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ELIMINATION OF CVB (*Chrysanthemum virus B*) FROM A RANGE OF CHRYSANTHEMUM VARIETIES BY APICAL MERISTEM CULTURE FOLLOWING ANTIVIRAL AGENT AND HEAT TREATMENTS

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ABSTRACT

CVB elimination for retaining healthy protocols from infected chrysanthemum plant was investigated through combined treatment of meristem culture with synthetic antiviral ribavirin or thermotherapy under *in vitro* conditions. The biological materials used for the experiment constituted of six commercial varieties: Dewi Sartika, Saraswati, Yellow Fiji, White Puma, Yellow Puma and White Reagent. Tissue culture initiation was conducted through plantlet establishment using MS supplemented with IAA. Ribavirin was added in media with the concentration of 40 mg/l on cv. Dewi Sartika, Saraswati and Yellow Fiji. Parallel with this step, heat treatment with different durations (1, 2, and 3 weeks) was also conducted on the plantlets on White Puma, Yellow Puma and White Reagent. Meristem culture was done following the chemo- and thermotherapy. The experiment resumed the failure of single treatment of meristem culture in eliminating CVB from the infected chrysanthemum plantlets. Under heat treatment, percentage of virus-free plantlets increased along with the duration of thermotherapy, though the survival rate of plantlets decreased in lengthened heat treatment. The best results regarding virus free plant percentage were obtained when meristem culture was applied following ribavirin or three weeks of heat treatment.

Key words: *Chrysanthemum virus-B* (CVB), chemotherapy, heat treatment, meristem culture, virus-free

INTRODUCTION

Chrysanthemum (*Dendranthema grandiflora [Ramat.]* Kitam) is one of the major cut flowers in the world. Chrysanthemum ranks the first of all cut flowers marketed

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every year from Indonesia (37.34 %) with the quantity of more than 66 million stalks in 2007 (Indonesian General Directorate of Horticulture 2008). Nowadays, however, chrysanthemum production has faced some constraints, and one of these was systemic disease attacks caused by viruses, viroids and phytoplasmic organisms. Up to present, Chrysanthemum Virus-B (CVB) is still one of the most common viruses found in commercial growers and have caused significant economic losses. (Marwoto *et al.* 2004).

Taxonomically, CVB belongs to carlavirus wide group. Genome of the virus consists of unipartite, single-stranded RNA with total genome size of 7.5 kb (Levay & Zavriev 1991). The nucleocapsid (virions) are filamentous with no protein envelop and usually straight with a clear modal length of 685 nm (Brunt *et al.* 1996). Aside from chrysanthemum (Compositae), CVB was also known systemically hosted in *Vicia faba* (Leguminosae), Nicotiana, Petunia and Tetragonia (Megan *et al.* 2001). The general symptoms of the infected plant were stunted in growth, chlorotic in leaf blades, organ structure malformation and discolored petal. Degeneration in proliferation level with slower multiplication rate was also observed in the infected planlet during *in vitro* culture (Marwoto *et al.* 2004).

Efforts have been made to get healthy protocols by eliminating virus from the infected plants. Several methods such as chemo-, thermotherapy and electrical charges 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 (Elia *et al.* 2008), ringspot virus in citrus (Sharma *et al.* 2007) and yellow leaf virus in sugarcane (Parmessur & Saumtally 2001). 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 (Senula *et al.* 2000*)*. 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 (Klein & Livingston 2008).

Heat treatment or thermotherapy was established for virus elimination related to characteristic of some viruses which showed declination in multiplication rate especially at high temperature. This method was successfully applied in the range of 35 - 40 ^{0}C for producing virus-free potato plant (Converse & Tanne 1984), alstromeria (Hakkaart & Versluijs 1988) and apple (Wang *et al.* 2006). However, both chemo- and thermotherapy methods, depended on plant genotypes and viruses. Varying degree of meristem cells deaths, phytotoxic causing an increase in culture time and the need for frequent transfers into fresh media coincided with the quantity of virus-free plants obtained (Chen & Sherwood 1991).

