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Elimination of Potyvirus and Carlavirus from Infected shallot bulbs

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Abstract

Viral diseases on shallot in Indonesia has been reported and have important role in yield loss. Viral diseases are easily transmitted via infected bulbs in generations and in regions. Cultivation of virus-free bulbs is considered the most effective method of controlling viral diseases, so the production of virus-free bulb are important and necessary. Elimination of viruses on local cultivars was achieved using shoot tip culture combined with thermotherapy. In this research, the method for elimination of *Potyvirus* and *Carlavirus* from shallot bulbs was developed using combination of thermotherapy (30 °C, 37 °C, in homogenous and heterogenous condition) with different size of explant shoot tip culture (1 mm, 2 mm, 3 mm). The result showed that virus-free plantlets could be derived from 1 mm-explant. Treatment combination of 2 mm-explant with homogenous temperture 37 °C was also able to eliminate virus.

Key words: Chemotherapy, shoot tip culture, thermotherapy, virus-free plantlets

Introduction

Shallot (*Allium cepa* var. *aggreggatum*) is one of important horticultural comodity in Indonesia. Viral diseases have been known to cause significant problem on shallot production. Although shallot can be propagated through generative or vegetative stages using true seeds or bulbs, respectively, however most farmers in Indonesia use bulbs as vegetative propagative material. This is due to difficulties to produce true shallot seed (TSS) in Indonesia because of climate condition. Several cultural practices have been applied by farmers in order to reduce virus infection. In general, farmers in Indonesia spray insecticides very intensively to control insect vector. Unfortunately, most of control practices are not effective and not friendly to environment.

The use of virus-free bulbs as initial planting material will help reduce diseases development and consequently improve crop production (Perroto *et al.,* 2010). *In vitro* propagation, using shoot tip culture, is the most widely used to obtain virus-free bulbs (Al Maarri *et al.*, 2012). Shoot tip culture can be combined with several techniques, such as thermotherapy, chemotherapy, electrotherapy, and cryotherapy to improve efficiency of virus elimination. Among them, thermotherapy and chemotherapy are the most common used in the treatment of virus elimination (Rout *et al.,* 2006). The effective virus elimination on local cultivars in Indonesia using shoot tip culture was not reported yet. Based on this, the efficient techniques getting virus-free bulbs should be developed to improve the quality of shallot productionin Indonesia. Therefore, research has been done to develop an efficient method based on combination of shoot tip culture and thermotherapy to get virus-free shallot bulbs.

Materials and methods

Plant materials: Local shallot cultivars *i.e.,* 'Bima Brebes' and 'Bima Curut' were obtained from Tenguli village in Brebes district, Province of Central Java, Indonesia. The bulbs were 60 -70 days in storage after they were collected for virus detection. One hundred bulb samples from each cultivar were grown in cold room (16 ^oC) for one week. Leaf samples were then collected for virus detection using dot blot immunobiding assay (DIBA) according to protocol described by Asniwita *et al.* (2012). Specific antibody to *Onion yellow dwarf virus* (OYDV), *Garlic common latent virus* (GCLV), and *Shallot latent virus* (SLV) was used separately in the assay. The disease incidence of OYDV, SLV, and GCLV on cv. Bima Brebes and cv. Bima Curut *i.e*. 100, 98, and 93 % based on DIBA results. The infected bulbs were then used as initial materials for shoot tip culture.

Bulb and explant sterilization: Shallot bulbs were washed and cleaned up prior to shoot tip culture. The outer layer and necrotic stem base of the mature bulb were removed. The bulbs were washed using detergent and rinsed with tap water. The bulbs were then soaked into 2 g L^{-1} fungicide (mancozeb 80 %) and 2 g L^{-1} bactericide (streptomycin sulphate 20 %) then incubated overnight . Afterward, bulbs were soaked into 20% sodium hypochlorite and shaked at 150 rpm for 20 min. These was followed by rinsing 3 times in sterile distilated water in laminar air flow, two outer layers were removed, then soaked again into 10 % sodium hypochlorite for 10 min and rinsed for 3 times. The bulbs were excised until 5 mm, the cuts was dipped into 5 % sodium hypochlorite for 5 min then planted in MS0 medium to prevent explant damage. The 5 mm explants were excised into 3 mm, 2 mm, and 1 mm using sterile needles under binocular microscope. Shoot-inducing medium was made by MS (*Murashige*-skoog) medium added with 2-Isopentanyladenine (2 ppm), Gibberellic acid (0.3 ppm), 0.3 % sucrose, and Gelrite

