

HOT WATER TREATMENT OF CUTTING-CANE INFECTED WITH *SUGARCANE STREAK MOSAIC VIRUS (SCSMV)*

Tri Asmira Damayanti¹, Lilik Koesmihartono Putra² and Giyanto¹

¹Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University,
Jl. Kamper Darmaga Campus Bogor 16680, West Java, Indonesia

Email: triadys@ipb.ac.id/triadys@yahoo.com

²Indonesia Sugar Research Institute (ISRI)

Jl. Pahlawan No. 25, Pasuruan 67126, East Java, Indonesia

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ABSTRACT

The thermal inactivation point (TIP) of SCSMV was determined by heating the infected plant sap for 10 min at 50°C, 55°C, 60°C, and 65°C. The sap was inoculated mechanically on indicator plant *Dactylactonium aegypticum*. The virus was detected by a reverse transcription polymerase chain reaction (RT-PCR) using specific primer to amplify a part of the coat protein (CP) gene. The SCSMV was detected on plants inoculated with 50°C and 55°C heat-treated sap, and was not detected in plants inoculated with sap heat-treated at higher temperature. This TIP was used as a guideline to treat the infected cutting-canes by hot water treatment (HWT) to evaluate its effectiveness in eliminating SCSMV. The temperature ranged from 52°C to 55°C and heat treatments were 10, 20, and 30 min submersion time. The results showed that elevating temperature and submersion time affected the viability and severity significantly. At an upper temperature of 53°C with longer than 20 min submersion time, plant death was observed. Although the virus was not completely eliminated from the cutting-canes, HWT at 53°C for 10 min was able to reduce the disease severity and maintain the plant's viability. The impact of HWT before planting will reduce the virus titer in plants and disease severity at the critical time of 3-4 months after planting of sugarcane thus minimizing the effect of virus infection in sugarcane.

Key words : cutting cane, TIP, virus elimination, severity

INTRODUCTION

During the period from 1995-2001, sugar production in Indonesia decreased from 2.1 million ton to 1.72 million tons and national production decreased every year until 2006 when only 2.3 million tons were produced (Dewan Gula Indonesia; Kompas 2007). This was below the national consumption. To cover the deficit, government imported sugar amounting to 200.000 tons. Since 2002, the Indonesian government proclaimed an acceleration program to increase sugar production with the main goal to achieve sugar self-sufficiency for direct consumption in 2009 and for total sugar consumption in 2014 (Departemen Perindustrian, 2009). Some constraints to sugar production include problems in plant pest and diseases which may become a serious threat to sugar production (ISRI, 2008).

Recently, an outbreak of streak mosaic disease on sugarcane occurred in many plantations in Java Island. Based on our previous studies, the cause of the streak mosaic disease is *Sugarcane streak mosaic virus* (SCSMV), a *Potyviriidae* member (Damayanti and Putra, 2010). There was no report of SCSMV in Indonesia previously; it is considered an exotic virus. The emergence of a new virus disease might be a consequence of the introduction of cutting canes from other countries and a

sufficient detection method was not available. The SCSMV was first time reported in Pakistan and India (Hall *et al.*, 1998; Hema *et al.*, 1999). In India, its incidence is up to 100% and can infect sugarcane together with *Sugarcane mosaic virus* (SCMV) (Hema *et al.*, 2003; Chatenet *et al.*, 2005). In Indonesia, it was estimated that SCSMV is distributed in more than 50% of sugarcane plantations in Java (ISRI, 2010), with incidence up to 62% (Damayanti and Putra, 2010). The effect of SCSMV infection on sugarcane var. PS 864 could reduce the sugar yield up to 22% with incidence up to 50% (Asnawi, 2009).

Basic studies related with a new pathogen such its identity, its bio-ecological characters including its transmission modes, vector, host range and bio-molecular characters need to be conducted to get useful information as a basic consideration to decide on an appropriate management strategy of the disease. Various control methods can be chosen such as culture practices, utilization of chemicals, biological control, physical therapy, utilization of plant extract which act as antiviral substances and genetic resistance. Among those methods, control attempts which are environmentally safe and sustainable are preferred over synthetic chemical use. To improve the effectiveness of controlling pathogens, an integration of some control methods will generally be effective in reducing the impact of the infection (Schumann and D'Arcy, 2007).

