

# **UNIVERSITI PUTRA MALAYSIA**

# DETECTION, CHARACTERIZATION AND PATHOGENICITY OF Coleus blumei viroid IN Coleus blumei BENTH. IN PENINSULAR MALAYSIA

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FP 2019 26



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NURUL NAJWA BINTI CHE ROSLAN

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

January 2019

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

## DETECTION, CHARACTERIZATION AND PATHOGENICITY OF Coleus blumei viroid IN Coleus blumei BENTH. IN PENINSULAR MALAYSIA

By

### NURUL NAJWA BINTI CHE ROSLAN

January 2019

#### Chairman: Associate Professor Ganesan Vadamalai, PhD Faculty: Agriculture

Plectranthus scutellarioides or synonym known as Coleus blumei is a popular ornamental plant in Malaysia grown for beautification as garden plants and mostly used for landscape due to their brightly colour foliage. However, this plant is susceptible to infection of six variants of *Coleus blumei viroid* (CbVd-1 to CbVd-6) of genus *Coleviroid* from family *Pospiviroidae*. CbVd is distributed worldwide, but yet to be reported in Malaysia. Inadequate information on viroid disease in ornamental plant species, specifically C. blumei in Malaysia is of concern for the landscape industry. In view of this, the objectives of this study were (a) to detect and characterize of CbVd from C. blumei benth. in Peninsular Malaysia using RT-PCR, cloning and sequencing, (b) to study the pathogenicity of CbVd-1 and CbVd-5 on C. blumei cultivar 'Dipt in wine'. Altogether, 49 samples of C. blumei showing viroid-like symptoms such as stunted growth, faded leaf colour and reduction in leaf size and nonsymptomatic consists mixed cultivars were sampled from Perlis, Kedah, Perak, Selangor, Melaka, Kelantan and Johor. RT-PCR was used to detect CbVd using three sets of CbVd primers (universal CbVd primer, specific CbVd1K and specific CbVd-5 primer). Results of molecular detection by RT-PCR showed that 47 of 49 samples were positive for CbVd (CbVd universal primer), 45 samples were positive with CbVd-1 (CbVd1K primer) and 41 samples were positive with CbVd-5 (CbVd-5 primer). Meanwhile, mixed infection of both CbVd-1 and CbVd-5 variants were detected in 40 out of 49 collected samples. All nucleotides of CbVd-1 and CbVd-5 positive samples were 249-250 nt and 274 nt in length. Sequence analysis of both CbVd-1 (MF176948-MF176951) and CbVd-5 clones (MF176952-MF176955) revealed 99% to 100% sequence similarity to CbVd-1 clone 1, complete genome (GenBank Accession No. DQ178399.1) and Korea (GenBank Accession No. EU410620.1) and CbVd-5 clone 1, complete genome (GenBank Accession No. FJ151370.1) from China. Results from phylogenetic analysis of the Malaysian CbVd-1 with isolates from Korea, India, China, Brazil and Germany showed that the Malaysian isolates formed same clade with Korea (EU410620), India (AB740017 and AB740018), China (DQ178397- DQ178399) and Brazil (X69293) meanwhile CbVd-



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5 isolates with isolates from China (FJ151370-FJ151372 and NC012127) and Japan (LC068970). Pathogenicity study showed that *C. blumei* cultivar 'Dipt in wine' seedlings inoculated with leaf sap and plasmid containing a CbVd-1 (CbVd-1\_J1MY) and CbVd-5 insert (CbVd-5\_R3MY) expressed only leaf colour fading symptom meanwhile inoculated seedlings with leaf sap and plasmid containing combination of both CbVd variants (CbVd-1\_J1MY and CbVd-5\_R3MY) showed leaf colour fading and stunting over 12 weeks observation period. CbVd-1, CbVd-5 and combination of both variant were detected in *C. blumei* cultivar 'Dipt in wine' seedlings between 3 to 9 weeks post-inoculation by RT-PCR. Sequencing of the amplicons confirmed the presence of CbVd-1, CbVd-5 and mixed infection with more than 95% sequence similarity with CbVd-1 (CbVd-1\_J1MY) and CbVd-5 variants (CbVd-5\_R3MY) that were used for the inoculation. In summary, CbVd-1 and CbVd-5 were present in *C. blumei* cultivar 'Dipt in wine'. This is the first report of CbVd-1 and CbVd-5 in Malaysia.



