Elimination of mosaic disease caused by potyvirus and fabavirus on patchouli using synthetic antiviral

Sukamto[1*], *Indijarto Budi* Rahardjo[2], *Kurniawan* Budiarto[2], *Styowati Retno* Djiwanti[1], *Rita* Noveriza[1], and *Endang* Hadipoentyanti[1]

[1]Indonesian Center Research for Estate Crops, National Research and Innovation Agency, Cibinong Science Center, Jl. Raya Jakarta – Bogor, Cibinong, Bogor (16915), Indonesia
[2]Indonesian Center Research for Horticultura Crops, National Research and Innovation Agency, Cibinong Science Center, Jl. Raya Jakarta – Bogor, Cibinong, Bogor (16915), Indonesia

Abstract. Patchouli growers in Indonesia were constrained by detrimentally systemic diseases caused by viruses. Potyvirus and Fabavirus were pathogenic viruses that commonly found in several production centers. Virus elimination for retaining healthy protocols was investigated and one of the promising methods through mersitem culture and antiviral treatments. The research was conducted to find out the effects of ribavirin treatments following meristem culture on the existence of Potyvirus and Fabavirus on the infected patchouli plants. The research was conducted at The Indonesian Spices and Medicinal Research Institute from September 2018 to August 2019. A factorial experiment with 10 replications established to facilitate the combination of two factors. The first factor was two commercial patchouli cultivars, namely Patchoulina 1 and Patchoulina 2. While the second factor dealt with the concentration of ribavirin i.e. 0, 5, 10, 15 and 20 ppm. The results showed that the potyvirus was still detected based on ELISA analysis after the treatments of meristem culture and ribavirin in any concentrations. However, the application ribavirin at 20 ppm following meristem culture effectively eliminated fabavirus form both the tested patchouli cultivars. These partial virus elimination within the plantlet gave significant growth improvement on plantlet height, number of leaves and number of auxiliary shoots after 8 weeks subculture.

1 Introduction

Patchouli (*Pogostemon cablin* Benth.) is a plant which can be extracted for essential oil and one of Indonesian most important commodity for export purpose. Patchouli oil is extracted through oil distillation process or other methods such as solvent extraction, solvent-free hydrodestillation and supercritical CO_2 [1,2]. The patchouli oil is used for fragrance products and as a raw material for a number of roducts such as antiseptics, aromatherapy, cosmetics, pesticides and a fixative to bind other essential oils, like an ingredient in Asian and Arabian traditional medicine [3,4]. The plant is native to tropical regions of Asia, and is now

* Corresponding author: sukamto@brin.go.id

extensively cultivated in China, Indonesia, India, Malaysia, Mauritius, Taiwan, the Philippines, Thailand, and Vietnam, as well as West Africa [5].

In a common practice, the cuttings can be firstly harvested at 6 months after planting. The subsequent harvest periods are in the interval of 3-4 months depending the genotype and growth performance [6]. Patchouli productions in Indonesia are unstable and so vary in quality and quantity. The plants actually can be maintained for more than one year, but the yields usually start to decline after 3-4 harvesting cyles [7]. The main constraint in patchouli cultivation in Indonesia is the lack of good varieties, the occurrence of allelopathy and the presence of pests and diseases caused by various organisms, including viruses [8].

Some viruses have been reported naturally infecting patchouli plants, such as *Tobacco Necrosis Virus* (TNV), *Patchouli Virus X* (PatVX), *Patchouli Mild Mozaic Virus* (PaMMV), *Patchouli Mottle Virus* (PaMoV), *Peanut Stripe Potyvirus* (PStV), *Pepper Ringspot Virus* (PRV) and *Cucumber Mozaic Virus* (CMV) [9]. In Indonesia, at least 6 viruses have been identified in several patchouli production centers, such as *Potyvirus* (TeMV, PStV), *Fabavirus* (*Broad Bean Wilt Virus 2*- BBWV2), Potexvirus (*Cymbidium Mozaic Virus*, CymMV), CMV and *Tobacco Mozaic Virus* (TMV). Among the identified viruses, Potyvirus is the dominantly found in most of patchouli plantation [8].