2 g L^{-1}. Root-inducing medium was made by MS medium with gelrite 2 g L^{-1} and 0.3 % sucrose.

Thermotherapy treatment: The treatment consisted of two temperatures *i.e.* 30 °C and 37 °C, and 25 °C as a control treatment. Two conditions of thermotherapy was examined, *i.e.* incubation in homogenous and in heterogenous condition. For heterogenous condition, the explant was incubated in 25 °C for the first 2 weeks, then in 30 °C for the next week, then in 37 °C for the following week; whereas for homogenous condition, explants were incubated in homogenous temperature (30 °C, 37 °C, or 25 °C) for 4 weeks. All plantlets survived from thermotherapy treatment was tested for present of *Potyvirus* and *Carlavirus* using RT-PCR as following.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Method: Two pairs of primer were used separately to amplify *Potyvirus* (U341/Poty1) and *Carlavirus* (AlcarF/Poty1) (Chen *et al.*, 2001; Langeveld *et al.*, 1991) (Table 1). Total RNA was extracted from leaf samples using Doyle and Doyle (1987) with modification. This research was used 0.1 g leaf samples and added with 1% CTAB buffer. The solution then incubated at 60 ºC for 30 min. The total RNA was used for reverse transcription reaction to form cDNA (complementary DNA) using reverse transcriptase enzyme. Each reverse transcription reaction consisted of: 10 x Buffer RT, 0.1 M DTT, 10 mM dNTP mix, 20 U/ µL RNAse inhibitor, 100 U/µL M-MuLV, 10 µM Poty 1 primer, 3 µL of total RNA, and the reaction was adjusted to 10 µl with nuclease free water.. The mixtures were added into clean 250 µL tube, then 3 µL of total RNA was added. Reverse transcription reaction was performed in automated thermal cycler (Gene Amp PCR System 9700; PE Applied Biosystem, USA) with one cycle program at temperature 65 °C for 5 min, 42 ºC for 60 min, and 70 °C for 10 min.. The synthesized cDNA was then used as DNA template in the amplification reaction. cDNA amplification reaction per tube consisted of: 12.5 µL Go Taq Green (Thermoscientific), 9.5 µL nuclease-free water, 10 µM for each primer (forward and reverse), and 1 µL of cDNA.

Amplification of cDNA viruses was performed ini GeneAmp PCR System 9700 started with pre-heating cycle for 5 min at 94 °C, followed by 35 cycles of denaturation (20 sec at 94 °C), annealing (1 min at 54 °C), and extension (3 min at 72 °C). The last stage was ended at 72 °C for 1 min and cooled down to 4 °C. The amplicon then analyzed by electrophoresis using 1% agarose gel in 0.5x TBE (Tris-Boric acid-EDTA) buffer. The electrophoresis was performed at 50 V for 50 min, then the gel was soaked on to 0.1% EtBr for 10 min, washed with H_2O for 5 min, and visualized under UV transilluminator.

Statistical analysis: The experiment was conducted using factorial completely block design with two factors for each cultivars, *i.e.* explant size (1 mm, 2 mm, and 3 mm) and temperatures (30 °C and 37 °C, control 25 °C), 3 replications, 10 explants per treatment. All data were subjected to analysis of variance, significant differences of the means were evaluated using Duncan's multiple range test or Student's *t*-test. Characteristic of plantlet growth that observed are percentage of survival plantlets, average number of leaves, and average number of roots.