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## PENGESANAN, PENCIRIAN DAN KEPATOGENAN Coleus blumei viroid PADA Coleus blumei BENTH. DI SEMENANJUNG MALAYSIA

Oleh

### NURUL NAJWA BINTI CHE ROSLAN

Januari 2019

#### Pengerusi: Profesor Madya Ganesan Vadamalai, PhD Fakulti: Pertanian

Plectranthus scutellarioides atau lebih dikenali sebagai Coleus blumei merupakan tumbuhan hiasan yang popular ditanam di Malaysia untuk tujuan keindahan sebagai tumbuhan taman dan kebanyakannya digunakan untuk landskap disebabkan daunnya yang berwarna terang. Walau bagaimanapun, tumbuhan ini terdedah kepada jangkitan enam varian Coleus blumei viroid (CbVd-1 hingga CbVd-5) daripada genus Coleviroid dari keluarga Pospiviroidae. CbVd telah tersebar di seluruh dunia, namun belum dilaporkan di Malaysia. Maklumat yang tidak mencukupi mengenai penyakit viroid dalam spesis tanaman hiasan, khususnya C. blumei menjadi kebimbangan terhadap industri landskap di Malaysia. Oleh yang demikian, objektif kajian ini adalah (a) Untuk mengesan dan mencirikan CbVd dari C. blumei benth. di Semenanjung Malaysia menggunakan RT-PCR, pengklonan dan penjujukan, (b) Untuk menguji kepatogenan CbVd-1 dan CbVd-5 pada anak benih C. blumei kultivar Dipt in wine'. Kesemuanya 49 sampel C. blumei menunjukkan gejala jangkitan viroid seperti pertumbuhan terbantut, pudar warna daun dan pengurangan saiz daun serta tidak menunjukkan sebarang gejala jangkitan viroid, yang terdiri daripada pelbagai jenis kultivar telah disampel daripada Perlis, Kedah, Perak, Selangor, Melaka, Kelantan dan Johor. RT-PCR telah digunakan untuk mengesan CbVd melalui penggunaan tiga pasang CbVd primer (universal CbVd primer, spesifik CbVd1K dan spesifik CbVd 5 primer). Hasil pengesanan molekul oleh RT-PCR menunjukkan bahawa 47 daripada 49 sampel adalah positif dengan CbVd-1 (CbVd1K primer) dan 41 sampel positif dengan CbVd-5 (CbVd 5 primer). Manakala, 40 daripada 49 sampel positif dengan kedua-dua varian CbVd-1 dan CbVd-5. Kesemua sampel yang positif dengan CbVd-1 mempunyai saiz diantara 249 nt hingga 250 nt dan CbVd-5 dengan 274 nt. Analisis jujukan kedua-dua klon CbVd-1 (MF176948-MF176951) dan CbVd-5 (MF176952-MF176955) menunjukan kadar persamaan 99% hingga 100% kepada CbVd-1 klon 1, genom lengkap yang dilaporkan di China (GenBank Accession No. DQ178399.1) dan Korea (GenBank Accession No. EU410620.1) dan CbVd-5 klon 1, genom lengkap (GenBank Accession No. FJ151370.1) yang dilaporkan di China. Hasil daripada analisis filogenik, isolat CbVd-1 Malaysia dengan isolat dari Korea, India, China,