The family Potyviridae consists of 6 genera based on their transmission; fungi (Bymovirus), whiteflies (Ipomovirus), aphids (Macluravirus, Potyvirus) or mites (Rymovirus, Tritimovirus) and Potyvirus is the largest among the six genera in the family Potyviridae [10]. These viruses are 720–850 nm in length and are transmitted by aphids. They can also be easily transmitted by mechanical means. The genome of the virus is linear, single-stranded positive sense monopartite or bipartite RNA of 8,500–12,000 nucleotides with a poly (A) tail at the 39 end and probably a genome-linked protein (VPg) at its 59 end [9,10]. The most commonly found Potyvirus that accosiated with patchouli in Indonesia is identifed as *Telosma mosaic virus* (TeMV) and closely related to *Passion fruit Woodiness Virus* (PaWP) [8]. The diseased plant showed typical symptoms of yellow mosaic without malformation of the leaves, pale green mosaic with malformation of the leaves, severe mosaic, thickened leaves or stunted and retardation of the plant growth [8].

Aside from Potyvirus, *Broad Bean Wilt Virus 2* (BBWV2) was also reported to attack patchouli plants in Indonesia. The symptoms are varied from weak intensity of yellowish green or light green to dark green with thicker leaf blade and malformation on the leaf shape [11,12]. Both virus groups have contributed to the declination of patchouli yield and quality in most production centers.

Efforts have been made to get the healthy protocols by eliminating virus from the infected plants. Several methods such as electrical charges, heat treatment, meristem culture and the use of synthetic antiviral have been successfully conducted for virus elimination in some crops. Numerous chemicals have been tested for antiviral activity, but few were effective. The most substance is the synthetic analogue of guanosine, ribavirin (1-beta-D-ribofuranosyl-1-H-1,2,4-triazole-3- carboxamide) added to the media in the range of 30-50 mg/l was affective against *Potato virus X* (PVX), *Potato Virus Y* (PVY), *Potato Virus S* (PVS) and *Potato Virus M* (PVM) in potato [13,14], *Carnation Mottle Virus* (CarMV) in carnation [15], *Onion Yellow Dwarf Virus* (OYDV) in onion [16], *Satsuma Dwarf Virus* (SDV) in citrus [17] and *Chrysanthemum Virus-B* (CVB) in chrysanthemum [18]. Ribavirin is a member of the nucleoside anti metabolite compound that interferes with duplication of viral genetic material. Though not effective against all viruses, ribavirin is remarkable as a small molecule for its wide range of activity; including important activities against both DNA and RNA viruses [19]. Ribavirin's carboxamide group can make the native nucleoside drug resemble adenosine or guanosine, depending on its rotation. For this reason, when ribavirin is incorporated into RNA, as a base analog of either adenine or guanine, it pairs equally well with either uracil or cytosine, inducing mutations in RNA-dependent replication in RNA

viruses. Such hypermutation can be lethal to RNA viruses [20]. The successful chemotherapy method, however, depended on plant genotypes, viruses and phytotoxic that might cause in an increase in culture time and the need for frequent transfers into fresh media [21].

The application of antiviral ribavirin in culture media of patchouli was expected to reduce or even eliminate virus particles within the plants and produce free-virus protocols for mother stock plants. The healthy stock plants would improve the growth performance of the cuttings for mass oil production. Based on the reasons and promising antiviral effects, the research was conducted to find out the effects of ribavirin application in the media on the existence of potyvirus and fabavirus in infected patchouli plants.

