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Optimization of Sterilization Method and Callus Induction of *Salacca glabrescens*

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Abstract. *Salacca glabrescens* ST1 is one of the new developed clones from local *Salacca* species in Terengganu, Malaysia. Cultures were initiated from the immature inflorescences of *S. glabrescens*. Sterilization method was optimized to eliminate contamination problems in cultures. Three methods of sterilization were tested and method C obtained the highest percentage of aseptic cultures (95.33±0.54%). The immature inflorescence of *S. glabrescens* were cultured in two different basal media; MS (Murashige and Skoog, 1962) and Y3 (Eeuwens, 1976) media containing different concentrations of IBA, GA3, TDZ, 2,4-D, Picloram, NAA, and BA. The explants produced oof-white, friable callus after 10 days of culture. Callus induction frequency varied among types of basal media and treatments. The compositions of salts and vitamins in Y3 medium were found to produce superior results than MS medium. Significant callus formation were observed in explants cultured in Y3 media containing 0.2ppm TDZ, 4.0ppm 2,4-D and 2ppm picloram and Y3 media containing 1.0ppm NAA, 0.5ppm BA, and 1.5ppm 2,4-D. The results achieved suggested that immature inflorescences of *S. glabrescens* can be alternative sources of explants for the induction of callus formation and somatic embryogenesis.

Keywords: *Salacca glabrescens*, immature inflorescence, callus induction, Y3 media, MS media, sterilization method

1. Introduction

Salacca glabrescens is a palm species from Arecaceae family and is native to Indonesia and Malaysia. Salak is widely cultivated in the wetter parts of the Indo-Malay region and found as an understorey palm in Java and Southern Sumatra. Researchers have proven that salak fruits are rich with nutritional and sensory values. Several *in vivo* studies have shown that snake fruit has bioactive properties and positively affect plasma lipid profile and antioxidant activity in rats plasma composition (Leontowicz *et al*., 2006; Leontowicz *et al*., 2007 and Aralas *et al*., 2009). The *Salacca glabrescens* plant is usually dioecious with male and female spadices on separate plants. The tubular corolla of the female flower is yellow-green outside, dark red inside and has a triocular ovary with a short trifid red style and six staminodes. It can be propagated from seeds, suckers, layering stem or stem cuttings. Currently, the salak plants are being propagated using the traditional method where the apical dominance of mother plants were removed in order to induce auxillary branching and 5 – 10 new plants were obtained (Hashim, 2005). However, this method still cannot meet the demand for more planting materials to open up a new plantation (MARDI, 2009).

One alternative is to multiply the selected lines using *in vitro* technique. Micropropagation is a rapid propagation technique, but the greatest problem is contamination with fungi and bacteria. With this situation, aseptic technique must be established to sterilize the explants. A sterilization procedure must be established after the explant has been selected. Success at this stage firstly requires that explants should be transferred to the cultural environment, free from obvious microbial contaminants (George *et al.*, 2008). There are also successful plant regeneration from unfertilized ovaries of a sugar beet breeding line was achieved (Gurel *et al*., 1998). It was reported that between two methods of embryo culture, a segment of ovary containing embryo showed higher germination frequency with better root and shoot growth than the culture of naked

embryo alone of distant hybrid in Indica rice (Bindeshwar Prasad Sah, 2007). The objective of this study is to optimize the sterilization method and to induce callusing from ovary culture of *S. glabrescens*.

2. Materials and Method

2.1. Plant Material

Cultures were initiated from ovary of *S. glabrescens* ST1. Samples of *S. glabrescens* inflorescence were collected from Jerangau MARDI (Malaysian Agricultural Research and Development Institution) research station. The samples were stored in ice-box to maintain its freshness prior to culture.

2.2. Optimization of sterilization procedure

The inflorescences collected from the field were washed under running water for 15 to 20 minutes and soaked in Teepol® solution overnight in incubator shaker (130 rpm) to remove dirt and debris. The spadices were then rinsed with sterile distilled water 3 times in laminar air flow and subjected to three different method of sterilization (as described in Table 1).

