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Elimination of SCMV (*Sugarcane Mozaik Virus*) and Rapid Propagation of Virus-free Sugarcane (*Saccharum officinarum* L.) Using Somatic Embryogenesis

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Abstract

The use of apical buds and in-vitro shoot for elimination of SCMV and shoot proliferation in sugarcane was assessed. The purpose of this research was to determine the level of virus elimination and to obtain virus-free sugarcane. Research were using explants of apical buds and in vitro shoots of sugarcane PS-881 cultured on MS medium supplemented with antiviral acyclovir and ribavirin consisted of 0, 20 and 40 mg l^{-1} with incubation duration 4, 5 and 6 weeks. Elimination of virus-free plantlets was detected by DAS-ELISA and RT-PCR. The results showed that the detection of RT-PCR using apical explants treated with acyclovir 40 mg l^{-1} for 6 weeks was not effective to eliminate SCMV, while the use of in vitro shoot explants treated with 40 mg l^{-1} of acyclovir or 40 mg l^{-1} ribavirin eliminated the SCMV for about 100% resulting virus-free sugarcane plantlets. Sugarcane virus-free propagated through somatic embryogenesis on five different induction mediums. Callus induction and proliferation through somatic embryogenesis were obtained on MS nutrient medium with the addition of 3 mg l^{-1} 2,4-D + 1.5 mg l^{-1} BAP. The result of regeneration produced ± 11 virus-free plantlets within 8 weeks.

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Keywords: Virus-free sugarcane; SCMV; virus elimination; somatic embryogenesis

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1. Introduction

The cultivation of sugarcane (*Saccharum officinarum* L.) on commercial land often found mosaic disease caused by sugarcane mosaic virus (SCMV), leading to the decline of sugarcane potential productivity. The attack of sugarcane mosaic virus (SCMV) reported can decrease the production of sugarcane around 30-50% and may even reach 80%[1,2]. Controlling the virus was done through plant breeding using resistant varieties and genetic transformation[3], but has not been effective. Controlling of virus spread is the most effective to do by replacing of diseased seedlings with healthy seedlings originating from the disease-free seed stock[3,4]. Healthy plants that do not show symptoms of mosaic disease are not definitely free from SCMV virus particles in the body. Selection of appropriate sources of seed cane is very important considering the magnitude of mosaic virus spread, especially seeds derived from the previous crop residues, such as seeds of the cut back results[5].

The results of research to reduce viral diseases can be reduced by meristem culture[6,7,8], apical meristem[2,7], somatic embryogenesis[9] and the addition of chemotherapeutic[4]. Chemotherapeutic materials are widely used for the treatment of viral diseases which are dithiouracil[10,11,12], ribavirin and acyclovir[13,14,15,16].

Research on plant viral chemotherapy ever reported[17,18], using antiviral agent Ribavirin (Virazole). Besides ribavirin other chemicals that can be used as an antiviral agent are dithiourasil[10], amantadine, vidarabin, guanidine hydrochloride, acycloguanin or acyclovir[19]. Ribavirin is an antiviral compound that has a wide spectrum of DNA or RNA to inhibit the RNA polymerase enzyme so the process of mRNA translation is inhibited[13]. A high concentration of ribavirin can lead to phytotoxic which occurred growth weakened due to the death of meristem or decrease cellular metabolic processes on host plants. The provision of ribavirin with a concentration of 20 mg l^{-1} indicates the percentage elimination of the virus in Binella varieties of potato up to 87% while in the Burren varieties up to 82%. Results of the study showed that treatment cane thermotherapy dual therapy and chemotherapy with ribavirin concentration of 20 mg l^{-1} were able to eliminate 100% virus SCMV[20].

In addition to virus attack, not maximal production of sugarcane in Indonesia is the lack of stock of the provision of quality seeds in large quantities[21]. Quality seed cane obtained from sugar cane healthy without containing viral diseases, especially of sugarcane mosaic virus (SCMV). Propagation via somatic embryogenesis techniques have the potential to be developed because[22] propagated through somatic embryogenesis can generate unlimited number of plants in a short time.

