiotics were used in combination, as shown in Table 7. After soaking the explants into the mixture solution of antibiotic Levofloxacin (3.5%) and Streptomycin (1%), the highest percentage of survival reached 88%.

The other combinations however resulted in low percentage of survival explants. Even soaking the explants into solution overnight did not give better results. Antibiotic Levofloxacin should be used after freshly prepared. The solution was easily degraded, unless kept frozen in the freezer, however it should be used for not more than two days after preparation. Using the outdated antibiotics resulted in high rate of contamination.

CONCLUSION

From our research activities it can be concluded that long period of samples storage was important, since they were taken from long distance places and need some period of times before getting into the laboratory. After taking samples from the mother plants, the suckers of sago treated with alcohol showed the best method to obtain the fresh materials. The suckers still fresh four days later and the sterilized cultures obtained were also high. The best sterilization treatment was soaking the suckers in mixture solution of antibiotic Levofloxacin (3.5%) and Streptomycin (1%). The percentage of sterile suckers were 88%.

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