Table 1. Primers used for amplification of *Potyvirus* and *Carlavirus*

Primers	Sequences	Amplicon (bp)
U341	5'-CCGGAATTCATGRTITGGTGYATIGAIAAYGG-3'	*Potyvirus*,
Poty1	5'-GGATCCCGGGTTTTTTTTTTTTTTTTTV-3'	± 600-750
AlcarF	5'-TGCTGCYTTTGATACYTTCGAT-3'	*Carlavirus*,
Poty1	5'-GGATCCCGGGTTTTTTTTTTTTTTTTTV-3'	± 715

Results and Discussion

Shoot tip culture had been widely used to obtain virus-free plantlets. Combination with thermotherapy was a common method to improve the efficiency of those techniques. In our experiment, thermotherapy at 30 °C under heterogenous condition did not give significant effect on life percentage of cv. 'Bima Brebes'. In contrast, thermotherapy at 37 °C had deleterious effect on plantlets survival for both cultivars (Table 2). These result is similar with previous reports on the attempts to eliminate virus infection from garlic. Thermotherapy at 36 °C for 4-5 weeks on garlic resulted on reduction on regeneration potential of dome shaped structures (Ghaemizadeh *et al.*, 2014). Torres *et al.* (2000) also reported that increasing temperatures up to 40 °C could decrease survival percentage of plantlets and ability of plantlets to regenerate up to 20 %. In our experiment, plantlets growth at 37 °C were limited, leaves are deformed, and distorted in contrast

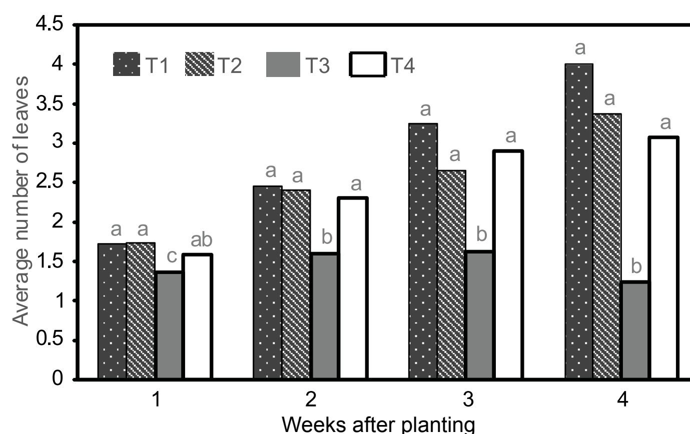

Fig. 1. Effect of temperature on average number of leaves cv. 'Bima Brebes'. T1: control; T2: 30°C; T3: 37°C; T4: heterogenous.

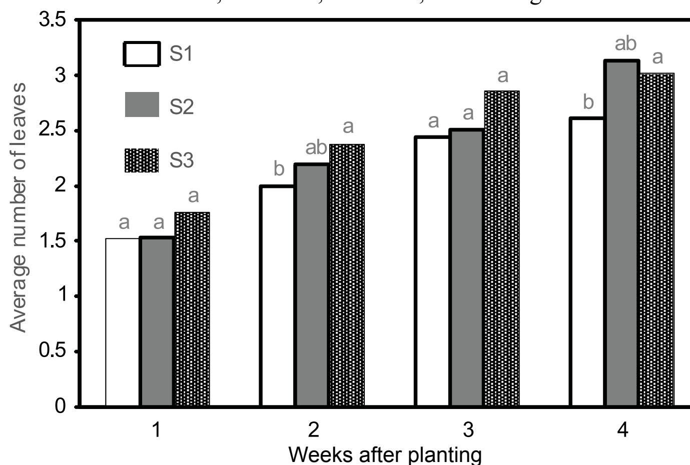

Fig. 2. Effect of explant size on average number of leaves cv. 'Bima Brebes'. S1: 1 mm-explant; S2: 2 mm-explant; S3: 3 mm-explant

with normal shoots produced by control plantlets. Plantlets showed vitrified and the leaves colour became light green to yellow. These might be the result of deficiency in chlorophyl a and b which can inhibit the plantlet growth (Rasco & Patena, 1997).