Brazil dan German menunjukkan isolat Malaysia membentuk klad yang sama dengan Korea (EU410620), India (AB740017 dan AB740018), China (DQ178397-DQ178399) dan Brazil (X69293) manakala isolat CbVd-5 dengan isolat dari China (FJ151370-FJ151372 dan NC012127) dan Jepun (LC068970). Kajian patogenisiti menunjukkan anak benih C. blumei kultivar 'Dipt in wine' yang diinokulasi dengan sap daun dan plasmid yang mengandungi selitan CbVd-1 (CbVd-1\_J1MY) dan CbVd-5 (CbVd-5 R3MY) hanya menunjukan gejala pudar warna daun. Manakala, bagi anak benih yang diinokulasikan dengan gabungan varian CbVd-1 dan CbVd-5 (CbVd-1\_J1MY and CbVd-5\_R3MY) menunjukkan gejala pudar warna daun dan pertumbuhan terbantut sepanjang 12 minggu pemerhatian. CbVd-1, CbVd-5 dan gabungan kedua-dua varian telah dikesan dalam anak benih C. blumei cultivar 'Dipt in wine' yang diinokulasi di antara 3 hingga 9 minggu per-inokulasi oleh RT-PCR. Penjujukan amplikon mengesahkan kehadiran CbVd-1, CbVd-5 dan campuran jangkitan CbVd-1 dan CbVd-5 dengan persamaan lebih 95% pada varian CbVd-1 (CbVd-1\_J1MY) dan CbVd-5 (CbVd-5\_R3MY) yang digunakan untuk inokulasi. Secara ringkas, CbVd-1 dan CbVd-5 dikesan pada C. blumei di Semenanjung Malaysia dan mereka mampu berreplikasi dalam C. blumei cultivar 'Dipt in wine'. Laporan ini merupakan laporan pertama varian CbVd-1 dan CbVd-5 di Malaysia.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follow:

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- 3.9 RT-PCR assay of total nucleic acid extract from symptomatic samples collected from Perak using specific CbVd 5 primer showed bands at the region between 250 nt to 300 nt on 2.0% agarose gel electrophoresis
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- 4.1 Three-week-old *C. blumei* cultivar 'Dipt in wine' seedlings were 52 bought from nursery
- 4.2 Inoculation of CbVd inoculums on *C. blumei* cv. Dipt in wine 54 seedlings. A) Carborandum dusted young leaf of old *C. blumei* seedlings were rubbed with cotton wool. B) Leaves were washed with distilled water. C) Cotton wool with plasmid (CbVd-1, CbVd-5 and combination of both CbVd-1 and CbVd-5) were rubbed onto two to three young leaves. D) Cotton wool with leaf sap (CbVd-1, CbVd-5 and mixed infection) were rubbed onto two to three young leaves of seedlings. E) Inoculated seedling were kept in glasshouse

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- 4.3 Leaf colour fading symptom induced on *C. blumei* cv. 'Dipt in wine' inoculated with CbVd plasmids clone. Figure (A): a) Inoculated seedling with CbVd-5 plasmid clones at 4 week post-inoculation showed leaf color fading, b) Healthy seedling; Figure (B): a) Inoculated seedling with CbVd-1 plasmid clone showed leaf colour fading symptom at 12 week post-inoculation, b) Healthy seedling; Figure (C): Inoculated seedling with mixed infection plasmid clone showed leaf colour fading symptom at 8 week post-inoculation, b) Healthy seedling; b) Healthy seedling
- 4.4 Leaf colour fading symptom induced on *C. blumei* cv. 'Dipt in wine' inoculated with CbVd leaf sap inoculum. Figure (A): a) Inoculated seedling with CbVd-5 leaf sap at 4 week post-inoculation showed leaf color fading, b) Healthy seedling; Figure (B): a) Inoculated seedling with CbVd-1 leaf sap showed leaf colour fading symptom at 8 week post-inoculation, b) Healthy seedling; Figure (C): Inoculated seedling with mixed infection leaf sap showed leaf colour fading symptom at 8 week post-inoculation, b) Healthy seedling; Figure (C): Inoculated seedling with mixed infection leaf sap showed leaf colour fading symptom at 8 week post-inoculation, b) Healthy seedling;
- 4.5 RT-PCR assays with specific CbVd 1K primer of nucleic acid extracted from *C. blumei* cultivar 'Dipt in wine' seedlings inoculated with CbVd-1 plasmid at 3, 6 and 9 week postinoculation. The size of amplicon was 249 nt. M- 100 bp molecular marker. NTC-Non-template control. +VE- Positive sample with CbVd-1 (249 nt)
- 4.6 RT-PCR assays with specific CbVd 5 primer of nucleic acid 62 extracted from *C. blumei* cultivar 'Dipt in wine' seedlings inoculated with CbVd-5 plasmid at 3, 6 and 9 week post-inoculation. The size of amplicon was 274 nt. M- 100 bp molecular marker. NTC-Non-template control. +VE- Positive sample with CbVd-5 (274 nt)
- 4.7 RT-PCR assays with universal CbVd primer of nucleic acid extracted from *C. blumei* cultivar 'Dipt in wine' seedlings inoculated with mixed infection plasmid at 3, 6 and 9 week postinoculation. The size of amplicon were between 250 to 300 nt. M-100 bp molecular marker. NTC-Non-template control. +VE-Positive sample with mixed infection (249 nt and 274 nt)
- 4.8 RT-PCR assays with specific CbVd 1K primer of nucleic acid extracted from *C. blumei* cultivar 'Dipt in wine' seedlings inoculated with CbVd-1 leaf sap at 3, 6 and 9 week postinoculation. The size of amplicon was 249 nt. M- 100 bp molecular marker. NTC-Non-template control. +VE- Positive sample with CbVd-1 (249 nt)