2 Materials and methods

The research was conducted in the laboratory of tissue culture and virology at The Indonesian Spices and Medicinal Research Institute from September 2018 to August 2019. A complete factorial experiment with 10 replications was designed to accomplish the combination of two factors. The first factor was two commercial patchouli cultivars, namely Patchoulina 1 and Patchoulina 2. Patchouli cv. Sidakalang was used for the negative control during ELISA analysis. While the second factor dealt with the concentration of virazol (1-beta-D-ribofuranosyl-1-H-1,2,4-triazole-3- carboxamide = Ribavirin), i.e. 0, 5, 10, 15 and 20 ppm.

The infected cutting samples were collected from farmers' field. The cuttings were then, replanted in 15 cm pot and maintained in protected screen house. After 6 weeks, the plants were pinched and the new emerging lateral growths served for explants. Shoot induction was conducted by inoculating apicals into MS + BAP 0,5 mg/l. Subculture on the same media were carried out 3 times to obtain uniform plantlets and sufficient samples for the experiment. Six weeks after subculture, approximately < 0.5 mm apical meristem was dissected under the binocular microscope, and inoculated into MS media containing NAA for callus induction. The callus was then subcultured into the same media and incubated into light condition for further proliferation and shoot induction. The new emerging shoots were deflasked into regeneration media to obtain complete plantlets.

After 6 weeks incubation, the plantlets were transferred into treatments media, consisting MS + 0.5 mg/l BA supplemented with ribavirin with various concentrations according to treatments. The plantlets were then maintained under 16 h light of 1000 lux and 18-22^{0}C culture environment. After 8 weeks cultures, randomly plantlet samples in every treatment combinations were selected for potyvirus and fabavirus rapid detection using direct ELISA. The procedure for direct ELISA detection was presented into the following descriptions.

- 100 µl potyvirus/fabavirus IgG (AGDIA, USA) was mixed 1 : 200 with the coating buffer (Na_2CO_3 + $NaHCO_3$ + NaN_3) and over nightly incubated in the temperature of 4 ^{0}C.
- The microplates were then, rinsed twice with PBS Tween (NaCl + KH_2PO_4 + Na_2HPO_4 + KCl + NaN_3 + Tween 20 + H_2O) buffer in three minutes each.
- 0.2 g of leaf samples were extracted and buffered with 1 ml mixture of PBST + 0,02 % PVP (1 : 5). The 100 µl of leaf extracts were then, incubated for 2 h in 37 ^{0}C and rinsed with PBS Tween buffer.
- After labeled with Alkaline Phosphatase enzyme, 100 µl of IgG potyvirus/fabavirus was pipetted into microplates and mixed with ECI (PBST + 0,2 % BSA) with the composition of 1 : 200. The mixture was incubated for 2 h in 37 ^{0}C. After 2 h incubation, the microplates were then rinsed with PBS Tween buffer.
- 100 µl substrate buffer containing 4-nitrophenylphospate was put into microplates and incubated in the room temperature. After the substrate changed in color into yellow, the reactions were then ceased with 25 µl NaOH 3M.
- Color intensities of the substrate were measured using ELISA reader (Minireader II Dynatech) on 405 nm wavelength. The plant sample was dedicated virus-free if three

times of the substrate absorbent measured from the ELISA reader was less than the value of positive control.

The observation was also conducted on plantlet growth performances after ribavirin treatments. All the gathered data were analyze using ANOVA and the mean comparison was carried out based on Least Significant Difference (LSD, $\alpha \leq 5\%$).

3 Results and discussion

3.1 ELISA assessment of treated plantlets

3.1.1 Potyvirus

The ELISA assessment on the ribavirin-treated plantlet towards potyvirus and fabavirus revealed different results. Meristem culture followed by ribavirin application was not able to reduce or even eliminate potyvirus particles on the infected Patchoulina 1 and Patchoulina 2 plantlets (Table 1). All the treated plantlet of both cultivars in all ribavirin concentrations still positively contained virus particles based on ELISA assessment. The failure of meristem culture and antiviral application in reducing or eliminating potyvirus on patchouli plantlets might due to several factors. First, the applied meristem culture was not able and not sufficient enough to isolate virus-free meristematic area of shoot apical [22]. The maximum size or thickness of isolated meristematic dome that could be acquired in the study was 0.5 mm, whereas the ideal size for isolating virus-free meristematic area was less than 0.2 mm [23,24].