The combination of both chemo- and thermotherapy with meristem culture was dedicated to encounter the technical constraints in the single application of respective techniques. These methods were also reported to be more effective for virus destruction on sweet potato, nicotiana, lemon and cucumber (Zaitlin & Palukaitis 2000). The research was then conducted to find out the effect of ribavirin application in the media and heat treatment at different durations followed by meristem culture on the existence of CVB in infected chrysanthemum plants.

MATERIALS AND METHODS

The research was conducted at The Indonesian Ornamental Crops Research Institute (IOCRI). Six CVB infected chrysanthemum varieties i.e Dewi Sartika, Saraswati, Yellow Fiji, White Puma, Yellow Puma and White Reagent were equally divided into two parallel experiments. The first three varieties served as chemotherapy using Ribavirin, while the rest underwent the heat treatments. Fifteen cutting samples from each variety were collected and replanted in 15 cm pot. These were then, maintained in growth chamber for 16 h. After 2 weeks, the plants were pinched and the new emerging lateral growths served as explants. Shoot induction was conducted by inoculating apicals into ½ MS + 0.5 mg/l IAA. The shoots were then subcultured into ½ MS + 0.1 mg/l IAA to obtain uniform plantlets.

In Vitro Treatment of Ribavirin

After three weeks incubation, the plantlets were transferred into treatments media, consisting of ½ MS + 0.1 mg/l IAA and ½ MS + 0.1 mg/l IAA + 40 mg/l ribavirin. Two weeks after incubation, meristematic apical of plantlets (< 0.2 mm) were dissected using binocular microscope and transferred into MS media for shoot induction. The dissections of meristematic apical of plantlets were repeated three times from newly emerging shoots.

In Vitro Heat Treatment

One week before heat treatments, the plantlets were preconditioned in the incubator with the daily temperature of 30 - 35 ^{0}C. The temperature of the incubator was then increased up to 38 - 40 ^{0}C with the duration of 1, 2 and 3 weeks. After the heat treatments, meristematic apical of plantlets was dissected and inoculated into MS media for shoot induction.

ELISA Bioassay

After transferred into regeneration media, phenotypic performance of plantlet was recorded. Randomly plantlet samples in every treatment were also selected for CVB rapid detection using direct ELISA method (Clark & Adam 1977). An amount of 100 µl CVB IgG (AGDIA, USA) was mixed with a ratio of 1 : 200 of the coating buffer (Na_2CO_3 + $NaHCO_3$ + NaN_3) and overnightly incubated at 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 of 3 minutes each. Leaf samples of 0.2 g 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 CVB was pipetted into microplates and mixed with ECI (PBST + 0,2 % BSA) with a ratio 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. An amount of 100 µl substrate buffer containing 4-nitrophenylphospate was placed into microplates and incubated at room temperature. After the substrate color changed into yellow, the reactions was then ceased with 25 µl NaOH 3M. Color intensities of the substrate were measured using ELISA reader (Minireader II Dynatech) on 410 nm wavelength. Virus free samples were obtained when the absorbent values were three times less than that of positive control.

RESULTS AND DISCUSSION

Chemotherapy by Antiviral Ribavirin

Plantlet performances were affected by the application of antiviral ribavirin and meristem tip culture, however,there were no specific interaction between cultivars tested and antiviral treatments. The increase of regeneration capacity was significant in plant height, number of leaves and number of nodes of ribavirin-treated plantlets, when samples were supplemented with 40 ppm ribavirin compared to untreated plants, thus hastened multiplication rate of the plantlet of all varieties tested (Table 1).

Table 1. Plantlet height, number of leaves and nodes after 30 days of subculture, and multiplication rate of chrysanthemum varieties treated with ribavirin.