For a long time, hot water treatment (HWT) has been utilized to get disease-free plant propagation materials. By using plant tissue culture and chemotherapy in combination with HWT, the effectiveness of eliminating almost all of the pathogens is increased (Mink *et al.*, 1998). Kim *et al.*, (2003) reported that HWT at 75°C for 72 hr and at 85°C for 24 h were able to inactivate *Cucumber green mottle mosaic virus* (CGMMV) on cucumber seeds. Further, 51% and 50% of garlic cloves were virus free of *Garlic yellow streak virus* (GYSV) when plantlets subjected to hot air treatment in a growth chamber at 36°C for 30 and 40 days, respectively and almost all plantlets were virus free when the treatment was more than 60 days (Conci and Nome, 1991). The elimination of plant viruses by HWT is usually based on its thermal inactivation point (TIP), however the TIP of SCSMV is still unknown up to the present. The TIP *in vitro* is higher than the plant thermal-death point (Nyland and Goheen, 1969). However, the optimization of either temperature or submersion time, can make HWT a useful therapy to eliminate viruses especially for perennial plants or vegetative propagation materials such as sugarcane and chrysanthemum.

Resistant varieties against SCSMV are not yet available and SCSMV free cane setts are hard to find in the fields at the present. Thus, this work sought to determine the TIP of SCSMV and use it as a guideline to evaluate the effectiveness of HWT on SCSMV infected cutting cane in an attempt to minimize the impact of the infection on sugarcane.

MATERIALS AND METHODS

Source of Inoculum

SCSMV infected cutting canes PS 864 variety were obtained from the Indonesia Sugar Research Institute (ISRI), Pasuruan, East Java. PS 864 is categorized as a susceptible variety which is cultivated widely in sugarcane plantations in Indonesia. The cutting canes (setts) were planted in plastic pots (Ø 50 cm) containing sterile soil and manure. The young leaves were used as a source of inocula.

Planting of *Dactylactonium aegypticum*

Grass seeds were planted in plastic pots (Ø 30 cm) containing sterile soil. Plants were maintained under screen house conditions until ready for further use. About 10-20 grasses/pot of 45

days old plants were inoculated with heat treated sap mechanically. All of the inoculated grasses were harvested and 0.1g of the composite samples of each treatment were used for RT-PCR detection.

Determination of Thermal Inactivation Point (TIP)

Infected sugarcane leaves were ground in phosphate buffer pH 7 (Merck, Germany) (1:5 w/v) to make plant sap. Plant sap was heat treated at 50°C, 55°C, 60°C, and 65°C for 10 min.

Mechanical Inoculation

Grass leaves were gently dusted with Carborundum 600 mesh (Nacalai Tesque, Japan) prior rub-inoculation. The infected plant sap (1:5 w/v) in phosphate buffer (pH 7.0) (Merck, Germany) was heat-treated for 10 min at each temperature and then used to inoculate the leaves. Plants were grown in a screen house under natural conditions for 4 weeks post rub-inoculation.

Nucleic Acid Extraction

Total RNA was extracted using RNeasy kit (Qiagen) according to manufacture's recommendation.

cDNA construction

Total RNA (3 ul) was used as a template for cDNA construction. To the eppendorf tube, 1 ul of 10 uM the 3'-primer d(T)₂₀, 0.5 ul of 10 mM dNTP and sterile water up to 7 ul were added. The premix was denatured for 5 min on 65°C, and chilled on ice. Then, 1 ul of 10 x RT buffer, 0.5 ul (20U) of RNase Inhibitor (New England Biolabs, UK), 1 ul of 50 mM DTT and 0.5 ul (100U) of M-MuLV (New England Biolabs, UK) were added and the mixture was incubated for 1 hr at 42°C, and for 5 min at 95°C to inactivate the M-MuLV transcriptase.