An average number of leaves in plantlets cv. 'Bima

Table 2. Effect of thermotherapy to life percentage of plantlet cv. Bima Brebes and cv. Bima Curut

Cultivars	Treatment	Mean values
'Bima Curut'	Control vs 30 °C	0.265
	Control vs 37 °C	0.013*
	Control vs heterogenous	0.37
	37 °C vs 30 °C	0.017*
	30 °C vs heterogenous	0.274
	37 °C vs heterogenous	0.021*
'Bima Brebes'	Control vs 30 °C	0.211
	Control vs 37 °C	0.043*
	Control vs heterogenous	0.091
	37 °C vs 30 °C	0.04*
	30 °C vs heterogenous	0.065
	37 °C vs heterogenous	0.051

*Significantly different at 5% level using Student's T-test

Brebes' (Fig. 1 and Fig. 2.) and cv. 'Bima Curut' (Fig. 3. And Fig. 4.) were not affected by combination of explant size and temperatures, although untreated plantlets and plantlets treated at heterogenous condition had better growth. Plantlets developed from 3 mm-explant had the best leaves development. Fayek *et al. (*2009) described that the larger explant size in meristem culture gave a higher results in all characteristic of plant growth.

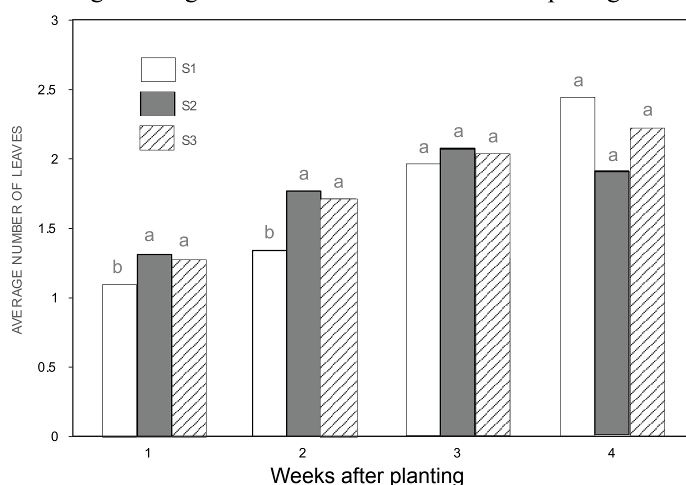

Fig. 3. Effect of temperatures on average number of leaves plantlets cv. 'Bima Curut'. T1: control; T2: 30°C; T3: 37°C; T4: heterogenous.

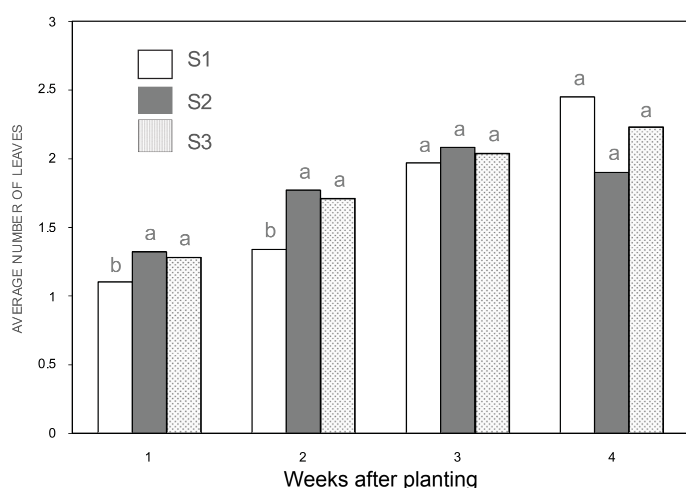

Fig. 4. Effect of explant size on average number of leaves plantlets cv. 'Bima Curut'. S1: 1 mm-explant; S2: 2 mm-explant; S3: 3 mm-explant

Furthermore, Ali *et al.* (2013) described that meristem is the delicate part of the plant. These part is easily injured or even died in high temperature. Therefore, explants could not survived well when exposed to high temperature. Plantlets cv. 'Bima Curut' from 1 mm-explant in 4 weeks treatment were taller than plantlets from 3 mm and 2 mm. Although there is no significant difference between each explant, the smaller explant size produce leaves per plant better than larger explant . According to Taskin *et al. (*2013), the smaller explant size of garlic carried out either free or very low concentration of viruses, therefore the competition are less and in turn plantlets were able to grow better.