2.3. Media Compositions and Growth Conditions

The basal media used for this experiment were MS media (Murashige and Skoog, 1962) and Y3 media (Eeuwens, 1976). The media contained components which can be divided into four groups: macronutrients, iron salts, micronutrients, and vitamins and supplements (Table 2). The medium was autoclaved at 121°C for 20 min. 1% silver nitrate was added into the medium under aseptic condition. The cultures were maintained in a dark culture room. The room temperature was 25±2˚C. The combinations of plant growth regulators for callus induction experiment are shown in Table 3.

Table 1: Method of sterilization for inflorescence of *S. glabrescens*

Method A	Method B	Method C
Wash the inflorescence under running water (15-20minutes) ↓ Soak in detergent overnight in incubator shaker ↓ Wash with sterile distilled water (3 times) ↓ Soak mercuric chloride (10-15 minutes) ↓ Under aseptic conditions, soak the inflorescence in 70% ethanol for 30 seconds and treat with commercial sodium hypochlorite 30% for 15minutes ↓ Wash with sterile distilled water ↓ Dissect the spadices and collect the inflorescences ↓ Treat the inflorescences with argentum nitrate ↓ Culture the inflorescence on Y3 hormone free medium	Wash the inflorescence under running water (15-20minutes) ↓ Soak in detergent overnight in incubator shaker ↓ Wash with sterile distilled water (3 times) ↓ Wash with 50% commercial Sodium hypochlorite for 20 minutes. Rinse with sterile distilled water Dissect the spadices and collect the inflorescences ↓ Under aseptic conditions, soak the inflorescences in 70% ethanol for 30 seconds and treat with commercial bleach 20%+ Tween-20 (15-20 minutes) ↓ Rinse with sterile distilled water ↓ Culture the inflorescences on Y3 hormone free medium.	Wash the inflorescence under running water (15-20minutes) ↓ Soak in detergent overnight in incubator shaker ↓ Wash with sterile distilled water (3 times) ↓ Soak in benlate 1% for 5 minutes ↓ Wash with sterile distilled water ↓ Dissect the spadices and collect the inflorescences ↓ Under aseptic conditions, soak the inflorescences in 70% ethanol for 30 seconds and treat with commercial bleach 20% + Tween-20 (15-20minutes) ↓ Rinse with sterile distilled water ↓ Culture the inflorescences on Y3 hormone free medium.

Table 2: Compositions of MS (Murashige and Skoog, 1962) and Y3 (1976) basal media

Component	Y3	MS
Macroelements (mg/l)		
KNO_3	2020	1900
KCl	1492	-
KH_2PO_4	-	170
NH_4Cl	535	-
NH_4NO_3	-	1650
$NaH_2PO4.2H_2O$	312	-
$CaCl_2.2H_2O$	294	440
$MgSO_4.7H_2O$	247	370
Iron salts (mg/l)		
$Na_2EDTA2H_2O$	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8
Microelements (mg/l)		

$MnSO_4.4H_2O$	11.2	22.3
KI	8.3	0.83
$ZnSO_4.7H_2O$	7.2	8.6
H_3BO_3	3.1	6.2
$CoCl_2.6H_2O$	0.24	-
$Na_2MoO_4.2H_2O$	0.24	0.25
$CuSO_4.5H_2O$	0.16	0.025
$NiCl_2.6H_2O$	0.024	-
Supplements (mg/l)		
Meso inositol	100	100
Thiamine-HCl	0.5	0.1
Pyridoxine-HCl	0.05	0.5
Calcium pentothenate	0.05	-
Nicotinic acid	0.05	0.5
Biotin (B-complex)	0.05	-
Gibberelic acid	0.038	-
Glycine		2
Sucrose (mg/l)	45000	70000
Coconut water	5%	-
Activated charcoal (mg/l)	1000	-
Agar (g/l)	7.5	7.5
pH	5.5	5.8

Table 3: Combinations of plant growth regulators for callus induction of *S. glabrescens*