Many factors influence the successful of somatic embryogenesis among other things exogenous auxin[23], the composition of auxin and cytokinin[24], the source of explants[7,23,25], nitrogen and sucrose[26]. Several studies have reported that the addition of auxin 2,4-D alone increased callus initiation[27]. The addition of 2,4-D with a concentration of 3-4 mg l^{-1} can induce callus better than addition of 2,4-D on the concentration of 1-1.5 mg l^{-1}. A combination of 2,4-D and BAP is the most effective way to induce somatic embryos[7,24]. Regeneration can be obtained by reducing or eliminating auxin concentration of media[28]. The research goal is to get the type of explants and appropriate concentration of antiviral for elimination SCMV and virus-free sugarcane propagation method through somatic embryogenesis.

2. Methods

2.1 Plant Material.

Plant material using the cane tops of PS 881 variety ± 15 cm, 5 months grown in the fields that infected SCMV disease plants, originally from Jatiroto PTPN-XI field. The cane top is removed until obtain apical buds ± for explants. Apical buds explants cultured, height of shoots up to 2 cm used as shoot explants in vitro. Plant material for multiplication through somatic embryogenesis using virus- free shoot in vitro, cut into ± 0.5 cm in the basal of explants.

2.2 Virus Elimination

Apical buds explants cultured in a MS medium + PVP 300 mg l^{-1} + chemotherapy acyclovir by the concentration: K0: 0 mg l^{-1}, K1: 20 mg l^{-1}, K2: 40 mg l^{-1} with incubation duration L1: 4 weeks, L2: 5 weeks, L3: 6 weeks. Experiments using virus elimination treatment repeated three times. Shoot explants in vitro cultures in MS

medium + chemotherapy A1: acyclovir, A2: ribavirin by concentration: K0: 0 mg l^{-1} K1: 20 mg l^{-1}, K2: 40 mg l^{-1}. Experiments using virus elimination treatment repeated three times.

2.3 Shoot and Root Induction Medium

Eliminated virus explants for 6 months subcultured on shooting MS medium consist of PVP 300 mg l^{-1} + arginine 50 mg l^{-1} + BA 2 mg l^{-1} + kinetin 0.5 mg l^{-1}. Plantlets were subcultured into rooting medium after height shoot up to ± 2 cm consist of ½ MS medium.

2.4 DAS-ELISA Analysis

Sample preparation. Sample of in vitro leaves were collected 0.2 g, crushed using liquid nitrogen until smooth, then dissolved in SBI buffer by comparison 1 : 3.

2.5 Serology Test

Testing was done using ELISA *Sugarcane* Mosaic Virus Kit from AC diagnostics, Inc.

2.6 Data Interpretation

Interpretation was done by measuring absorbance value in 405 nm to ELISA reader. Determining the positive sample of infected plants were done with a cut off value method, determine the mean absorbance value of negative control added by 3 x standard deviation. Percentage of *Sugarcane Mosaic Virus* (SCMV) elimination was calculated by:

$$P = \frac{n}{N} x100$$

- P = Percentage of elimination
- n = Number of positive ELISA plants
- N = Number of tested plants

2.7 RT-PCR Analysis

RNA isolation was done by using RNAprep Pure Plant Kit TianGen product. cDNA synthase was done by using Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR product was visualitated by electroforesis to 1% agarose gel.

2.8 Callus Induction

Regeneration through somatic embryogenesis was done through 3 stages: I. Callus induction, II. Proliferation, and III. Regeneration. Induction medium consist of:

Table 1. Composition of callus induction medium.

Code	Callus induction medium compositions
A	MS 0 without plant growth regulator
B	MS 0 +2,4-D 3 mg l^{-1}
C	MS 0 + 2,4-D 4.5 mg l^{-1} -1
D	MS 0 + 2,4-D 3 mg l^{-1} + BAP 1.5 mg l^{-1}
E	MS 0 + 2,4-D 4.5 mg l^{-1} + BAP 1.5 mg l^{-1}

Explants were incubated in dark condition during 30 days, until embryogenic callus were formed. Embryogenic callus formed were subcultured in proliferation medium. Proliferation medium composition used MS medium containing 2,4-D 1.5 mg l^{-1} + CH 300 mg l^{-1} + prolin 560 mg l^{-1} + 30 g l^{-1} sucrose + 2.5 g l^{-1} phytagel. Embryogenic callus were incubated for 30 days in the proliferation stage, then subcultured to regeneration medium with MS medium containing 30 g l^{-1} sucrose + 2.5 g l^{-1} phytagel without addition of PGR. Callus morphology and somatic embryogenesis stages were observed by binocular microscope.