Observation on root formation indicated which was affected by temperatures (Fig. 5. and Fig. 7.), but explant size was not

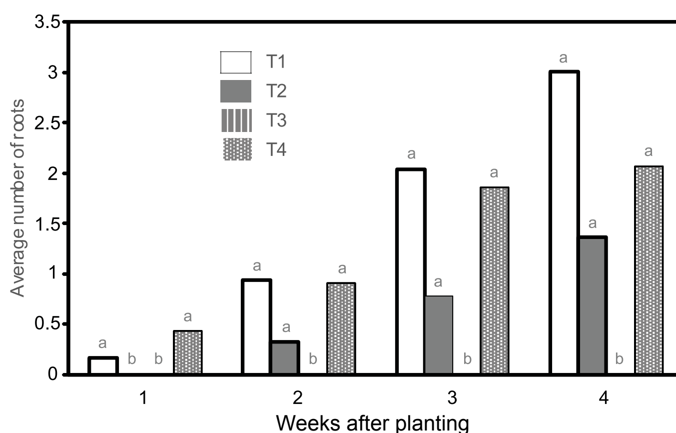

Fig. 5. Effect of temperature on average number of roots cv. 'Bima Brebes'. T1: control; T2: 30°C; T3: 37°C; T4: heterogenous

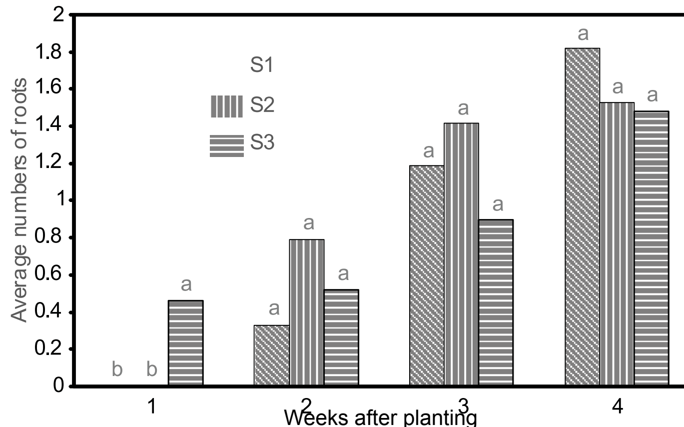

Fig. 6. Effect of explant size on average number of roots cv. 'Bima Brebes'. S1: 1 mm-explant; S2: 2 mm-explant; S3: 3 mm-explant.

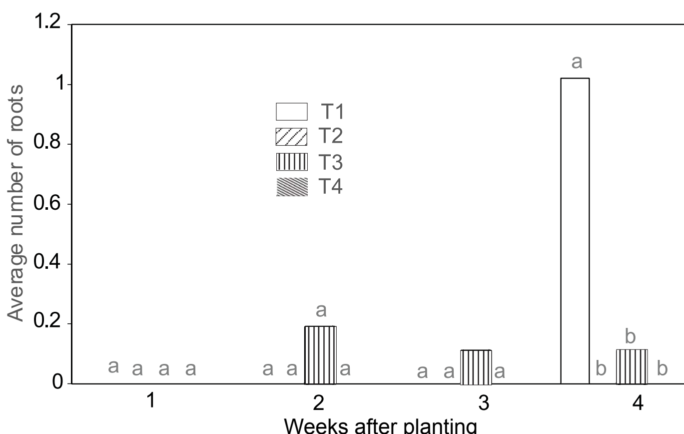

Fig. 7. Effect of temperature on average number of roots cv. 'Bima Curut'. T1: control; T2: 30°C; T3: 37°C; T4: heterogenous.