Basal Culture Media	Control (No Plant Growth Regulator)	0.4ppm Iba + 0.5ppm GA_3	0.2ppm TDZ + 4ppm 2,4-D + 2ppm Picloram	1ppm NAA + 0.5ppm BA + 1.5ppm 2,4-D
MS	MS0	MSA	MSB	MSB
Y3	Y30	Y3A	Y3 B	Y3C

Each treatment consisted of 30 replicates and each replicate contained 5 explants

3. Results and Discussion

3.1. Optimization of sterilization method

S. glabrescens ST1 developed by MARDI have high commercial values. However, shortness of female plants and conventional breeding method that takes years before the plants can produce fruits is the reasons why this exotic fruit cannot be fully commercialized in Malaysia. The protocol for *in vitro* mass propagation of this particular Salacca species is therefore crucial to produce a large amount of female plants. Contamination in cultures is one of the limiting factors in plant tissue culture protocols. Contaminations by bacteria and fungus affect the percentage of aseptic cultures. In method B and C, the inflorescences containing ovaries were extracted from the spadices before they were subjected to another stage of sterilization. Based on this experiment, method B (94.08±0.78%) and C (95.33±2.16%) significantly ($P<0.05$) gave the higher percentage of aseptic cultures compared to method A. Ghoreyshi *et al*. (2010) reported explants that were surface sterilized in series or stages resulted in higher percentage of aseptic cultures. Direct contact of the explants to the sterilant during the second stage of sterilization increased the percentage of aseptic cultures. This significant result may be to the direct contact of the sterilant to the explants (inflorescence) (Figure 2(b)) and the use of silver nitrate in the culture medium (Rostami and Shahsavar, 2009). Method C gave the highest percentage of aseptic cultures and therefore was chosen as the sterilization method for the callus induction experiment.

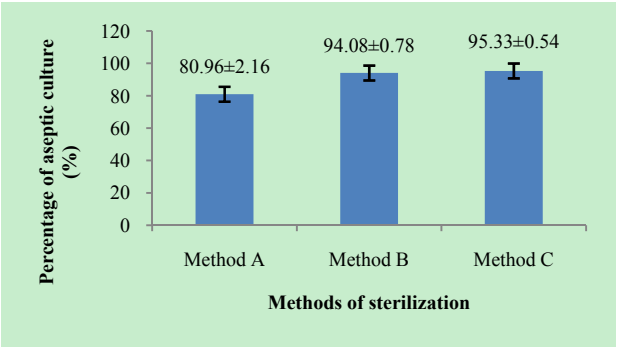


Figure 1: The effect of three different methods of sterilization on the percentage of aseptic cultures

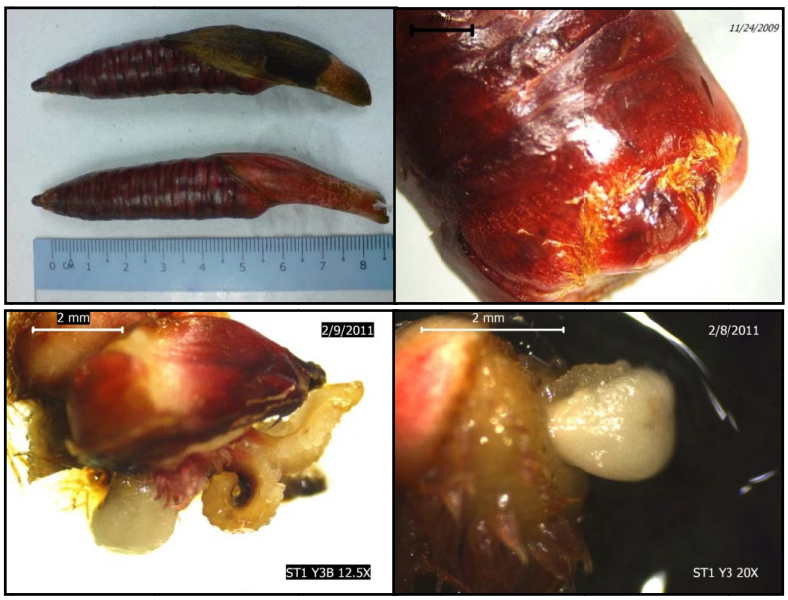
3.2. Callus induction of *S. glabrescens*