RT-PCR

cDNA (1 ul) was added to a PCR cocktail (2.5 ul of 10x PCR buffer, 0.5 ul of 10 mM dNTP, forward and reverse primers (10 uM) 1 ul each, Taq polymerase 2.5 U (New England Biolabs, UK) and adjusted with sterile water up to 25 ul). The forward primer SCSMV-cpF (5'-GTGGGTTTCAGTTCTCGGTTTCGTAGC-3') and the reverse primer SCSMV-AP3' (5'-TTTTTTCCTCCTCACGGGGCAGGTTGATTG-3') (Hema *et al.*, 2003) was used to amplify a 500 bp DNA fragment of partial coat protein gene (CP) and the 3' terminal of SCSMV. PCR condition was 35 cycles at 94°C for 30 sec, 47°C for 1 min, and 72°C for 2 min and a final extension on 72°C for 10 min.

DNA Visualisation

PCR products were separated by agarose gel electrophoresis using 1.2% gel agarose in Tris/Borate/EDTA (TBE) containing ethidium bromide (0.5 ug/ml) for 30 minutes on 70 Volt. Ladder 100 bp DNA (New England Biolabs, UK) was used as a marker. DNA visualisation was done under a UV illuminator and was documented by using a digital camera.

Hot water treatment (HWT) of sugarcane setts and its viability and severity

The thermal inactivation point of SCSMV was 55°C and the temperature was modified between 52-55°C. The sugarcane setts PS 864 variety was used to evaluate the effectiveness of HWT in reducing SCSMV in this variety. Setts were subjected to HWT at 52°C, 53°C, 54°C, and 55°C for

10, 20, and 30 min of each treatment. After HWT, setts were grown in sterile soil and maintained in the screen house under natural conditions. Parameter assessments were viability, disease incidence and severity at 2 months after planting.

Disease severity rating was performed with mock plants (healthy plants) with a standard made by ISRI. The first visible dewlap leaf (FVD) and spindle leaves of each plant were examined for mosaic symptoms at 2 months after planting by recording the percentage of leaf areas showing mosaic symptoms based on a score of 0-8 (Table 1).

Table 1. Scoring system of mosaic symptoms

Score	Approximately % of leaf area covered by mosaic symptoms*
0	0 %
1	0.1 – 5.0 %
2	5.1 – 10.0 %
3	10.1 – 20.0 %
4	20.1 – 30.0 %
5	30.1 – 40.0 %
6	40.1 – 50.0 %
7	50.1 – 75.0 %
8	75.1 – 100.0 %

*The first and second visible dewlap leaves and spindle leaves

Disease severity was counted using the following formula :

$$DS = \frac{\sum (n_i \cdot v_i)}{N \cdot Z} \times 100\%$$

where :

- DS = Disease severity (%)
- n = number leaves with a certain score
- v = score
- N = number leaves observed
- Z = the highest score

Data Analysis

The experimental design used in the experiments was randomized complete design with 10 cutting canes per treatment and three replicates. Data was analyzed with analysis of variance (ANOVA) using SAS software version 6.13 (SAS Institute, Gary, NC, USA) and was followed by Duncan's Multiple Range Test (DMRT) ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Determination of TIP

Until 4 weeks post inoculation (wpi) the inoculated *D. aegypticum* showed no obvious symptoms as well as control plants. However, after 4 weeks, the tested plants became dry and finally induced plant death (data not shown).

The total RNA from infected plants was extracted and detected by RT-PCR using primer specific to partial SCSMV CP gene. RT-PCR successfully amplified a 500 bp DNA fragment from inoculated samples of 50°C and 55°C treatments. However, the virus was undetectable from inoculated samples of 60°C and 65°C treatments (Figure 1). It was suggested that the virus was still infectious at 50°C to 55°C treatments. This result indicated that the SCSMV's TIP was between 55-60°C.

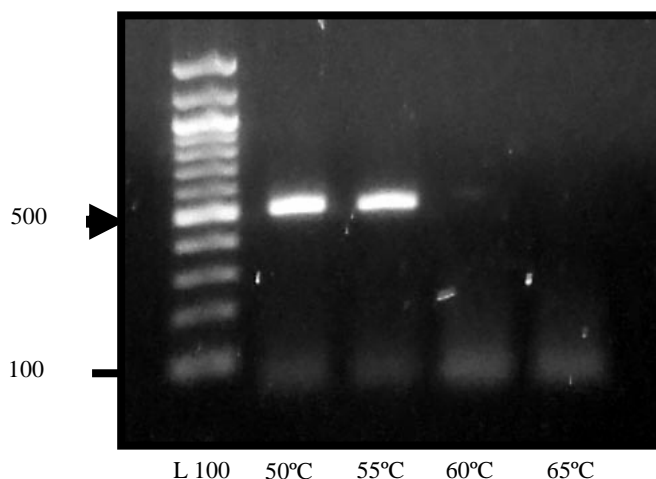


Fig. 1. Gel electrophoresis of SCSMV DNA PCR product (arrowhead). Total RNA was extracted from *D. aegypticum* inoculated with heat treated sap at 50-65°C. Ladder DNA 100 bp (NEB) on the left