affected (Fig. 6. and Fig. 8.). The earliest root development was observed on untreated plantlets and plantlets cv. 'Bima Brebes' treated by heterogenous condition. The poorest root formation was observed on plantlet treated at 37 °C (Fig. 5). Lower temperature tend to give a beneficial effecton root formation, meanwhile high temperature can inhibit root formation. Lower temperature might affect cell proliferation and concentration of endogenous gibberelin. Within treatment, plantlets cv. 'Bima Curut' treated with 37 °C formed roots earlier than plantlets treated with other treatment (Fig. 7). These might be due to genotype, exogenous plant hormones, and ability to form roots in

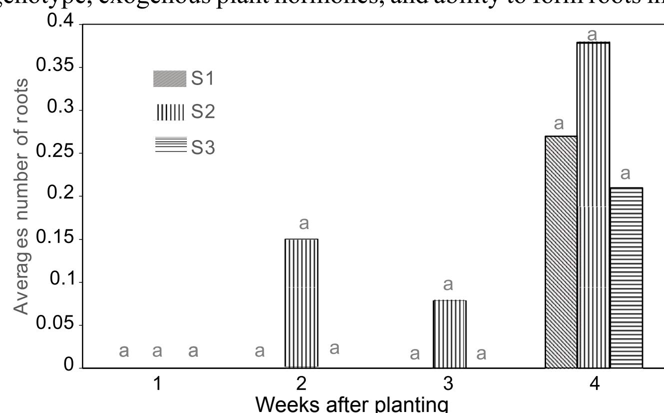

Fig. 8. Effect of explant size on average number of roots cv. 'Bima Curut'. S1: 1 mm-explant; S2: 2 mm-explant; S3: 3 mm-explant.

different temperatures. Some genotype of plants are able to form roots earlier with increasing of temperature (Dinarti *et al.,* 2011).

Virus incidence on plantlets was reduced by explant size. Neither *Potyvirus* nor *Carlavirus* was detected on both cultivars of 1 mm-explants (Fig. 9) dan (Fig. 10). It is known that 1 mm-explant consisted of one apical dome and one leaf primodia. These region has a high cell proliferation, and may prevent the multiplication of viruses. It is also hypothesized that vascular system was not found in the meristem so that virus could not reach this region (Panattoni *et al.*, 2013). The used of 1-mm explant increasing efficiency on virus elimination, but this method had some difficulties *i.e* the rate of survival planlets are sometimes associated with explant size, smallest explant could lead hyper-hydrocity and vitrification that limits successful establishment of tissue culture plants when transferring into greenhouse (Manjunathagowda *et al.,* 2017).

Combination treatment of temperature and explant size gave different result for plantlets derived from 2 and 3 mm-explants (Fig. 9) and (Fig. 10). In this case, *Potyvirus* seems to be more difficult to be eliminated from the plantlets compared to *Carlavirus*. Both viruses were successfully eliminated from these plantlets when treated with 37 °C. Although thermotherapy 37 °C potentially eliminated viruses, however, it was also deletarious effect on plantlets growth. These fact indicated that 37 °C might be maximum limits for physiological tolerance for shallots tissue culture.