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- 4.9 RT-PCR assays with specific CbVd 5 primer of nucleic acid extracted from *C. blumei* cultivar 'Dipt in wine' seedlings inoculated with CbVd-5 leaf sap at 3, 6 and 9 week postinoculation. The size of amplicon was 274 nt. M- 100 bp molecular marker. NTC-Non-template control. +VE- Positive sample with CbVd-5 (274 nt)
- 4.10 RT-PCR assays with universal CbVd primer of nucleic acid extracted from *C. blumei* cultivar 'Dipt in wine' seedlings inoculated with mixed infection leaf sap at 3, 6 and 9 week post-inoculation. The size of amplicon were between 250 to 300 nt. M- 100 bp molecular marker. NTC-Non-template control. +VE-Positive sample with mixed infection (249 nt and 274 nt)



# LIST OF ABBREVIATIONS

%	Percentage
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
AMV-RT	Avian myeloblastosis virus reverse transcriptase
ANOVA	Analysis of variant
ASBVd	Avocado sunblotch viroid
Blast	Basic local alignment search tool
bp	Base pair
ĊA	Chlorophorm: isoAmyl alcohol
CbVd	Coleus blumei viroid
CbVd-1	Coleus blumei viroid-1
CbVd-2	Coleus blumei viroid-2
CbVd-3	Coleus blumei viroid-3
CbVd-4	Coleus blumei viroid-4
CbVd-5	Coleus blumei viroid-5
CbVd-6	Coleus blumei viroid-6
CCCVd	Coconut cadang-cadang viroid
CChMVd	Chrysanthemum chlorotic mottle viroid
CCR	Conserved central region
cDNA	Complementary deoxyribonucleic acid
CEVd-A	Citrus exocortis viroid –A
cm	Centimeter
CRD	Completely randomized design
СТАВ	Cetyl trimethylammonium bromide
CTiVd	Coconut tinangaja viroid (CTiVd)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELVd	Eggplant latent viroid
EtBr	Ethidium bromide
EtOH	Ethanol
g	Gram
g	Relative centrifugal force
HLVd	Hop latent viroid
HSVd	Hop Stunt Viroid
Μ	Moles/ liter (Molarity)
Mg	Milligram
MgCl2	Magnesium chloride
min	Minute
mL	Milliliter
mM	Millimolar
Multiplex RT-PCR	Multiplex reverse transcriptase polymerase chain
	reaction
NaCl	Sodium chloride

ng	Nanogram
nt	Nucleotide
Р	Pathogenicity
PLMVd	Peach latent mosaic viroid
pmole	Picomole
PSTVd	Potato tuber spindle tuber viroid
RNA	Ribonucleic acid
RNAsin	RNase inhibitor
R-PAGE	Return-polyacrylamide gel electrophoresis
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
sec	second
TASVd	Tomato apical stunt viroid
TBE	Tris-borate EDTA
TL	Terminal left
TPMVd	Tomato planta macho viroid
TR	Terminal right
Tris-HCL	Tris(hydroxymethyl) aminomethane hydrochloride
UV	Ultraviolet
V	Variable
V	Voltage
X-gal	5-bromo-4-chloro-3-Indolyl –D-galactopyranoside
μĽ	Microliter
μM	Micromolar

C

#### **CHAPTER 1**

#### INTRODUCTION

*Plectranthus scutellarioides* or namely known as *Coleus blumei* is one of the flowering plant from family *Lamiaceae*. This plant was originated from Africa and Indonesia but it is now distributed throughout the world including Malaysia. *C. blumei* is widely grown in many countries as bedding and garden plant. This ornamental plant can be raised through seed and stem cutting (Toogood, 1999; Rogers, 2008; Manoj *et al.*, 2015).