3. Results and Discussion

The result of ELISA on plantlets originally from apical shoots of PS 881 variety showed that the effectivity of acyclovir concentration and incubation duration effecting different response on SCMV elimination virus. The higher concentration of acyclovir and the longest incubation duration, the higher effectivitiness of elimination ability. The results of research showed that SCMV elimination virus using acyclovir 40 mg l^{-1} with incubation duration 4, 5, 6 weeks on apical shoots explants were able to eliminate virus 100% (Table 2). Oana *et al.* explained that chemoteraphy is the appropriate methode to obtain virus- free plants from infected mother plants by inhibited virus replication, virus inter cells transport, and between plant tissues.

Table 2. Percentage virus elimination at different acyclovir concentrations and incubation duration based on ELISA test (Cut off value: 0.206; n=2)

Treatments	Absorbance value	Interpretation	Percentage of virus elimination
Control (+)	1.0000 ± 0.0560	(+)	0%
Control (-)	0.0900 ± 0.0045	(-)	100%
Acyclovir 0 mg l^{-1}, 4 weeks	0.137 ± 0.125	(+)	0%
Acyclovir 0 mg l^{-1}, 5 weeks	0.122 ± 0.052	(+)	0%
Acyclovir 0 mg l^{-1}, 6 weeks	0.109 ± 0.108	(+)	0%
Acyclovir 20 mg l^{-1}, 4 weeks	0.106 ± 0.117	(+)	0%
Acyclovir 20 mg l^{-1}, 5 weeks	0.082 ± 0.119	(+)	0%
Acyclovir 20 mg l^{-1}, 6 weeks	0.082 ± 0.037	(+)	0%
Acyclovir 40 mg l^{-1}, 4 weeks	0.090 ± 0.023	(-)	100%
Acyclovir 40 mg l^{-1}, 5 weeks	0.050 ± 0.006	(-)	100%
Acyclovir 40 mg l^{-1}, 6 weeks	0.050 ± 0.052	(-)	100%

ELISA analysis on plantlet originally from shoot in vitro explants showed that addition of acyclovir as well as ribavirin at 20 mg l^{-1} concentration were able to eliminate SCMV virus 100% as well as 40 mg l^{-1} concentration (Table 3.). Virus elimination using ribavirin also reported by some researcher at the meristem culture of apple, plum, apricot, potato, sweet potato, and sugarcane up to 65–100%[4,12,15].

Table 3. Percentage of virus elimination at different acyclovir dan ribavirin concentration based on ELISA test (Cut off value: 0.206; n=2)

Treatments	Absorbance value	Interpretation	Percentage of virus elimination
Positive control	1.0000 ± 0.0560	(+)	0%
Negative control	0.0900 ± 0.0045	(–)	100%
Acyclovir 0 mg l^{-1}	0.2050 ± 0.0014	(+)	0%
Acyclovir 20 mg l^{-1}	0.0930 ± 0.0127	(–)	100%
Acyclovir 40 mg l^{-1}	0.0870 ± 0.0057	(–)	100%
Ribavirin 0 mg l^{-1}	0.2095 ± 0.0049	(+)	0%
Ribavirin 20 mg l^{-1}	0.0490 ± 0.0042	(–)	100%
Ribavirin 40 mg l^{-1}	0.0750 ± 0.0042	(–)	100%

Electroforesis result showed that sugarcane sample containing SCMV was able to amplified the DNA with the size 900 bp. The result of RT-PCR analysis in apical buds explants incubated by acyclovir concentration with different incubation duration were able to eliminate virus (Fig. 1a), whereas using in vitro shoot explants incubated by acyclovir or ribavirin concentration at 20 mg l^{-1} level were able to eliminate virus as shown as electroforesis results (Fig. 1b).