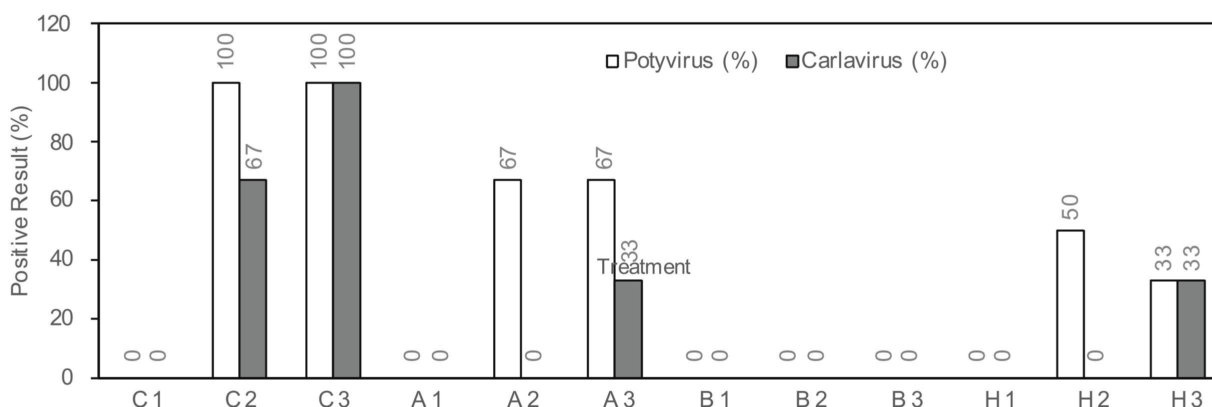

Fig. 9. Virus incidence of plantlets cv. 'Bima Brebes' given treatment combination of thermotherapy and explant size. C1: control 1 mm, C2: control 2 mm, C3: control 3 mm, A1: 30 °C 1 mm, A2: 30 °C 2 mm, A3: 30 °C 3 mm, B1: 37°C 1 mm, B2: 37 °C 2 mm, B3: 37°C 3 mm, H1: heterogenous 1 mm, H2: heterogenous 2 mm, and H3: heterogenous 3 mm.

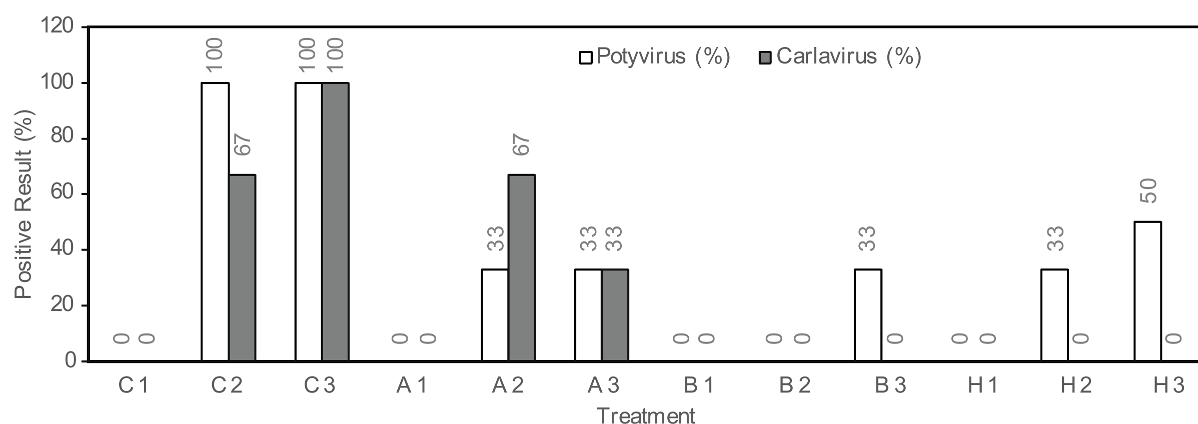

Fig. 10. Virus incidence of plantlets cv. 'Bima Curut' given treatment combination of thermotherapy and explant size. C1: control 1 mm, C2: control 2 mm, C3: control 3 mm, A1: 30 °C 1 mm, A2: 30 °C 2 mm, A3: 30 °C 3 mm, B1: 37 °C 1 mm, B2: 37°C 2 mm, B3: 37 °C 3 mm, H1: heterogenous 1 mm, H2: heterogenous 2 mm, and H3: heterogenous 3 mm.

In conclusion, elimination of the virus was successfully achieved on plantlets of 'Bima Brebes' and 'Bima Curut' developed from 1 mm-explant with or without thermotherapy. Bigger size of explants (2 mm and 3 mm) requires thermotherapy to reduce virus incidence. When this technique proceeded, the best treatment combination for both cultivars was using 2 mm-explant in heterogenous condition.

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