C. blumei is susceptible to various pests and diseases including viroid diseases. Coleus blumei viroid (CbVd) from the genus Coleviroid is reported worldwide to cause infection in C. blumei. The infection of this viroid on C. blumei resulted in symptomatic or asymptomatic infection depending on the cultivars (Fonseca *et al.*, 1989; Spieker, 1996a). The symptoms induced by CbVd includes stunting, chlorosis or purple pigmentation on leaf margin, leaf color fading and reduction in leaf size (Fonseca *et al.*, 1989; Spieker, 1996a; Singh and Boucher, 1991; Chung and Choi, 2008). CbVd can be transmitted among coleus through seeds, mechanical and grafting inoculation.

*C. blumei* has been reported worldwide infected with six main CbVd variants which are CbVd-1 to CbVd-6. However, there is no report on CbVd infecting *C. blumei* in Malaysia. Previous studies has proven the imported seed from Japan and United State available in commercial market in China, Korea, and Canada have been infected with CbVd (Singh and Boucher, 1990; Chung and Choi, 2008, Hou *et al.*, 2009a). Since CbVd are transmitted through seed, there is a probability that CbVd are widely present in Malaysia as most of the *C. blumei* planting materials in Malaysia are imported. In addition, most nurseries in Malaysia propagate this species through stem cutting.

There are many molecular diagnostic tools that can be used for viroids detection in plants as well as CbVd in *C. blumei*. Previous studies showed detection of CbVd mainly conducted using Polyacrylamide gel electrophoresis (PAGE) variations such as two dimensional polyacrylamide gel electrophoresis (2D-PAGE) and return polyacrylamide gel electrophoresis (R-PAGE) (Singh *et al.*, 1991; Hou *et al.*, 2009b; Jiang *et al.*, 2013), molecular hybridization such as northern blot hybridization and dot-blot hybridization (Li *et al.*, 2006), RT-PCR (Jiang *et al.*, 2011) microarray (Zhang *et al.*, 2013) and next generation sequencing (Zhang *et al.*, 2014). However, in this research, RT-PCR will be used for detection of CbVd in *C. blumei* in Peninsular Malaysia.

This study was conducted to confirm the present of CbVd in *C. blumei* in Peninsular Malaysia. In addition, this study will prove the Koch postulate of CbVd. The

outcomes of the present study will enable the development of disease management strategies in order to avoid a possible outbreak of CbVd in Malaysia. In view of this, the objectives of this study are:

- 1. To detect and characterize CbVd from *C. blumei* Benth. in Peninsular Malaysia using RT-PCR, cloning and sequencing.
- 2. To test the pathogenicity of CbVd variants on *C. blumei* cultivar 'Dipt in wine'



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The student was born in Sungai Petani, Kedah. She is the second child of three in her family. She received his early education in Sek.Keb. Pantai Prai, Sungai Petani from 1997-2002 and graduated from Sek. Men. Teknik Alor Setar, Kedah in 2007. She further his study in Diploma of Food Estate Management at Universiti Putra Malaysia Campus Bintulu, Sarawak from 2008-2011. In 2011, she enrolled in Bachelor of Agricultural Science at Universiti Putra Malaysia, Serdang, Selangor and graduate with First Class Honour in 2015. Her final year project was Identification and Characterization of Viroids from Coleus blumei Species in Malaysia under supervision of Assoc. Prof. Dr. Ganesan Vadamalai, Faculty of Agriculture, UPM. During her internship in Malaysia Field Research Station (Dupont Malaysia Sdn. Bhd.), Seberang Perai, Peneng she had conducted a project on Pesticide Trials on Vegetables Pest and Paddy and Rearing Insects Program under supervision of Mr. Hj. Rashid Ahmad, manager of research centre. She persued her master's study degree at Faculty of Agriculture under supervision of Assoc. Prof. Dr. Ganesan Vadamalai, Faculty of Agriculture, UPM on September 2015. During the course of his studies, he worked as graduate research fellow and assisted supervisor in lab practical. She was a recipient for MyBrain 15 scholarship under Ministry of Education in 2016.

## PUBLICATION

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