Application of HWT on infected cutting cane

The thermal inactivation point was used as a guideline to evaluate the effectiveness of HWT from 55-60°C with submersion times of 10, 20, 30, 60 and 120 min in eliminating the SCSMV from infected cutting canes. HWT for up to 30 min at 55°C caused more than 60% of cane setts did not grow, while HWT at 60°C for up to 10 min submersion time caused the buds of all tested setts to die. All tested cutting canes treated by either HWT at 55°C longer than 30 min or on 60°C longer than 10 min submersion time could not withstand (data not shown). Further, the HWT was optimized by using lower temperatures from 52°C-55°C and shorter submersion times of 10, 20, and 30 min (Table 2).

Effect on viability

The cane setts were obtained from SCSMV infected plants. The HWT at 52°C for 10 and 20 min had no effect on the incidence or viability, while 30 min submersion time caused viability of cane setts to decrease up to 10%. At 53°C for 10 min submersion time, all tested cane setts still had 100% viability, while 10 min longer submersion time decreased viability by up to 30%. A similar trend was observed for other treatments from 54°C-55°C in comparison with the cane setts control (Table 2). It is suggested that the elevation of temperature and submersion time causes cane setts to lose their viability.

Effect on incubation period

The first time appearance of symptom of all setts which were subjected by HWT tends to longer than control plants. It was revealed that increasing temperature and submersion time affect to

incubation period. There was 2-9 days delay time the first symptom by HWT in compare to those of control plants although there were not significant differences (Table 2).

Effect on disease severity

Severity of all plants tested showed significantly lower than control plants (Table 2). The HWT on 52°C for 10 min showed severity significantly higher than other treatments, while among other treatments were not significantly different. By elevated temperature and submersion time reduced the severity significantly in compared to those of control plants.

HWT can effectively eliminate pathogens from bulbs, and sereh disease from sugarcane setts (Mink *et al* 1998). Long hot water treatment (LHWT) at 50°C for 2 hr completely eliminated several causal agents of sugarcane diseases such as ratoon stunting disease (*Leifsonia xyli* subsp. *xyli*) (Irawan *et al.*, 1985; Mirzawan and Samoedi, 1995), leaf scald (*Xanthomonas albilineans*), and smut (*Ustilago scitaminea*) in sugarcane setts (Putra, 1997). In addition, LHWT not only effectively controlled ratoon stunting, but also could improve the quality of planting materials (Putra *et al.*, 2009) and could increase sugar yield on several sugarcane varieties (Suwarno and Legowo, 1990). Further, short hot water treatment (SHWT) at 52°C for 30 min can eliminate the causal agent of sereh disease (Handojo, 1982) but is unable to eliminate *L. xyli* from cane setts (Irawan *et al.*, 1985). In addition, these treatments did not effectively control mosaic disease.

Table 2. Effect of hot water treatment on setts viability, incubation period and disease severity.

Temperature -Time (°C – min)	Viability (%)	Incubation Period (days)	Disease Severity (%)
52-10	100 ± 0.00a*	16.0 ± 2.1a*	19.13 ± 5.73a*
52-20	100 ± 0.00a	16.0 ± 2.0a	15.93 ± 3.16b
52-30	90 ± 0.32ab	17.0 ± 5.7a	16.12 ± 5.80bc
53-10	100 ± 0.00a	17.0 ± 1.8a	16.00 ± 4.94bcd
53-20	70 ± 0.48abc	17.0 ± 8.4a	15.80 ± 8.31bc
53-30	70 ± 0.48abc	19.0 ± 9.2a	15.60 ± 8.42cde
54-10	70 ± 0.48abc	21.0 ± 10.1a	13.30 ± 6.80cde
54-20	60 ± 0.52abc	21.0 ± 11.0a	12.72 ± 6.70de
54-30	40 ± 0.52c	21.0 ± 10.9a	12.40 ± 6.46e
55-10	60 ± 0.52abc	23.0 ± 11.9a	11.60 ± 7.15e
55-20	50 ± 0.53bc	23.0 ± 12.2a	9.70 ± 5.60e
55-30	40 ± 0.52c	23.0 ± 11.8a	9.90 ± 5.30e
Control	100 ± 0.00a	14.0 ± 1.4a	60.00 ± 6.96f