Table 1. Percentage of potyvirus-free plantlets of Patchouli cv. Patchoulina 1 and 2 treated by different concentrations of ribavirin.

Ribavirin concentration (%)	Percentage of virus-free plantlets (%)	
	Patchoulina 1	Patchoulina 2
0	0	0
5	0	0
10	0	0
15	0	0
20	0	0

Other possible factor is that the concentration of ribavirin used in the study was not sufficient to give disturbance effects on the virus replication within the plants. Several reports indicated that the low concentration of antiviral within the media failed to reduce or even to eliminate virus particles within the plants [16]. The successful reported virus elimination using ribavirin on crops were mostly in the concentration range of 40-60% [25,26], though other studies also reported that some viruses were also successfully eliminated by ribavirin application at the concentration less than 30% [27,28].

Another potential factors in relation to the failure of ribavirin treatment on potyvirus elimination in this study was insufficient duration of plantlet exposure to ribavirin. The chemical ribavirin need certain period to be metabolically involved in plant biochemical reactions, thus intefered virus cycle and replication pathway. The effectivity of antiviral in eliminating plant viruses was also determined by the exposure period of explant to antiviral agents [29]. Though there was no specific recommended period stated, the period of treatment might compensate the low antiviral concentration against virus replications.

3.1.2 Fabavirus

Unlike on that potyvirus, the effect of meristem culture and ribavirin treatments on the existence of fabavirus particles within the infected patchouli plants were obvious. At Patchoulina 1, the meristem culture freed the plants from the fabavirus particles less than Patchoulina 2, as indicated on 0% ribavirin concentration treatment (Table 2). The difference on the percentage of virus free plants after meristem culture application between two cultivars was due to plant genotypes. Several studies have reported that the effectivity of meristem culture in eliminating virus particle within the plant were determined by several factors, and one of them is the persistency of virus [30,31] and the persistency of virus persistency was related with the plant genotypes [16].

Table 2. Percentage of fabavirus-free plantlets of Patchouli cv. Patchoulina 1 and 2 treated by different concentrations of ribavirin.

Ribavirin concentration (ppm)	Percentage of virus-free plantlets (%)	
	Patchoulina 1	Patchoulina 2
0	25	75
5	50	100
10	50	100
15	75	100
20	100	100

Table 2 also shows that the effects of ribavirin at different concentrations in reducing virus particles was determined by effectivity of meristem culture as the initial step. At Patchoulina 1, meristem culture was able to free 25% plantlets, yet 75% in Patchoulina 2. The higher initial reduction in virus particle in Patchoulina 2 has made less ribavirin concentrations (5ppm) in totally freed the plantlets from fabavirus. While in Patchoulina 1, the 100% virus free plantlets were obtained at 20 ppm ribavirin. The successful elimination of fabavirus particle indicated that the fabavirus infecting the patchouli plantlets was sensitive to ribavirin. The applied ribavirin was able to interfere virus cycle and disrupt the virus multiplication. According to some reports, the mode of action of ribavirin on the retardation of virus multiplication was through several ways. First, ribavirin was phosphorylated into ribavirin monophosphate. These interediate compound was then bound with inosine monophosphate dehydrogenase (IMPDH) and become an inhibitor of the respected enzyme. The enzyme was actually play important role in the biosynthesis of de novo purine [32]. The second, ribavirin was able inhibit the virus RNA synthesis. Ribavirin has native nucleosides resemble to adenosine or guanosine on purine RNA nucleotide. These functional sites were phosphorylated into ribavirin triphosphate (RTP) and bound with RNA-dependent RNA polymerase. The newly compound acted as enzyme inhibitor on the process of base nucleotide polymerization [33]. Lastly, the RTP that was an analog of purine guanosine triphosphate was inserted to the virus genome and resulted in mutation on transcription and translation on the virus RNA. These condition was considered lethal for virus [20].