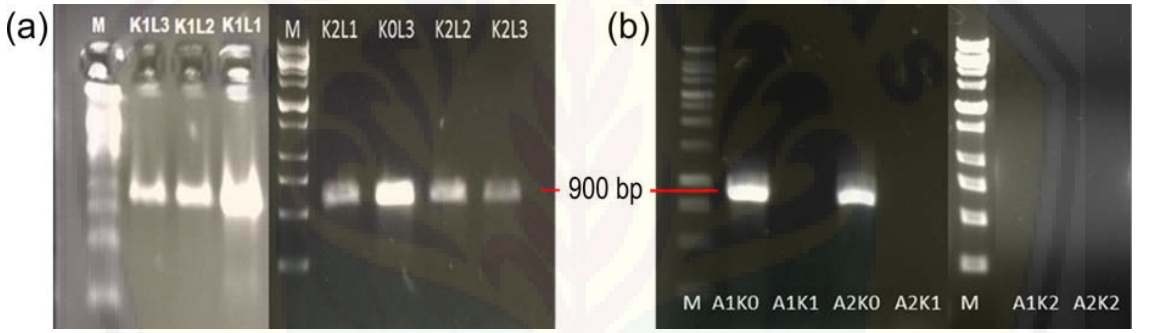

Fig. 1. cDNA electroforesis results of RT-PCR with the pair of SCMV-CP-F and SCMV-CP-R and cDNA sugarcane template from the antiviral therapy result, amplify the DNA with the size 900 bp. (a) Acyclovir therapy on apical buds explants; (b) Acyclovir and ribavirin therapy on in vitro shoot explants. M: DNA marker 1 kb, K_1L_3: acyclovir 20 mg l^{-1}, 6 weeks inkubation ; K_1L_2: acyclovir 20 mg l^{-1} 5 weeks inkubation; K_1L_1: acyclovir 20 mg l^{-1}, 4 weeks inkubation; K_2L_1; acyclovir 40 mg l^{-1}, 4 weeks inkubation; K0L3: acyclovir 0 mg l^{-1}, 6 weeks inkubation; K2L2: acyclovir 40 mg l^{-1}, 5 weeks inkubation; K2L3: acyclovir 40 mg l^{-1}, 6 weeks inkubation; A1K0: acyclovir 0 mg l^{-1}; A1K1: acyclovir 20 mg l^{-1}; A2K0: ribavirin 0 mg l^{-1}; A2K1: ribavirin 20 mg l^{-1} ; A1K2: acyclovir 40 mg l^{-1}; A2K2: ribavirin 40 mg l^{-1}.

Detection of the virus content by ELISA techniques need to be equipped with testing RT-PCR. The result of research showed that ELISA test in apical buds with chemotherapy treatment acyclovir 40 mg l^{-1} was able to eliminate the virus content SCMV, but based on RT-PCR result, bands of the virus was still detectable Fig. 1a. While the ELISA detection in shoots in vitro cultured using chemotherapy treatment by adding acyclovir or ribavirin 20 and 40 mg l^{-1} was able to eliminate the virus SCMV content, as well as detection using RT-PCR (Fig. 1b). From this study, the virus-free shoots of SCMV in vitro were obtained, then the virus-free shoot were multiplied through somatic embryogenesis.

Multiplication through in direct somatic embryogenesis carried out by callus induction stage, proliferation and regeneration. The observation day of callus induction showed that explants in vitro grown in five types of medium compositions induction gives a different response. The fastest day of callus induction was 7 days at medium containing in B: 3 mg l^{-1} 2,4-D while the A medium without addition PGR, no callus formed, but leads to the formation of organs (Fig. 2 and Fig. 3A). It showed that the addition of 2,4-D in callus induction medium was able to trigger a callus initiation process[22].

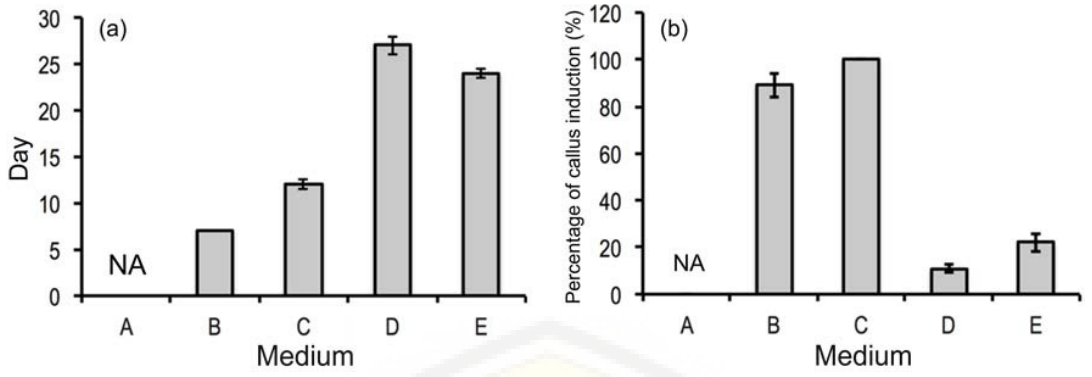

Fig. 2. Days of callus induction (a) and percentage of callus induction; (b) of PS 881 variety (NA = Not Available).