*Number in columns followed by the same letter are not significantly different ($\alpha = 0.05$)

In this study, HWT did not completely eliminate the SCSMV as effectively as it eliminated fungi and bacteria. However, a previous report by Benda (1971) showed that serial heat treatment including pre-treatment 52°C for 20 min in the first day followed by 57.3°C for 20 min in the second day and 57°C for 20 min in the third day, can cure the SCMV infected cutting canes. While in case of garlic cloves, hot air treatment at 36°C for 30 and 40 days resulted 50% and 51% cloves free GYSV and plantlet free virus for treatment more than 60 days (Conci and Nome, 1991). The biological character of virus, its distribution in infected tissues, type of plant tissues, temperature and submersion

time might affect the effectiveness of HWT. Therefore, in the case of SCSMV additional trials to optimize the appropriate temperature and submersion time need to be conducted.

Heat affects viral replication and virus movement. It has been reported that treated plant in sustained temperature of 37°C or above would completely inhibit multiplication of many viruses (Kassanis 1957 in Hadidi *et al.*, 1998). Heat can also cause inactivation of the virus in the early phase resulting in earlier reduction in SCMV titer (Balamuralikrishnan *et al.*, 2003). These might explain why HWT causes the incubation period to extend and reduces disease severity than control. Since the antisera against SCSMV is not yet available commercially, the effect of HWT on the virus titer could not be quantified. Further, based on the intensity of the DNA band of each treatment could not be differentiated by RT-PCR (data not shown).

Sugarcane are maintained and propagated by using setts which lead to high virus infestation over time. Hence the SCSMV titer needs to be reduced and subsequently eliminated. Although none of the treatments produced virus free plants, treatment at 53°C for 10 min might be the best treatment to reduce the severity drastically and delay the appearance of phenotype symptoms without obvious effect on the viability. It is suggested that reduction of disease severity might be related to reduction of the virus titer as previously reported on SCMV (Balamuralikrishnan *et al.*, 2003). Further, HWT at 55°C with submersion time between 20 to 30 min might the best treatment to get the source of meristem tip culture with lowest severity. El-Nasr *et al.* (1989) reported that SCMV could eliminated by HWT at 55°C and 57°C followed by meristem tip culture, while serial HWT was effective in the elimination of the virus (Benda *et al.*, 1989). The early reduction in virus titer could be utilized to obtain more virus free plants in meristem tip culture. The SCSMV infection affected the production of tiller numbers (Asnawi, 2009) during the tillering period at 3-4 months after planting. This period is also a critical time for sugarcane to respond to the pathogen infection. Thus, HWT before planting would suppress the effect of SCSMV infection during the critical time. The maximum suppression of virus might lead to suppression of disease severity and extension of the incubation period which would eventually allow plants to overcome the infection.

The application of HWT in large scale plantations might be time consuming and expensive. However, HWT is worth considering to mitigate the impact of the virus at the plantation scale, especially during the tillering period. Further, HWT in combination with micro-propagation using meristem tip culture, might lead to complete virus elimination to preserve more virus-free plants. To improve the effectiveness of HWT, the present researches related with utilization of beneficial microbes such as PGPR, endophytic and decomposer bacteria to improve plant health and increase systemic resistance against SCSMV infection are on-going.

CONCLUSION

The thermal inactivation point of SCSMV is between 55°C to 60°C, and it is higher than the plant thermal death point for sugarcane. All heat treatments did not completely eliminate SCSMV, however HWT at 53°C for 10 min drastically reduced disease severity while maintaining 100% plant viability. The earlier suppression of virus before planting by HWT would minimize the effect of SCSMV infection during the tillering period.

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