3.2 Plantlet growth performance

The growth performance of patchouli plantlets after meristem culture and ribavirin treatments were presented in Table 3 and 4. Table 3 shows that the effects of meristem culture and ribavirin treatments were clearly shown after 8 weeks subculture on Patchoulina 1. Similar phenomena were also observed on Patchoulina 2 (Table 4). The meristem culture and ribavirin treatments had not shown on the plantlet growth improvement yet. No significant

difference was observed on plantlet height, number of leaves and number of auxiliary shoots in any ribavirin treatments after 2 weeks subculture.

Table 3. Average of plantlet height, number of leaves and number of auxiliary shoots of Patchoulina 1 at 2 and 8 weeks after subculture (WAS).

Ribavirin concentration	Growth parameters at... weeks after subculture (WAS)					
	2			8 WAS		
	Plantlet height (cm)	Number of leaves	Number of auxilary shoots	Plant height (cm)	Number of leaves	Number of auxilary shoots
0 ppm	6.03 a	10.40 a	1.33 a	22.29 bc	34.20 b	2.07 bc
5 ppm	6.17 a	12.93 a	1.73 a	23.81 b	35.73 b	2.33 bc
10 ppm	6.31 a	13.07 a	1.80 a	23.44 b	37.67 b	2.27 bc
15 ppm	6.49 a	12.63 a	1.87 a	25.51 b	40.07 b	2.53 b
20 ppm	7.03 a	13.53 a	1.40 a	30.95 a	59.27 a	3.13 a
CV	0.88	1.14	0.22	3.76	10.17	0.46

Table 4. Average of plantlet height, number of leaves and number of auxiliary shoots of Patchoulina 2 at 2 and 8 weeks after subculture (WAS).

Ribavirin concentration	Growth parameters at... weeks after subculture (WAS)					
	2			8 WAS		
	Plant height (cm)	Number of leaves	Number of auxiliary shoots	Plant height (cm)	Number of leaves	Number of auxilary shoots
0 ppm	3.35 ab	1.43 a	11.73 a	20.81 b	2.07 ab	30.53 b
5 ppm	4.44 b	1.60 a	11.67 a	21.01 b	2.07 ab	35.53 b
10 ppm	4.97 ab	1.73 a	12.00 a	20.85 b	2.27 ab	36.87 b
15 ppm	5.37 ab	1.80 a	12.60 a	22.29 b	2.07 ab	38.40 b
20 ppm	8.56 a	1.80 a	14.73 a	30.42 a	2.93 a	58.13 a
CV	1.73	0.18	1.21	3.94	0.39	9.54

After 8 weeks subcultures, plantlets of the studied cultivars showed different growth performances especially those with ribavirin treatments. Plantlet height, number of leaves and number of auxiliary shoots were detected higher on ribavirin-treated plantlets, though the values were still insignificantly different up to the concentration of 15 ppm. At 20 ppm ribavirin, the plantlets showed more obvious improvement with significant higher plantlets and more number of leaves and auxiliary shoots compared to lesser ribavirin treatments. The improved plantlets growth was presumably due to the reduction of severity of the virus infection [34]. In this case, 20 ppm ribavirin could partially eliminate virus containments within the plantlets, especially Fabavirus. These also proved that though potyvirus were still unable to be eliminated by ribavirin treatment up to 20 ppm, the partial virus elimination can promote better plant performance in the case of if more than viruses attacked the plant [35].

4 Conclusions

The treatments of meristem culture and ribavirin had different effectivities on the potyvirus and fabavirus containment on Patchouli plantlets. The applied meristem culture and ribavirin in any concentrations were unable to eliminate potyvirus. While, the application ribavirin at 20 ppm following meristem culture effectively eliminated fabavirus form both the tested patchouli cultivars. These partial virus elimination within the plantlet gave significant

growth improvement on plantlet height, number of leaves and number of auxiliary shoots after 8 weeks subculture.

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