Good response to the percentage of callus induction demonstrated by addition of only 2,4-D compared with combination of 2,4-D and BAP at the level concentrations of 2,4-D 3 mg l^{-1} and 4.5 mg l^{-1}. The percentage of callus induction on the 2,4-D concentration of 3 mg l^{-1} and 4.5 mg l^{-1} up to 89% and 100% (Fig. 3). A similar observation was reported that addition of 2,4-D alone showed the best results for callus induction compared with a combination of 2,4-D and BAP[28]. However, another study reported that additional 2,4-D, BAP and casein hydrolyzate produced callus up to 83-86%[29]. The morphology of embryogenic callus PS 881 sugarcane can be seen in Fig. 3.

The growth of explants in vitro buds on different medium composition showed a different response. Explants grown on MS medium without plant growth regulator induced the formation of shoots (Fig. 3A), while explants grown on the medium groups of 2,4-D alone B (2,4-D 3 mg l^{-1}) induced looks translucent brown, wet and emerge from the callus explants browning while the C medium (2,4-D 4.5 mg l^{-1}) callus look clear, glossy, slightly browned and at the end of the proliferation coincided with the emergence of shoots that growth on callus clumps.

The same thing was found in callus contained on the medium groups of 2,4-D and BAP . The addition of 2,4-D 3 mg l^{-1} + BAP 1.5 mg l^{-1} (D) produced colored translucent callus, crumb, slightly brownish, while the addition of 2,4-D 4.5 mg l^{-1} + BAP 1.5 mg l^{-1} (E) produced visible colored translucent, partially visible slick white, not transparent and compact callus as well as some parts of the blackish brown showed browning section (Fig. 3E).

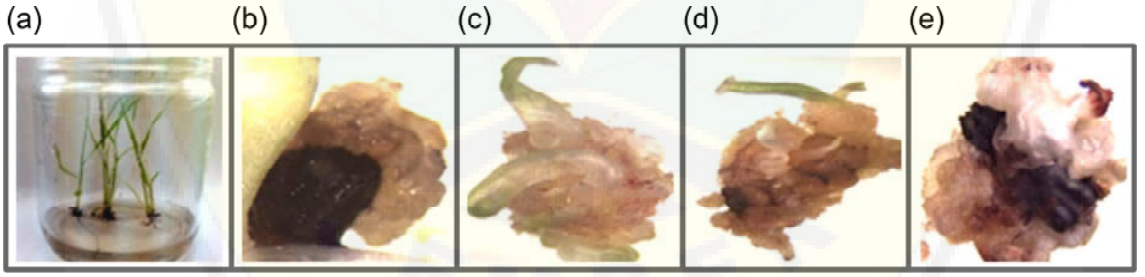

Fig. 3. Morphology of PS881 callus at different composition medium: (A) MS; (B) 2,4-D 3 mg l^{-1}; (C) 2,4-D 4.5 mg l^{-1}; (D) 2,4-D 3 mg l^{-1} + BAP 1.5 mg l^{-1}; (E) 2,4-D 4.5 mg l^{-1} + BAP 1.5 mg l^{-1}.

4. Conclusions

The detection of RT-PCR using apical explants treated with acyclovir 40 mg l^{-1} for 6 weeks was not effective to eliminate SCMV, while the use of in vitro shoot explants treated with 40 mg l^{-1} of acyclovir or 40 mg l^{-1} ribavirin eliminated the SCMV for about 100% resulting virus-free sugarcane plantlets. Callus induction and proliferation through somatic embryogenesis were obtained on MS nutrient medium with the addition of 3 mg l^{-1}2,4-D + 1.5 mg l^{-1} BAP. The result of regeneration produced ± 21virus-freeplantlets within 8 weeks.

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