Treatments	Plantlet height*) (cm)		Number of leaves*)		Number of nodes*)		Multiplication rate*)	
Ribavirin treatments on media								
MS + 0.1 mg/l IAA + ribavirin	9.5	a	12.6	a	12.7	a	10.75	a
MS + 0.1 mg/l IAA	6.8	b	6.4	b	6.3	b	7.41	b
Chrysanthemum varieties								
Dewi Sartika	9.2	a	12.7	a	11.7	a	10.22	a
Saraswati	7.5	ab	10.2	ab	10.5	ab	8.75	ab
Yellow Fiji	6.0	b	9.1	b	9.2	b	6.16	b

* Note : Values followed by different letters in the same column differ significantly at LSD 5%.

Faster multiplication rate of ribavirin-treated plantlets indicated that cell totipotency was retained. The most putative concerns of these phenomena were related to viricidist effect of ribavirin. The antiviral might block virus replication and although existing virus might remain in the original stem sections, the new outgrowths would be virus-free or contain only very low amounts of the virus (Simpkins *et al.* 1981). Ribavirin might be active in its triphosphate form, which inhibits the 5' capping

of viral RNAs (Dawson & Lozoya Saldana 1984). It inhibited virus replication at the early stage by impairing synthesis of RNA- dependent RNA polymerase and at a later stage by impairing synthesis of the coat protein (Schuster & Huber 1991). With the absent of or low concentration viral particles, the interfering physiological orientation of embryonic derived cells were overruled to the maximum growth of genotype response.

During the experiment, no necrotic symptom used as indication of phytotoxicity was found in all varieties. Though vegetal tissues tolerance to ribavirin is genotype dependent (Sidwell *et al.* 2005), the chemical concentration added into the media (40 mg/l) was considered proportional for chrysanthemum. These might refer to the fact that with such concentration range, phytotoxic symptoms were observed in other crops during virus elimination thus, inhibited or decreased plant cellular metabolic activity when applied in higher concentration than 30 mg/l (Verma *et al. 2005;* Parmessur & Saumtally *2001).*

Application of Thermotherapy

The survival and death rates of plantlets in every duration of thermal treatments were varied among cultivars tested, though the trend was similar. During the first week of heat treatments, 24 - 36 % of treated plantlets were ceased, with cv. White Reagent showing the least number of death plantlets compared to the others. The number of death plantlets was slightly decreased in the following week, 12 14 % (Fig. 1). A short preconditioning incubation (one week with gradual increase at 30 -35 ^{0}C) before the thermotherapy was apparently not sufficient for the plantlet to make suitable adaptation on higher temperature of heat treatment. Though the heat tolerance of plants was indigenously specific, the mechanical adaptation of plants was time-depended on the degree of transition in regular change. The plantlet should be provided with sufficient precondition period with small and regular increase of temperature approaching the level of heat treatment to hinder high plantlet death at the early stage (Manganaris *et al.* 2003). Through the small gradual increase of the temperature in longer period, the plantlet could establish physiological adaptation to the temperature level of 35 to 40 ^{0}C.

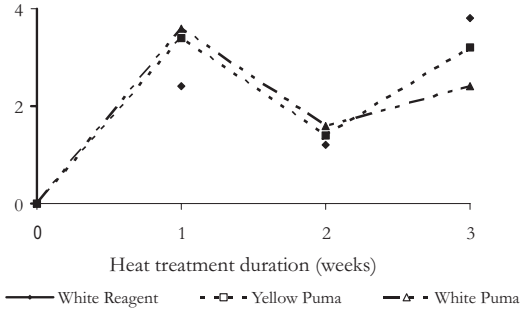

Figure 1. Plantlet death rates of three chrysanthemum cultivars after one, two and three weeks of heat treatments.