The cultures were initiated from the inflorescence of *S. glabrescens* cultured in MS and Y3 basal media supplemented with three different hormonal treatments. The explant was cut into two sections and placed on the media. After 10 days of culture, the ovaries started to swell and produced white, translucent, and friable callus. No callus was observed in control and MSA treatment. Explants in treatment MSB, MSC, Y3A, Y3B, and Y3C developed callus but the callus induction frequency varied among types of basal medium and treatments. Table 4 showed that callus induction frequencies depended on the basal media and hormonal treatment. Significant ($P<0.005$) callus formation was observed in treatment Y3B (Y3 media containing 0.2ppm TDZ, 4.0ppm 2,4-D and 2ppm picloram) and treatment Y3C (Y3 media containing 1.0ppm NAA, 0.5ppm BA, and 1.5ppm 2,4-D), with 37% and 11% respectively.

Table 4: The effect of plant growth different treatments and basal media on callus induction frequencies after

Basal Media	Treatment (%)			
	Control	0.4ppm IBA+ 0.5ppm GA_3	0.2ppm TDZ + 4ppm 2,4-D+2ppm Picloram	1ppm NAA + 0.5ppm BA +1.5ppm 2,4-D
MS	0%	0%	10%	8%
Y3	0%	3%	37%	11%

The highest percentage of callusing was observed in Y3B (37%). Y3 media was found to be more suitable for callus induction *S. glabrescence* compared to MS media. This similar result was obtained when stem, leaf, and inflorescence explants of *Cocos nucifera* L cultured in Y3 media. These explants formed layer of white callus within a month after culture initiation. The compositions of macro and micro elements in Y3 media has been reported to be more suitable for palm species compared to White, Heller, or Murashige and Skoog (Eeuwens, 1976). The growth of callus was also affected by the concentration of 2,4-D. 10% cultures showed callusing at lower concentration (1.5 ppm) while at higher concentration (4 ppm), the frequency of explants producing callus (37%) was increased (Table 4). 4 weeks after initiation, the callus were transferred into fresh medium to prevent browning and nutrient depletion. After 10 weeks of culture initiation and 2 subcultures, the presence of BA and NAA in Y3 media promoted induction of bright yellow callus. The friable callus organized and became bright yellow compact callus and crystallize callus formed on the surface (Figure 2 (f)).



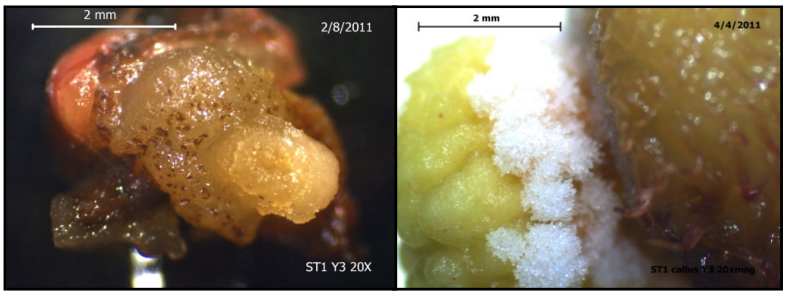


Figure 2: (a) Spadices containing inflorescences of S. glabrescens, (b) Arrow shows inflorescence of S. glabrescens, (c) Callusing on the swollen ovary and stigma, (d) Callus on the surface of swollen ovary, (e) Off-white and friable callus originating from the base of the explants, (f) crystallize callus formed on the surface of yellow compact callus.

4. Conclusion

The sterilization method for inflorescence has been optimized and the results achieved suggested that inflorescence of *S. glabrescence* can be used as explants for callus initiation and somatic embryogenesis. From this experiment, we now know that Y3 media supplemented with 0.2ppm TDZ, 4.0ppm 2,4-D, and 2ppm Picloram is suitable for callus induction and somatic embryogenesis of *S. glabrescens*. However, the hormonal treatment needs to be improved to increase the quality and quantity of embryogenic callus and initiation of somatic embryoids.

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