Conversely from the first and second weeks, cv. White Reagent showed higher plantlet death rates in the third week compared to cv. White Puma and Yellow Puma (Fig. 1). This period was considered critical, since plantlet death rates of all cultivars tested were also highest in these week (24 - 38 %). Wang (2006) concluded that inside the culture flask or *in vitro* condition, a protected environment might lead to narrower adaptation of plantlets to the extreme conditions such as high temperature. This finding suggested that at the third week the toleration limit of chrysanthemum plantlet to heat treatments was obtained.

After heat treatments, apical meristematic tissue of individual plantlets was inoculated into shoot induction media. Period taken for bud initiation and plantlet height after three weeks transferring into regeneration media were observed different referring to the length of thermal treatments. Shortest period for bud initiation and tallest plantlets were shown by the three weeks heat treatments in all varieties tested (Table 2). Slower growth rate on plantlets treated by one and two weeks thermotherapy indicated that physiologically, the plantlets were still in their suboptimal potential. These growth retardations were predictably affected by virus particles remained in the tissues which systemic persistently interfered with plant metabolism (Marwoto *et al.* 2004; Bhatthacharyya *et al.* 1990)

Table 2. Buds initiation and plantlet height after three weeks transferring of three chrysanthemum varieties under different period of heat treatments.

Cultivars	Period of heat treatments (weeks)		
	1	2	3
Bud initiation (days) *)			
White Reagent	63.8 a	64.4 a	49.8 b
White Puma	64.4 a	59.6 b	48.2 c
Yellow Puma	61.6 a	59.7 a	44.4 b
Plantlet height alter three weeks transfering into regeneration media (cm) *)			
White Reagent	4,32 a	5,11 a	6,34 b
White Puma	4,36 a	4.82 a	6.84 b
Yellow Puma	5,02 a	6,21 b	7,13 c

*) Remarks : Values followed by different letters in the same rows differ significantly at LSD 5 %.

ELISA bioassay

Virus detection using direct ELISA method was conducted to plant samples in all treatment combinations and spectro-photometerically revealed as absorbent values (Table 3). These values indicated the existence of virus particles in plant tissues. The percentage of CVB-free plantlets increased accordingly to the lengthened heat treatments and application of ribavirin within the media. Three weeks thermotherapy or supplemental antiviral followed by meristem culture successfully eliminated CVB from the infected chrysanthemum plantlets.

The success of chemo- and thermotherapy in eliminating CVB from infected plants in our works inferred that CVB particles and their persistence was highly affected by heat treatments at 38 - 40 ^{0}C and antiviral application (ribavirin 40 mg/l). The failure of single method of meristem culture for producing virus free plantlets as also presented in the absent of ribavirin treatment indicated that the isolation of meristematic sites was not sufficient to totally free the tissue from virus particles. Due to its small size, virus particles could traffic cell to cell even through branched plasmodesmata and infected the meristemal cells (Laimer 2003). Consequently, these naturally high antiviral-content tissues were also difficult to be isolated, since they were mostly microscopic and very sensitive (Brown *et al.* 1988). Thus, combination of thermo- or chemotherapy with meristem culture for virus elimination would be more promising, especially when mixed infection occurred or for those more persistent strains.

Table 3. CVB detection by direct ELISA and percentage of virus-free plantlets under thermo- and chemotherapy treatments.

Treatments	Absorbance values*)	Percentage of CVB-free plantlet *) (%)
Heat treatment		
- 1 week	0.06 – 0.11	41.6
- 2 weeks	0.03 – 0.09	72.3
- 3 weeks	0.02 – 0.07	100
Chemotherapy using ribavirin 40 mg/l		
- Without ribavirin	0.06 – 0.12	43.6
- With ribavirin	0.01 – 0.06	100
Positive control	0.22	0
Negative control	0.01	100

*) Remarks : values derived from 36 plantlet samples in each treatment combination

CONCLUSIONS

Under heat treatment, percentage of virus-free plantlets increased along with the duration of thermotherapy, while the survival rate of plantlets decreased in lengthened duration. The procedure of meristem culture application following ribavirin application or three weeks of heat treatment may be effective in eliminating CVB from the infected plantlets.

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